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Morphological and microsatellite analysis of the ancient Montenegrin olive variety 'Žutica' revealed different clones

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Morphological and microsatellite analysis of the ancient Montenegrin olive variety 'Žutica' revealed different clones

Abstract: The 'Žutica' represents the most common Montenegrin olive varieties mainly used for the production of olive oil and green and black fruit canning. Traditionally, the olive plants have been propagated vegetatively, and a small level of genetic polymorphism is expected among clones of the same variety. This topic was only partially studied in the Montenegrin olive 'Žutica'. Therefore, this study aimed to determine intra-varietal genetic variability in twenty-three 'Žutica' trees selected in situ, analyzing the variability of morphological traits and microsatellites. The Principal Component Analyses (PCA) with six axes explains the total cumulative variance of 91.3 %, with fruit and endocarp traits in the first three PC. The unweighted pair group method with arithmetic mean of twenty morphological traits grouped 'Žutica' trees into two clusters and five independent trees. Nine microsatellite primers amplified 31 fragments of which 22 were polymorphic and enabled the detection of nine different microsatellite profiles (potential different clones). A comparison of dendrogram groups based on morphological and microsatellite markers showed low cophenetic values in the determination of intra-varietal variability. The results showed that the old variety 'Žutica', from a relatively small geographic region, has a variable genetic base, which could be used in the selection of superior clones.

Key words: intra-varietal variability; morphological characterization; PCA; microsatellites; 'Žutica'

Morfološka in mikrosatelitska analiza stare črnogorske sorte oljke 'Žutica' sta odkrili variabilnost klonov

Izvleček: Sorta 'Žutica' predstavlja najbolj pogosto zastopano črnogorsko sorto oljke, ki se uporablja za pridelavo oljčnega olja ter za vlaganje zelenih in obarvanih plodov. Tradicionalno se oljko razmnožuje vegetativno, zato je pričakovati majhen genetski polimorfizem med kloni iste sorte. To je bilo pri črnogorski sorti 'Žutica' le delno proučeno in je predmet te raziskave. Za določitev znotraj sortne genetske variabilnosti z analizo morfoloških lastnosti in mikrosatelitov je bilo izbranih 23 dreves. Analiza glavnih komponent (PCA) je s prvimi šestimi osmi pojasnila 91,3 % celokupne variabilnosti, pri čemer so k vrednosti prvih treh osi najbolj prispevale lastnosti ploda in endokarpa. Združevanje z metodo netehtane aritmetične sredine na osnovi dvajsetih morfoloških lastnosti je drevesa 'Žutice' razvrstilo v dve skupini, pet dreves pa je ostalo nerazvrščenih. Z začetnimi oligonukleotidi za devet mikrosatelitskih lokusov se je pomnožilo 31 fragmentov, od katerih je bilo 22 polimorfnih, kar je omogočilo določitev devetih mikrosatelitskih profilov (potencialno različnih klonov). Pri primerjavi skupin iz dendrogramov, izdelanih na osnovi znotraj sortne variabilnosti morfoloških in mikrosatelitskih označevalcev, je bila ugotovljena majhna vrednost kofenetske korelacije. Rezultati so pokazali, da ima stara sorta 'Žutica', z relativno majhnega geografskega območja, raznoliko genetsko osnovo, kar bi lahko uporabili za izbor najboljših klonov.

Ključne besede: znotrajsortna raznolikost; morfološka karakterizacija; PCA; mikrosateliti; 'Žutica'

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1 INTRODUCTION

The olive *(Olea europaea* L.) is one of the most important and the oldest fruit trees in the Mediterranean Basin, probably domesticated in Chalcolithic Levant (Zohary et al., 2012). Olive trees are grown to produce high-quality fruit for consuming oil and table consumption, but also wood and ornamental design in the natural landscapes.

The cultivated olive has a very wide genetic background and a high number of olive varieties (presumed clones) are grown throughout the world. Several hundred, assumed olive clones are described within the Mediterranean region (Figueiredo et al., 2013). The number of cultivated varieties in olive germplasm is reported in Olive Germplasm Database (http://www.oleadb.it/olivodb.html). About 1,250 varieties in 54 countries conserved in over 100 collections, is probably an underestimate since there is a lack of information about local varieties, rarer cultivars widespread in the different olive growing areas. Indeed, a large number of synonyms (one genotype with several denominations) and homonyms (one denomination for several genotypes) in different areas of cultivation additionally hinder the identification of olive varieties (Caruso et al., 2014). That creates many complications in the classification of cultivated olive varieties due to the lack of standards reference variety (Figueiredo et al., 2013).

On the relatively small area of the East coast of the Adriatic sea bordered by Montenegro, olive cultivation has lasted for thousands of years. Nowadays, around 436,000 productive trees (MONSTAT, 2012) covering approximately 3,200 ha grow in this area (Lazović & Adakalić, 2012a). The growing of traditional varieties prevails, characterized mainly by the cultivation of numerous ancient trees representing autochthonous olive germplasm composed of twelve main cultivated varieties. The Coastal area is divided into Bar and Boka Kotorska Bay subareas, mainly due to the relief structure and to the olive assortment. In the Southern part (municipalities of Ulcinj, Bar and Budva) the indigenous old variety 'Žutica' predominates with around 97 % of total olive trees. In the area of Boka Kotorska Bay (municipalities of Tivat, Kotor and Herceg Novi) olive growing is based on 'Žutica' (36 %) and other varieties 'Crnica', 'Lumbardeška', 'Sitnica', 'Šarulja', etc. (Miranović, 2006; Lazović & Adakalić, 2012b; Lazović et al., 2014a).

During the long history of olive cultivation, polyclonality occurred and resulted in the formation of heterogeneous varieties (different genotypes or variety populations), and/or populations of clones (a mixture of clone variants) (Figueiredo et al., 2013; Caruso et al., 2014). Genetic variability of olive germplasm and intra-varietal diversity was reported in many olive varieties using morphological and molecular markers. Formerly, morphological and agronomical characterization had been widely used to evaluate diversity in the olive (Barranco et al., 2000; Cimato et al., 2001; Bassi et al., 2003; Rallo, 2005) with the addition of analyzing the data by PCA (Cantini et al., 1999; Trentacoste & Puertas, 2011). The introduction of DNA markers provided a sound discriminatory system for varietal identification and intra-varietal polymorphism detection, independent of environmental conditions. Therefore, DNA molecular markers are today widely used to complement morphological analysis (Trujillo et al., 2014) and to identify clones unambiguously. Polyclonal olive varieties have been detected using RAPD and AFLP (Figueiredo et al., 2013; Strikić et al., 2011; Banilas et al., 2003; Bandelj, 2005; Sanz-Cortés et al., 2003) or combining different molecular markers (Belaj et al., 2004; Gemas et al., 2004; Gomes et al., 2008; Martins-Lopes et al., 2009). Microsatellites have been primarily used to detect intra-varietal polymorphism and to identify clones (Lopes et al., 2004; Taamalli et al., 2007; Omrani-Sabbaghi et al., 2007; Noormohammadi et al., 2009; Rony et al., 2009; Muzzalupo et al., 2009). The new promising genotypes or superior clones were identified by microsatellite markers and morphological traits (Caruso et al., 2014; Zaher et al., 2011; Marra et al., 2014). SNP markers have recently been used in the genotyping and detection of polymorphisms in olives, as well as their association with important agronomic traits (Kaya et al., 2016, 2019). Therefore, the identification of clones today is mainly based on the study of molecular genetics techniques integrated with morphological traits.

The selection of clones with desirable fruit and oil quality is required since the quality of table olives and olive oil is significantly affected by genotype (Ipek et al., 2012). Due to the available genetic diversity in the olive, it is essential to select the clones with desirable traits such as low vigour, tolerance/resistance to low temperatures, salinity (Muzzalupo et al., 2010), drought stress (Caruso et al., 2014), etc. Some of these objectives could be taken into account in future selection programs of desirable genotypes to promote olive growing in Montenegro.

'Žutica' is the old dual purpose variety with olive oil content higher than 20 % and medium to large fruits in some trees. Those are advantages that could be implemented in the future olive growing and spreading of such clones. Previous research on intra-varietal variability in 'Žutica' was conducted by Lazović et al. (2002, 2016, 2018a). RAPD and SSR polymorphism was revealed, but the morphological properties of leaf, inflorescences, fruit and endocarp of the same clones were not analyzed. In some other researches, morpho-phenological and agronomic attributes (Lazović et al., 2014b, 2014c,

2018b; Adakalić & Lazović, 2018), as well as oil composition variability (Lazović et al., 2011; Adakalić & Lazović, 2018) of different clones of 'Žutica' were recorded.

So, the purpose of this paper was to present the mutual analysis of the data conducted from our research on autochthonous ancient olive variety 'Žutica' with the following objectives: (i) to characterize the 'Žutica' olive variety by analyzing morphological traits, (ii) to evaluate the genetic variability within the variety using microsatellite markers and (iii) to compare the efficiency of morphological and microsatellite markers to indicate the remarkable clones.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

Twenty-three olive trees (*O. europaea* L.) belonging to the 'Žutica' variety were studied. A larger number of samples from the Bar subarea were analyzed due to the higher representation in the cultivation of this olive variety in this subarea. Eighteen olive trees from Bar subarea are marked with codes: ZAV4, ZAV7, ZAV9, ZAV16, ZAV19, ZAV28, ZAV42, DAB1, DAB2, DAB3, DM5, SUS1, BARV, VALL, VAL2, PETR, REZ, IVA, and five olive trees from Boka Kotorska Bay coded: LUS14, LUS15, LUSM, GRB and HNSD. Sampling sites of collected olive material at two subareas are presented on the map (Fig 1).

2.2 MORPHOLOGICAL CHARACTERIZATION

To record the extent of morphological diversity over three growing seasons (from 2004 to 2008), a total of 20 morphological characters were studied based on olive descriptors (Barranco et al., 2000; EU/COI., 1997) recommendations. These included the characteristics of leaves (length-LL, width-LW and shape index-LI), internodes (length-INT), inflorescences (length-IL, number of flowers-NF, number of aborted flowers-NAF and percentage of aborted flowers-PAF, inflorescence density-ID), fruits (length-FL, width-FW, shape index -FI, mass-FM, pulp mass-PM and pulp percentage-PP) and endocarps (length-EL, width-EW, shape index -EI, mass-EM and pulp/endocarp ratio-P/E). Forty plant organ samples from the south-facing sides of trees were collected and characterized for each morphological measurement. Mean values, variability range, variation coefficient and minimum significant difference among analyzed characteristics for all parameters were reported.

2.3 DNA EXTRACTION AND MOLECULAR ANALYSIS

Total genomic DNA was isolated from fresh leaf material following the CTAB procedure described by Kump at al. (1992). DNA concentration was measured on a mini fluorometer TKO100 (Hoefer Scientific, San Francisco, USA) following the manufacturer's instructions. Nine microsatellite loci were tested using the following primer pairs: DCA3, DCA9, DCA11, DCA14 and DCA16 (Sefc et al., 2000), EMO3 and EMO90 (De la Rosa et al., 2002), GAPU101 (Carriero et al., 2002) and UDO99-19 (Cipriani et al., 2002). PCR amplifications were performed in a reaction volume of 10 µl containing 1 × PCR buffer (Promega, Manheim, Germany) [10 mM Tris-HCl (pH 8,3 at 20 °C); 1,5 mM $MgCl_2$; 50 mM

Figure 1: The Coastal area of Montenegro with two subareas, Bar (black ellipse) and Boka Kotorska Bay (red ellipse), showing the location of the 23 olive trees belonging to variety 'Žutica' examined. The green diamond symbol denotes sampling sites

KCl], 0.2 mM of each dNTP (Sigma-ALDRICH, St. Louis, USA), 0.2 µM initial concentration oligonucleotides, 0.25 µM universal M13(-21) (TGTAAAACGACGGCCAGT) primer (Schuelke, 2000), marked with fluorescent molecule FAM, VIC, PET or NED (Applied Biosystems), 0.25 U *Taq* polymerase (Promega, Manheim, Germany) and 20 ng of olive DNA. Amplifications were performed following the protocol of Bandelj et al. (2004) in a Thermal Cycler 2720 (Applied Biosystems), using the following cycling conditions: initial denaturation at 94 °C for 5 min followed by 5 cycles of 45 s at 94 °C, 30 s at 57 °C, 30 s at 72 °C (the annealing temperature was lowered for 1 °C with each cycle), followed by 25 cycles of 30 s at 94 °C, 30 s at 52 °C, 1.5 min at 72 °C and final extension step of 8 min at 72 °C. The resulting PCR products were separated on an ABI Prism 3130 DNA Genetic Analyzer (Applied Biosystems). Output data were analyzed using GeneMapper 4.1 software (Applied Biosystems).

2.4 DATA ANALYSIS

To determine the significant differences between examined olive trees, 20 morphological parameters were analyzed, and the ANOVA LSD test was used, with a threshold of $p < 0.05$, using the software Statistix 7.0 (General AOV, Florida, USA). The data were standardized and Principal Component Analysis (PCA) was performed and the scatter plot of the principal component according to variables and individuals was performed. Hierarchical cluster analysis was carried out using the unweighted pair group method using arithmetic average (UPGMA) and the dendrogram was created using the squared Euclidean distance as the similarity coefficient. The analyses were performed using the statistical software XLSTAT (Version 2015.5.01.22537).

Allele frequencies (p_i), number of effective alleles (n_e), observed (Ho) and expected (He) heterozygosity, fixation index (*F*) were analyzed by using GenAlEx 6.4 (Peakall & Smouse, 2006). Presence of null alleles (*r*) was calculated in IDENTITY 1.0 (Wagner & Sefc, 1999), Polymorphism Information Content (PIC) in CERVUS 3.0.3 (Kalinowski et al., 2007) and genotype specific alleles in MICROSAT 1.5 (Minch, 1997). Microsatellite polymorphisms were scored for the presence (1) or absence (0) of amplified bands and were used for estimation of the

Table 1: Analysis of 20 morphological characteristics evaluated in this study in 23 'Žutica' trees

Morphological characters	Maximum	Minimum	Average	CV (%) [a]	LSD [b] *p*-value
Leaf length – LL (cm)	6.40	4.86	5.81	8.020	0.0710ns
Leaf width – LW (cm)	1.39	1.13	1.25	8.399	0.0009**
Leaf shape index – LI (LL/LW)	5.16	4.26	4.63	11.758	0.2187ns
Internodes length – INT (cm)	1.75	1.12	1.52	18.118	0.0016**
Inflorescence length – IL (cm)	3.06	1.57	2.51	16.972	0.0000**
Number of flowers – NF	21.05	8.63	13.68	19.226	0.0000**
Number of aborted flowers – NAF	8.09	1.47	2.92	67.061	0.0064**
Percent of aborted flowers – PAF (%)	38.45	10.29	20.98	58.364	0.1717ns
Inflorescence density – ID (NF/IL)	6.94	4.81	5.45	13.637	0.0181*
Fruit length – FL (cm)	2.35	1.78	2.12	7.823	0.0026**
Fruit width – FW (cm)	1.72	1.40	1.57	7.339	0.0067**
Fruit shape index – FI (FL/FW)	1.53	1.23	1.33	6.726	0.0011**
Fruit mass – FM (g)	4.28	2.02	3.11	18.972	0.0000**
Pulp mass – PM (g)	3.72	1.76	2.71	19.873	0.0000**
Pulp percentage – PP (%)	89.33	84.39	87.08	2.430	0.0393*
Endocarp length – EL (cm)	1.55	1.16	1.38	8.631	0.0048**
Endocarp width – EW (cm)	0.81	0.63	0.72	8.012	0.0007**
Endocarp shape index – EI (EI/EW)	2.28	1.69	1.90	8.549	0.0000**
Endocarp mass – EM (g)	0.56	0.28	0.40	19.704	0.0438*
Pulp/endocarp ratio – P/E	8.37	5.41	6.83	17.679	0.0200*

[a] Coefficient of variance expressed in percentage.
[b] LSD Least Significant Difference test, *p*-values are **Significant at $p > 0.01$; *Significant at $p > 0.05$; ns–not significant ($p < 0.05$).

Dice similarity coefficients between clones. This genetic distance matrix was used to construct a dendrogram by using the UPGMA method in NTSYS 2.0 (Rohlf, 1998) software. The cophenetic value matrix of the clustering was used to test for the goodness of fit of the clustering to the similarity matrix by the Mantel statistics in the COPH and MXCOMP modules of NTSYS 2.0 (Rohlf, 1998) software.

3 RESULTS AND DISCUSSION

3.1 MORPHOLOGICAL CHARACTERISTICS

In terms of 20 morphological characteristics, there is a great amount of variation among the 23 olive trees analyzed. The morphological traits (Table 1 and Fig. 2) showed that 'Žutica' had moderately long internodes (EU/COI., 1997). According to the evaluation of Barranco et al. (2000) 'Žutica' had a leaf of medium length and width and elliptic-lancelet shape, medium length inflorescence, with a small number of flowers in the inflorescence and the medium number and percentage of aborted flowers in an inflorescence. Based on the mean inflorescence density, 'Žutica' represents a variety with a medium length and loose inflorescence (Cimato et al., 2001; Bassi et al., 2003). According to the methodology of Barranco et al. (2000) along with the variety, various environmental and agronomic factors influence the phenomenon of malformations of the female apparatus. Regarding that, 'Žutica' belongs to a category of medium percent of pistil abortion (20-60 %). Based on the same methodology the form of fruit was oval and of endocarp elliptic. The mean mass of the fruit and endocarp (3.11 and 0.40 g respectively) resulted in a favorable pulp mass (2.71 g), percentage (87.08 %) and the ratio of the pulp/endocarp (6.83). The favorable pulp/endocarp ratio upward of 6 (Barone et al., 1993) had 78.2 % of 'Žutica' trees.

All 20 morphological characteristics were included in the analysis of principal components (PCA) of morphological variability. The first four main components make up 78.7 % of the cumulative total variance, while six components explain 91.3 % of the variation. The eigenvalue of the components constitutes 30.2 %, 23.5 %, 13.1 %, 11.7 %, 6.7 % and 5.7 % of the total variance among the average values of the morphological characteristics of the studied 'Žutica' trees (Table 2 and Fig. 3). The first two or three components provide good data summation and separation of traits that mostly affect the clustering of the examined trees. The clustering was influenced by the properties of PC1 (FL, FW, FM, EL, EW, EM and PM), PC2 (FI and FE) and PC3 (LI, INT, PP and P/E), while some characteristics of the leaf (LL and LW) and all inflorescence traits were less important in grouping the trees into clusters belonging to the PC4, PC5 and PC6 component. Affiliation of fruit and endocarp characteristics (length, width and mass) in PC1 has been reported by several authors. In various olive varieties in Montenegro (Lazović & Adakalić, 2012b), Italy (Cantini et al., 1999), Argentina (Trentacoste & Puertas, 2011) and the Croatian variety 'Oblica' (Strikić et al., 2009), characteristics of fruit and endocarp mostly contribute to the grouping of analyzed individuals into clusters.

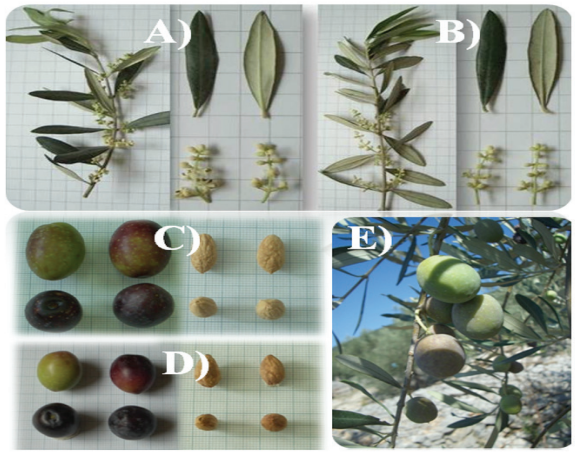

Figure 2: Vegetative and generative organs of some 'Žutica' trees: A) olive inflorescences with the lowest average inflorescence density (ID) determined in VALL (located nearby Ulcinj); B) olive twig with the longest internodes (INT) determined in PETR (Petrovac); C) fruit and endocarp of 'Žutica' tree coded VAL2 (nearby Ulcinj), which has the greatest values of fruit and endocarp mass (FM and EM); D) fruit and endocarp of DAB1 (nearby Bar), with the least values of fruit and endocarp mass; E) twig of LUSM (Lustica) with a few fruits.

Table 2: Total variance, cumulative variance and eigenvalues of the first six main components (PC) for 20 characteristics in 23 'Žutica' trees

Morphological characters	PC1	PC2	PC3	PC4	PC5	PC6
Leaf length – LL (cm)	-0.170	0.213	0.258	0.026	**0.892**	-0.085
Leaf width – LW (cm)	-0.095	-0.180	-0.453	0.068	**0.797**	0.171
Leaf shape index – LI (LL/LW)	-0.091	0.392	**0.730**	-0.041	0.077	-0.254
Internodes length – INT (cm)	0.180	0.061	**-0.627**	-0.001	0.401	0.538
Inflorescence length – IL (cm)	0.284	0.180	0.084	-0.141	0.011	**0.909**
Number of flowers – NF	0.217	0.079	0.092	0.442	-0.007	**0.819**
Number of aborted flowers – NAF	0.063	0.137	0.173	**0.910**	0.085	0.248
Percent of aborted flowers – PAF (%)	-0.039	0.176	0.194	**0.889**	0.066	-0.094
Inflorescence density – ID (NF/IL)	-0.068	-0.128	0.043	**0.867**	-0.073	-0.040
Fruit length – FL (cm)	**0.855**	-0.473	-0.041	0.015	0.037	0.068
Fruit width – FW (cm)	**0.866**	0.252	0.201	0.145	0.075	0.148
Fruit shape index – FI (FL/FW)	0.074	**-0.878**	-0.279	-0.122	-0.007	-0.093
Fruit mass – FM (g)	**0.881**	-0.033	0.341	0.043	-0.108	0.254
Pulp mass – PM (g)	**0.853**	-0.024	0.406	0.069	-0.104	0.253
Pulp percentage – PP (%)	0.208	0.114	**0.890**	0.238	0.026	0.193
Endocarp length – EL (cm)	**0.684**	-0.632	-0.117	-0.117	-0.140	-0.118
Endocarp width – EW (cm)	**0.843**	0.412	-0.071	-0.039	-0.078	0.035
Endocarp shape index – EI (EI/EW)	-0.117	**-0.941**	-0.067	-0.056	-0.050	-0.148
Endocarp mass – EM (g)	**0.862**	-0.095	-0.295	-0.179	-0.148	0.187
Pulp/endocarp ratio – P/E	0.184	0.135	**0.894**	0.266	0.009	0.162
Eigenvalue	6.049	4.711	2.637	2.355	1.356	1.155
Total variance (%)	30.245	23.554	13.187	11.774	6.780	5.777
Cumulative variance (%)	**30.245**	**53.800**	**66.987**	**78.761**	**85.541**	**91.318**

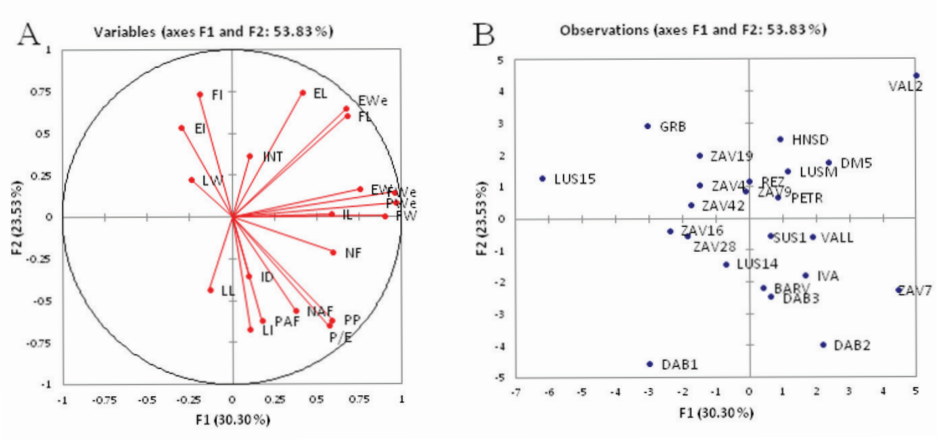

Figure 3: A: The scatter plot of the first two principal components (characteristics labels correspond to those in section 2.2 'Morphological characterization'); B: The scatter plot of 23 'Žutica' trees according to the plan generated by 1-2 axes of PCA (tree codes correspond to those in section 2.1 'Plant material').

Based on morphological characteristics, the trees were grouped into two clusters (Fig. 4) and five independent individuals (DAB1, LUS15, GRB, ZAV7 and VAL2). Very good matching of the morphological traits in the grouping of 'Žutica' trees in the clusters by using the squared Euclidean distance was confirmed by the high value of the cophenetic coefficient (r_{morph} = 0.91985). The clustering of 'Žutica' trees according to the fruit size was determined, and a partial clustering according to the geographical distribution. Trees belonging to the second cluster (DAB2, BARV, LUS14, DAB3, IVA, SUS1, VALL, DM5, HNSD and LUSM) had higher average values of fruit and pulp mass (3.35 and 2.95 g respectively), pulp percentage (88.20 %), and pulp/endocarp ratio (7.50) versus the first cluster. The great importance of fruit characteristics is also shown by the analysis of the principal components. Grouping of the trees according to the size of the fruit (Rotondi et al., 2003) indicates that the selection of clones in the past appears to be carried out according to this feature. The first cluster consists of eight trees (ZAV4, ZAV42, ZAV28, ZAV9, ZAV19, ZAV16, REZ and PETR) grown in the Bar subarea, and the second cluster includes ten trees grown in both olive growing areas, in Bar and Boka Kotorska Bay subareas. Contrary to our results, in the study on intra-varietal variability of Iranian varieties 'Zard' and 'Rowghani' (Hosseini-Mazinani et al., 2004) and Croatian 'Oblica' from several growing areas (Strikić et al., 2009), the authors did not find the grouping of individuals surveyed by areas of cultivation.

According to specific morphological characteristics (min. or max. values), five trees were separated from all 'Žutica' trees evaluated. Thus, DAB1 is characterized by the lowest values of some fruit and endocarp traits (FL, EL, EW and EM) and LUS15 by the lowest values of the internodes (INT), inflorescence (IL and NF) and fruit and endocarp (FW, FM, PM, PP and P/E) characteristics. GRB differs from other trees at the highest values of fruit and endocarp form (FI and EI). ZAV7 had the longest internodes (INT), the highest values of almost all inflorescence traits (NF, ID, NAF and PAF) and one fruit characteristics (FW), while VAL2 had the longest inflorescence (IL), the highest values of fruit and endocarp length and mass (FL, FM, EL, EM) and pulp mass (PM).

Very significant differences in morphological characteristics, especially in fruit characteristics, found in 'Žutica' trees indicate the need to form collections of these trees and study their characteristics under the same agro-ecological conditions. In such conditions, it would be of great importance to include these trees in further observation and study of other differences, such as productivity, olive oil quality, resistance, etc.

3.2. MICROSATELLITES ANALYSIS

With nine pairs of locus specific microsatellite primers, a total of 31 alleles (22 polymorphic and 9 monomorphic) were found in the 23 trees of 'Žutica' analyzed. Among the monomorphic alleles, allele 132 bp at the UDO99-19 locus was present in all 'Žutica' samples therefore this locus was excluded from further analysis (Table 3). The average number of alleles per each locus was 3.75, ranging from 2 at loci DCA14 and EMO3 to 7 at loci DCA9 and DCA11. These values are lower than the number of alleles detected by Lopes et al. (2004) and Caruso et al. (2014), but slightly higher than those detected by Muzzalupo et al. (2009) and Lazović et al. (2018a). The information value of the locus also depends on the frequency

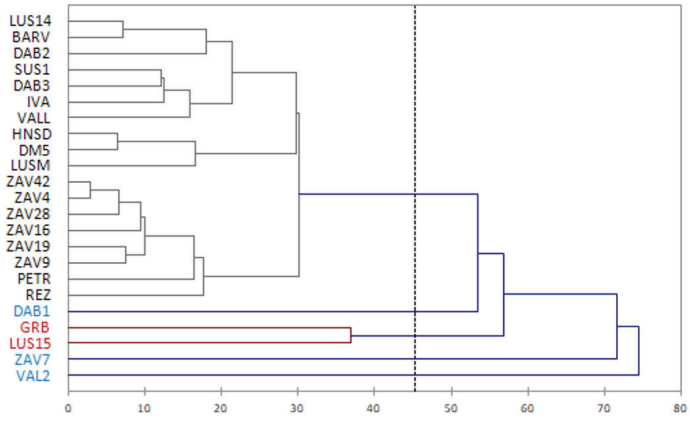

Figure 4: UPGMA dendrogram of 23 'Žutica' trees derived from 20 morphological traits. The dendrogram shows two clusters (black line and code colour) and five independent individuals (blue line and code colour) that include a pair of individuals with more or less similar morphological characteristics (red line and code colour).

of alleles, therefore the number of effective alleles was calculated, which differed from the actual number of alleles, and on average it was 2.21 per locus. The average observed heterozygosity (H_o) was 1,000, therefore no homozygous genotypes were observed. The average expected heterozygosity (H_e) was 0.542, ranging from 0.500 (DCA14 and EMO3) to 0.635 (DCA9). Similarly expected heterozygosity (0.57 and 0.41 respectively) has also been reported by Noormohammadi et al. (2009) and Rony et al. (2009) and higher values (0.67) by Lopes et al. (2004) in the studies of inter- and intra-cultivar variations. The tendency of higher observed (H_o) than expected (H_e) heterozygosity in all loci, is reflected in the negative fixation index (*F*) values, similar to the finding of Baldoni et al. (2009). Negative values of fixation index (average -0.860) resulted in the absence of null alleles (*r*) at all loci and it was on average -0.299. Contrary to our results, Lopes et al. (2004) recorded higher values of the expected than the observed heterozygosity for the locus DCA11 and the positive values of the probability of null alleles. Polymorphic information content (PIC), as a significant parameter of genetic diversity, refers to the informativeness of a particular locus. PIC values are derived from the frequency of alleles and are used as a benchmark for the use of locus for genetic mapping. In this study of genetically close plant material, the loci DCA9 and DCA11 could be included among the informative (PIC > 0.5). The PIC values varied from 0.375 (DCA14 and EMO3) to 0.567 (DCA9), with an average value of 0.435. In the study of clonal variation within the eight Sicilian cultivars, authors reported an average PIC value of 0.59 Caruso et al. (2014), and 0.51 within three Iranian cultivars (Noormohammadi et al., 2009).

Based on the presence (1) or absence (0) of a particular allele, a binary matrix was constructed, and Dice's coefficients of similarity were calculated. The very good similarity of the original data with the clustering of samples using the microsatellite markers confirms the calculation of the correlation coefficient between the cophenetic and the Dice's coefficients values in the amount of r_{SSR} = 0.98768. A dendrogram was constructed (Fig. 5) in which the 'Žutica' trees were grouped into 13 identical clones and 10 others with more or less genetic similarities. The greatest closeness (0.941) with this group had ZAV9, DAB3, IVA, DM5, and two pairs of DAB2 - VALL and SUS1 - REZ which had the same microsatellite profiles at this loci and ZAV19 on a higher genetic distance (0.882). The VAL2 coded olive tree has the lowest genetic similarity (0.619) to 'Žutica'. For this tree, Lazović et al. (2018a) suggest that the most likely is a putative seedling of 'Žutica'. This genetically most distant sample was different from the group of identical microsatellite profiles on 6 loci (8 alleles). Eight trees (ZAV9, DAB2, DAB3, DM5, SUS1, VALL, REZ and IVA) differ in one allele, and one tree (ZAV19) differs in two alleles from the group of identical microsatellite profiles. In the study of intra-varietal variability of Italian olive varieties, Cipriani et al. (2002) found differences in the lengths of alleles in one or two loci that were explained by somatic mutations, while Lopes et al. (2004) found the differences in 'Galega' represented with 1-2 to 10 different alleles. Simple sequence repeats (SSR) are reported as a very suitable tool for intra-varietal analysis (Caruso et al., 2014; Lopes et al., 2004). Based on the microsatellite analysis, no grouping according to the size of the fruit or geographic origin was observed, referring to a possible exchange of planting material among local producers in these areas during the long cultivation of olives in Montenegro.

3.3 COMPARISON BETWEEN MORPHOLOGICAL AND MOLECULAR MARKERS

Mantel's matrix correspondence test after 1.000 per-

Table 3: Genetic variability parameters of microsatellite loci in 23 'Žutica' trees: observed (Ho) and expected (He) heterozygosity, number of alleles (n), number of effective alleles (ne), polymorphism information content (PIC), fixation index (*F*) and presence of null alleles (*r*).

Locus	H_o	H_e	n	n_e	PIC	*F*	r
DCA3	1.000	0.521	3	2.09	0.406	-0.920	-0.315
DCA9	1.000	0.635	7	2.74	0.567	-0.574	-0.223
DCA11	1.000	0.614	7	2.59	0.541	-0.628	-0.239
DCA14	1.000	0.500	2	2.00	0.375	-1.000	-0.333
DCA16	1.000	0.521	3	2.09	0.406	-0.920	-0.315
EMO3	1.000	0.500	2	2.00	0.375	-1.000	-0.333
EMO90	1.000	0.521	3	2.09	0.406	-0.920	-0.315
GAPU101	1.000	0.521	3	2.09	0.406	-0.920	-0.315
Mean	1.000	0.542	3.75	2.21	0.435	-0.860	-0.299

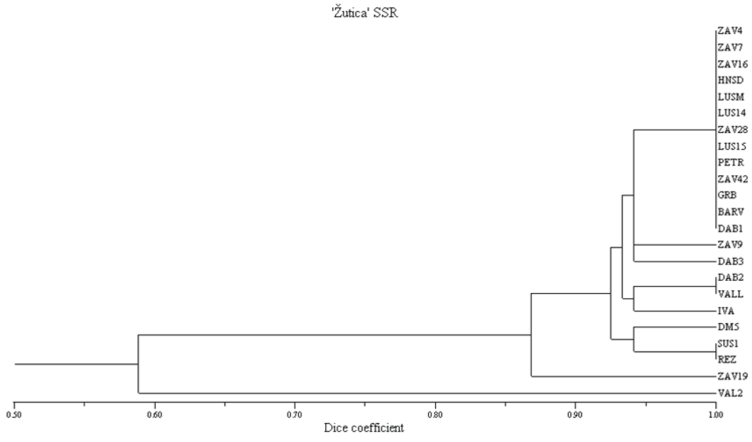

Figure 5: UPGMA dendrogram based on the results of microsatellite markers constructed according to the Dice coefficient of similarity in 23 'Žutica' trees

mutations was used to compare the distance matrices based on morphological traits and microsatellite markers. The correlation coefficient between the matrix based on morphological traits and microsatellite markers was relatively low ($r_{morph/SSR}$ = 0.17743). Such results are in favour of the fact that the morphological traits are largely under the influence of different environmental factors concerning the genetic basis of each individual, and for the detailed characterization of olive varieties, it is necessary to apply DNA analysis of polymorphism.

In our analysis, the sample VAL2 differs the most from others in terms of morphological and molecular markers. In addition to this tree, differentiation with microsatellite markers has been established in another nine trees. Six of them (DAB3, DAB2, VALL, IVA, DM5 and SUS1) belong to another cluster based on morphological traits, separated by higher average values of characteristics of fruit and pulp. The other three trees that are different from microsatellite analysis had some differences in the characteristics of leaves. Thus, ZAV9 had the smallest leaf shape index (LI), REZ maximum leaf width (LW) and ZAV19 without extreme values.

Each marker system measures different aspects of variability and this can explain the lack of consistency in genetic diversity and studies within the variety. The detected DNA variation, which is neutral, was often not correlated to the phenotypic and agronomical variation of olive varieties (Rao et al., 2009). However, despite the powerfulness of microsatellite markers to detect genetic variability and genetic relationships, they should not be seen as a substitute for traditional morpho-agronomic descriptors (Cantini et al., 2008).

4 CONCLUSIONS

In this paper, we have found morphological differences among investigated trees of the 'Žutica' olive variety, and microsatellite analysis allowed us the differentiation of 9 different profiles. In assessing the genetic variability of 'Žutica' trees, low morphological efficacy compared to molecular markers was recorded, which is confirmed by the low values of the cophenetic coefficients. Based on the established polymorphism of morphological and molecular markers, we can conclude that the main microsatellite profile was defined, but some differences point to a certain degree of genetic variability. That needs further examination with a larger number of individuals in situ and ex situ (in the olive collection) under the equal ecological/environmental factors. These results show great genetic diversity within 'Žutica' samples that should be preserved and further evaluated for the identification of distinct properties of defined genotype. This is a springboard for phenotyping for future research on potential resistance to diseases, pests, and abiotic stresses, olive oil quality improvement, and for breeding efforts to introduce clones with the desired traits Accordingly, the results show that old olive varieties that are analyzed by different marker systems enable a more complete understanding of the diversity within the olive varieties and the ways they can best be used for the selection of new genotypes, olive breeding and conservation strategies to improve olive growing in Montenegro.

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Growth assessment of juvenile oil palm (*Elaeis guineensis* Jacq.) intercropped with fruit vegetables in a rainforest zone of Nigeria

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Growth assessment of juvenile oil palm (*Elaeis guineensis* Jacq.) intercropped with fruit vegetables in a rainforest zone of Nigeria

Abstract: Improper intercropping of *Elaeis guineensis* with other crops has impaired the growth and development of the oil palm due to competition for environmental resources. The study was conducted to investigate the impact of intercropping on the growth of juvenile oil palm for 2 years. The research commenced during the rainy season of 2016 at an established juvenile oil palm plantation in Ala, Akure-North Local Government of Ondo State. Four fruit vegetables were intercropped separately within the alley of the plantation at 1, 2 or 3 m away from the oil palms in a randomized complete block design. Growth parameters of the juvenile oils were assessed. Results revealed that at 16 weeks after intercropping (WAI), the intercropped oil palm recorded better growth performance with higher canopy spread, number of frond, number of leaflets and trunk height (218.20, 37.00, 87.48 and 38.17 cm) respectively, than the sole oil palms (214.67, 32.83, 72.89 and 31.67 cm) respectively. There were no significant difference in all the growth parameters examined except canopy height ($p < 0.05$). Juvenile oil palm cultivated in rainforest agroecological zone of Nigeria can be intercropped with fruit vegetables without any deleterious effect when intercropped at minimum of 1 m away from the oil palms.

Key words: immature oil palm; fruit vegetables; intercroping; growth and development; weed menagement

Ocena rasti mladih oljnih palm (*Elaeis guineensis* Jacq.) z medsadnjo plodovk na območju deževnega gozda Nigerije

Izvleček: Neprimerna sadnja drugih kulturnih rastlin v nasade oljne palme (*Elaeis guineensis* Jacq.) zavira njeno rast in razvoj zaradi tekmovanja za okoljske vire.V raziskavi je bil preučevan vpliv medsadnje plodovk na rast mladih oljnih palm v dvoletnem poskusu. Poskus se je začel v deževni dobi 2016 v nasadu mladih oljnih palm v kraju Ala, Akure-North Local Government of Ondo State, Nigerija. Štiri plodovke so bile posajene med mlade oljne palme v razdaljah 1, 2 ali 3 m od oljnih palm v popolnem naključnem bločnem poskusu, v katerem so bili izmerejeni rastni parametri mladih oljnih palm. Rezultati so pokazali, da so imele oljne palme 16 tednov po medsadnji plodovk boljšo rast glede na obseg krošnje, število listov in lističev ter višino stebla (218,20; 37,00; 87,48 in 38,17 cm) kot palme, ki so rastle same (214,67; 32,83; 72,89 in 31,67 cm). Pri večini rastnih parametrov ni bilo značilnih razlik razen pri višini krošnje ($p < 0,05$). Med mlade oljne palme gojene v agroekoloških razmerah deževnega gozda v Nigeriji lahko posadimo plodovke brez škodljivih učinkov na rast palm, če so te posajene najmanj 1 m od palm.

Ključne besede: mlade oljne palme; plodovke; medsadnja; rast in razvoj; upravljanje plevelov

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1 INTRODUCTION

Growing a number of other food crops in association with juvenile oil palm trees is a widespread practice in most oil palm growing areas in the country. Oil palm is a wide spaced perennial crop with a long juvenile period of 3-5 years depending on cultivars (Igene et al., 2015). The space in the inter- and intra-rows can be put to use to create income during the long juvenile period of the crop. The spacing habit and growth pattern (9 × 9 m triangular spacing) of oil palm plantation permit a variety of annual and perennial crops to be cultivated with and under its canopy during the young stage of the oil palm (NIFOR, 2008).The benefits of intercropping with oil palms in the field according to NIFOR (2008) are adding value to the oil palm when the food crops are harvested and sold, and particularly so in the early years when the oil palm has not started to yield fruit bunches. Ibeawuchi (2007) reported that intercropping suppresses weeds and gives yield advantage and stable yield overtime. He opined that when suitable crops are grown in proximity, it promotes positive interaction among them.

Secondly, the planting of the food crops between inter-rows of the oil palm facilitates field maintenance of the plantations, resulting in reduction in overall maintenance cost. Hence, the double cost of maintenance is avoided (Igene et al., 2015).

Oil palm can successfully be intercropped with food crops. Nuertey et al. (2000) indicated that it is profitable to intercrop oil palm with food crops especially for the first three to four years when the palms are not fruiting as compared to sole cropping. Farmers are able to get enough money from the intercrop to sustain their family and also to maintain the farm.

The relative advantage of intercropping oil palm with food crops, suggests that intercropping systems may be most suitable for small-scale producers with limited resources to purchase large land to develop oil palm and food crops separately (Okyere et al., 2014).Intercropping is also important because it helps smallholder (SH) farmers who face labour constraints as they have the potential to reduce weeding frequency (Mashingaidze et al., 2000).

Nwawe et al. (2014) studied the economic analysis of economic analysis of oil palm and food crop enterprises in Edo and Delta State Nigeria; they stated that sustainable and stable mixed oil palm food crop enterprise in Nigeria requires that farmers are guided by for the choice of oil palm food crop combination.

Udosen et al. (2006) investigated the performance of oil palm under food crop combinations in four-year old oil palm in derived savannah zone of Nigeria. They asserted that there is benefit in appropriate cropping mixture with immature oil palm.

However, intercropping of juvenile oil palm with these food crops result in competition between the juvenile oil palm and the food crops for resources such as water, space and nutrients. This competition could result in adverse effect if there are limited resources in the environment. It is therefore important to assess the implication of the intercrop on the growth of the juvenile oil palm during the long juvenile phase of the crop. Therefore, the objective of this study is to evaluate the effects of intercropping on the growth of juvenile oil palm in rainforest agroecological zone of Nigeria. Hence, farmers can be educated on the proper way to carryout intercropping with juvenile oil palm in a way that would not cause deleterious effect on their growth.

2 MATERIALS AND METHODS

2.1 DESCRIPTION OF STUDY AREA

The field experiment was conducted within an established juvenile oil palm plantation located at Ala in Akure-North Local Government Area of Ondo State. The oil palm plantation is located at a coordinate range of Latitude 7.093^{0}N, Longitude 5.354^{0}E (N7^{0}5' 35.59837" E5^{0}21' 15.47179"), and Latitude 7.09302 Longitude 5.35422 (N7^{0}5' 34.8857" E5^{0}21' 15.19177") in the tropical rain forest region of Nigeria. It has two distinct seasons namely: dry and rainy seasons. Rainy season is between April and November and dry season is between November and March. Annual rainfall varies from 1150 to 2550 mm. Temperature is moderately high year round and range between 22 ^{0}C and 34 ^{0}C with daily average of 30 ^{0}C (Ogunrayi et al., 2016). Top soil (0-15 cm) was collected for soil test before and after intercropping to establish the physico-chemical properties of the soil. These properties include: soil pH, total carbon, organic matter, electrical conductivity, exchangeable cations (Na^+, Mg^{2+}, K^+, Ca^{2+} and titratable acidity or acid value), nitrate content, phosphorus content (Mussa et al, 2009), particle size (clay, sand and silt) according to the method of Kettler et al.(2001), and soil type (Olabisi et al., 2009). Electrical conductivity, total carbon and total organic matter were determined according to the methods of Wagh et al. 2013. Exchangeable cations, titratable acidity, nitrate and phosphate contents were determined according to the methods of Reeuwijk (2002), Czinkota et al. (2002), Ahmed (2009) and Mussa et al. (2009), respectively. Soil particle size was determined according to the method of Kettler et al. (2001).

The juvenile oil palm trees in the plantation were planted at a plant spacing of 6m × 6m.Four fruit vegetables: (i) tomato (accessions NGB 01665 and NG/AA/

SEP/09/053); (ii) pepper (NGB 01312 and NGB 01641); (iii) okra (NGB 01184); and (iv) eggplant (NGB 01737) were obtained at the National Centre for Genetic Resources and Biotechnology (NACGRAB) research institute, Ibadan, Oyo State and intercropped within alley of juvenile oil palms (240 m^2).The different accessions of the fruit vegetables were intercropped at 1, 2 or 3 m away from the juvenile oil palm at spacing of 1 × 1 m within the alley, in a randomized complete block design with four replicates each. The blocks were represented by the replicates and the treatments (intercropping distances) were assigned once within each block of the fruit vegetables-juvenile oil palm intercrop plots. The control plot was without juvenile oil palm.

The field experiment was carried when the juvenile oil palms were 2 years old.

Measurements were taken from sole and intercropped juvenile oil palms every 4 weeks (4 weeks, 8 weeks and 16 weeks after intercropping).

2.2 DATA COLLECTION AND PROCESSING

Growth variables collected on the juvenile oil palm include:

Number of fronds: This was determined by counting the number of fronds on each juvenile oil palm tree.

Average length of fronds: This was obtained by measuring with a meter rule the length of the fronds and finding the average.

Average number of leaflets: This was obtained by counting the number of leaflets on fronds and finding the average.

Average canopy spread determination: Canopy spread of the immature oil palms was measured according to Spoke Method (Blozan, 2004) with the use of graduated coloured plank or wooden rod (tar rod). Ten measurements were taken from the midpoint of the trunk to the outer extremities of the crown. These were averaged to get the result of the average canopy or crown spread. Canopy spread measurements were taken every 4 weeks (4, 8, 12 weeks and until final harvesting).

Trunk height: This was measured with the use of graduated coloured plank (tar rod) (Blozan, 2004).

Canopy height: It was also measured with the use of graduated coloured plank (tar rod) (Blozan, 2004).

Statistical analysis: T-test was used to analyze data gathered from the study using Statistical Package for Social Sciences (SPSS: version 17.0). Graphs were plotted using Origin (version 7.0) software.

3 RESULTS

The result on physical and chemical properties of the soil is shown in Table 1a-b.

The results on the effects of intercropping fruit vegetables with juvenile oil palm during second year of plantation establishment are given in the Figure 1-6.

Table 1a: Physical and chemical properties of the experimental plot before planting and after harvesting

Plot	Properties	pH value	Total organic carbon (%)	Organic matter (%)	Nitrate ion (mg/L)	Available phosphorus (%)	Particle size distribution		
							Sand (%)	Silt (%)	Clay (%)
Pre-planting	NGB 01665	6.02	1.47	2.542	1.44	1.84	47.60	12.48	39.92
	NG/AA/SEP/09/053	5.10	1.40	2.421	1.27	1.65	58.45	13.64	27.91
	NGB 01312	5.86	1.46	2.524	1.41	1.78	47.36	14.21	38.43
	NGB 01641	5.99	1.41	2.438	1.40	1.70	59.32	13.84	26.84
	NGB 01184	6.08	1.35	2.334	1.23	1.62	46.76	12.78	40.46
	NGB 01737	6.09	1.40	2.421	1.28	1.68	48.42	13.76	37.82
	Control (sole)	6.36	1.58	2.732	1.65	1.88	44.68	14.35	40.97
Post-harvest	NGB 01665	6.38	1.49	2.576	1.45	1.96	49.62	13.88	36.50
	NG/AA/SEP/09/053	6.23	1.36	2.351	1.25	1.66	50.84	13.90	35.26
	NGB 01312	2.04	2.64	4.565	2.78	2.98	48.66	13.69	37.65
	NGB 01641	4.86	1.34	2.317	1.23	1.60	43.98	14.62	41.40
	NGB 01184	5.62	1.40	2.421	1.27	1.67	45.86	13.86	40.28
	NGB 01737	6.02	1.41	2.438	1.40	1.72	47.73	14.36	37.91
	Control (sole)	6.23	1.50	2.594	1.46	1.97	42.87	14.38	42.75

Table 1b: Physical and chemical properties of the experimental plot before planting and after harvesting

Plot	Properties	Exchangeable cations			
		Ca^{2+}(mg kg^{-1})	Mg^{2+} (mg kg^{-1})	Na^{+} (mg kg^{-1})	K^{+} (mg kg^{-1})
Pre-planting	NGB 01665	1.41	0.19	0.84	0.191
	NG/AA/SEP/09/053	1.39	0.19	0.82	0.188
	NGB 01312	1.40	0.18	0.83	0.190
	NGB 01641	1.40	0.18	0.82	0.192
	NGB 01184	1.39	0.20	0.80	0.186
	NGB 01737	1.37	0.17	0.80	0.185
	Control (sole)	1.53	0.22	0.87	0.194
Post-planting	NGB 01665	1.42	0.20	0.85	0.192
	NG/AA/SEP/09/053	1.35	0.16	0.78	0.188
	NGB 01312	1.58	0.26	0.89	0.198
	NGB 01641	1.33	0.14	0.76	0.185
	NGB 01184	1.38	0.18	0.81	0.187
	NGB 01737	1.39	0.19	0.82	0.186
	Control (sole)	1.43	0.20	0.85	0.191

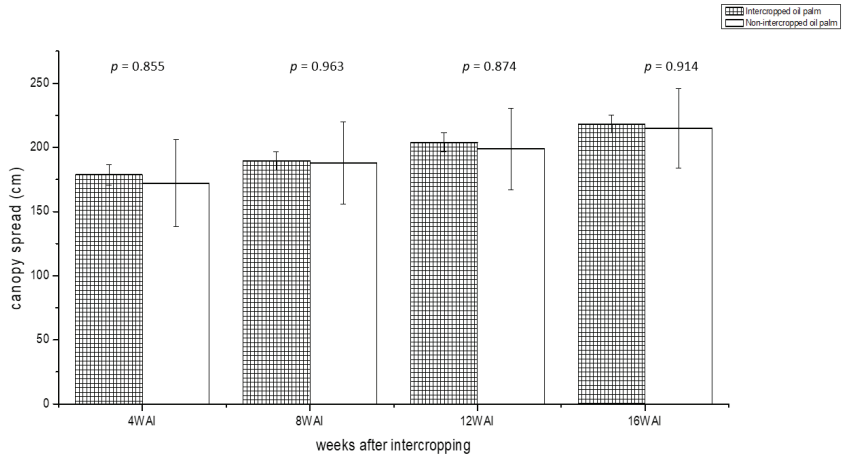

Figure 1: Canopy Spread of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n = 6) (2 years old)

Bars with the same letter are not significantly different at 95% degree of freedom ($p < 0.05$).

There was no significant difference between the canopy spread of the intercropped and sole juvenile oil palm at 4 weeks after intercropping through 16 weeks after intercropping, as none of the *p*-values are greater or equal to 0.05 as shown in Fig.1.

The number of frond of intercropped juvenile oil palm was higher and statistically significant from that of the sole juvenile oil palm at 4, 8 and 12 weeks after intercropping. However, the mean numbers of frond were not statistically different at 16 weeks after intercropping (Fig. 2).

Length of frond of intercropped and sole juvenile oil palms were not statistically different at 4 weeks after intercropping (WIA) through 16 WAI, with intercropped juvenile oil palm recording higher values (Fig. 3).

Fig. 4 shows that there was no statistical significant between the number of leaflets of intercropped and sole juvenile oil palm. However, the intercropped juvenile oil palm recorded higher mean number of leaflets at 4 WAI through 16 WAI.

The intercropped juvenile oil palm recording higher trunk height values; however, no significant difference was recorded at 4 WAI through 16 WAI as shown in Fig. 5.

The canopy heights of intercropped juvenile oil palms were higher than the sole juvenile oil palms; how-

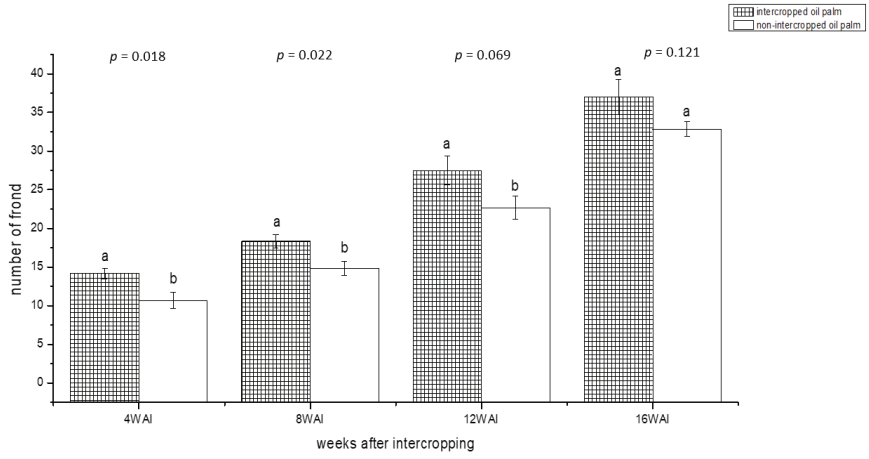

Figure 2: Number of frond of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n = 6) (2 years old)

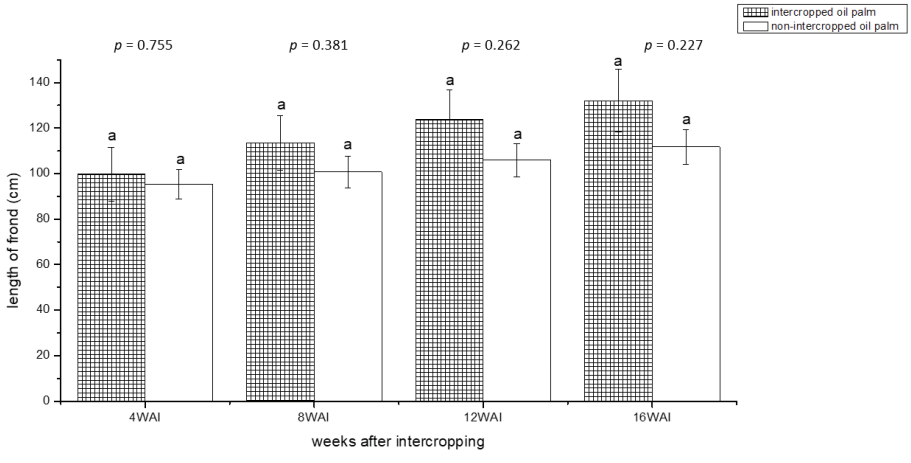

Figure 3: Length of frond of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n = 6) (2 years old)

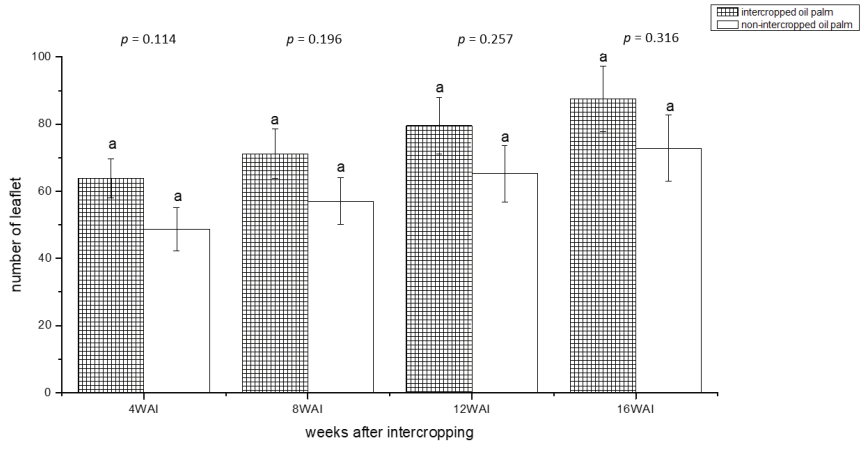

Figure 4: Number of leaflet of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n = 6) (2 years old)

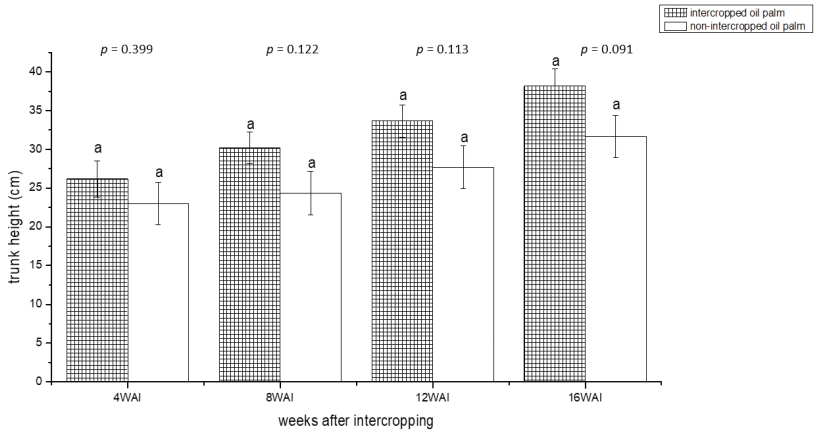

Figure 5: Trunk height of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n= 6)

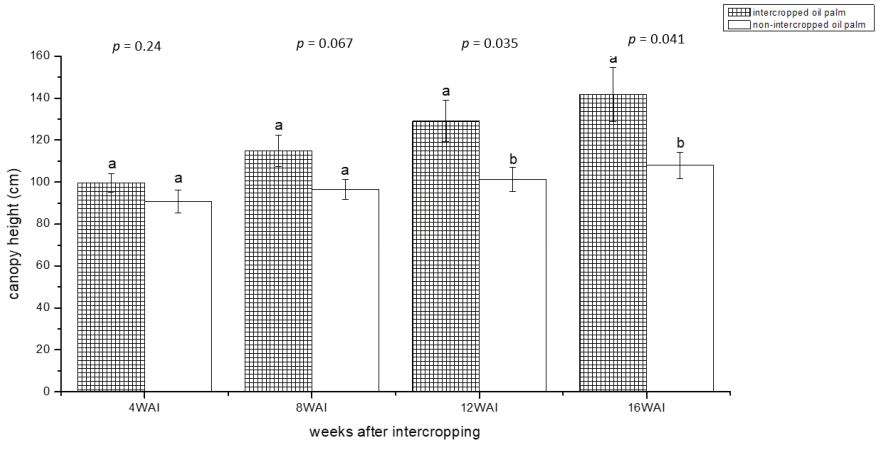

Figure 6: Canopy height of intercropped and sole juvenile oil palms after 4, 8, 12 and 16 weeks after intercropping (n= 6) (2 years old)

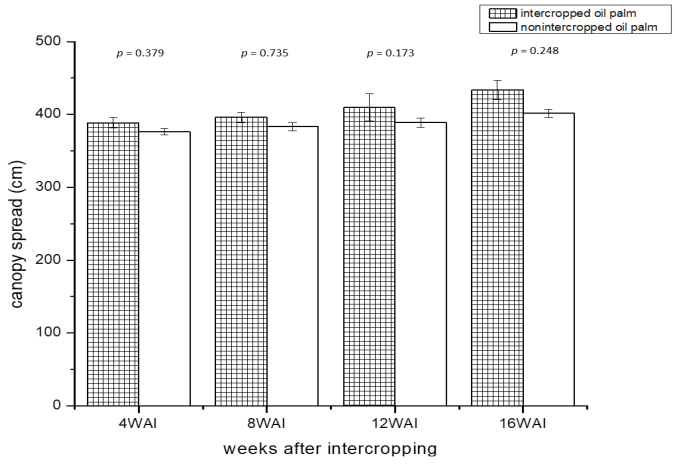

Figure 7: Effects of intercropping on canopy spread of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n = 6) (2 years old)

ever, they were not statistically significant at 4 and 8 WAI. At 12 and 16 WAI, there were significant differences (Fig. 6).

The results on the effects of intercropping fruit vegetables on the growth of juvenile oil palm during third year of plantation establishment are given in the Figure 7-12.

Bars with $p < 0.05$ are statistically significant at 95 % degree of confidence.

The intercropped oil palms recorded higher canopy spread at 4, 8, 12 and 16 weeks after intercropping (WAI). However there was no statistical significance between the canopy spread of intercropped and the sole juvenile oil palm from 4 WAI to 16 WAI as shown in Fig. 7.

At 4 WAI, the intercropped oil palm recorded higher number of fronds. The sole juvenile oil palms recorded higher number of fronds at 8 and 12 WAI. At 16WAI, the intercropped sole juvenile oil palm recorded the same mean number of fronds. The mean values of number of fronds for the intercropped and the non-intercropped juvenile oil palms were not statistically significant at 4 WAI through 16 WAI (Fig. 8).

At 4, 8 and 12 WAI, the sole juvenile oil palms recorded higher length of fronds and were statistically higher than the intercropped oil palms. At 16 WAI, the intercropped oil palms recorded higher length of fronds; however it was not statistically significant (Fig. 9).

At 4, 8, 12 and 16 WAI, the intercropped juvenile oil palm recorded higher number of leaflets. However, they were statistically significant at 4 and 8 WAI as shown in Fig. 10.

The intercropped juvenile oil palms recorded higher trunk height at 4, 8, 12 and 16 WAI; however they were

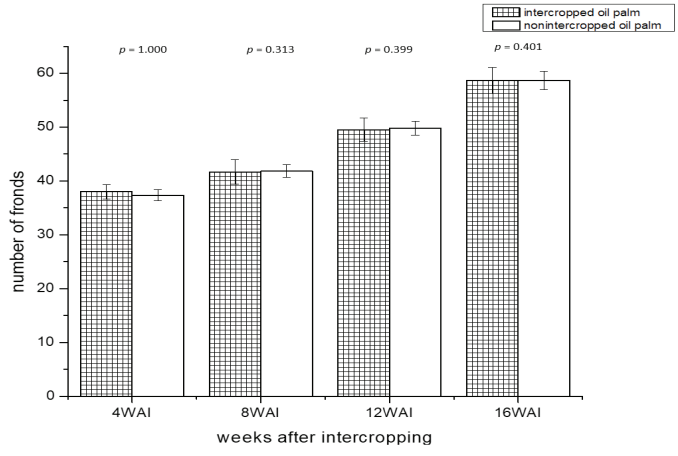

Figure 8: Effects of intercropping on number of fronds of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n = 6) (2 years old)

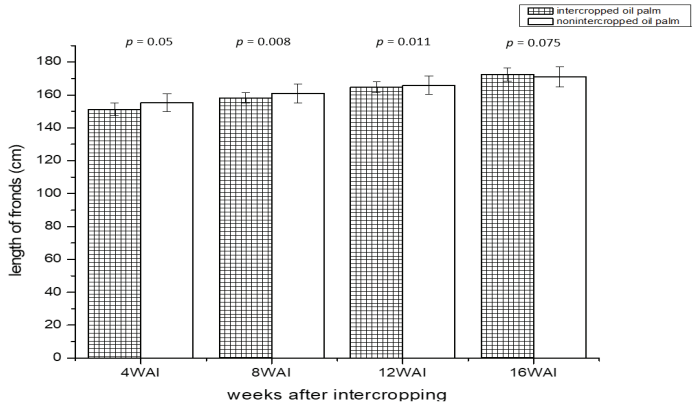

Figure 9: Effects of intercropping on length of fronds of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n = 6) (2 years old)

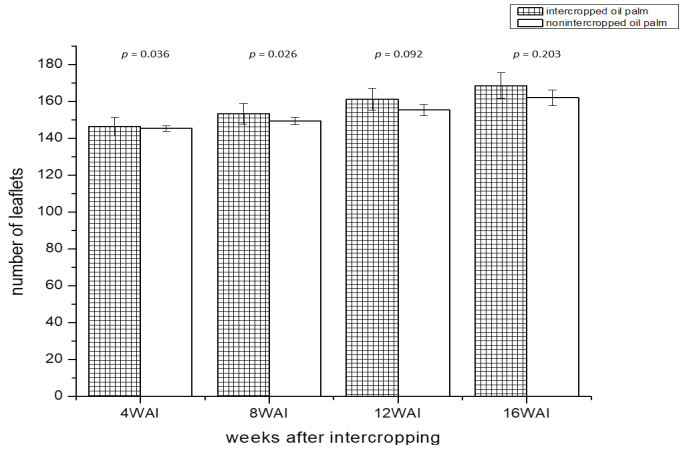

Figure 10: Effects of intercropping on number of leaflets of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n = 6)(2 years old)

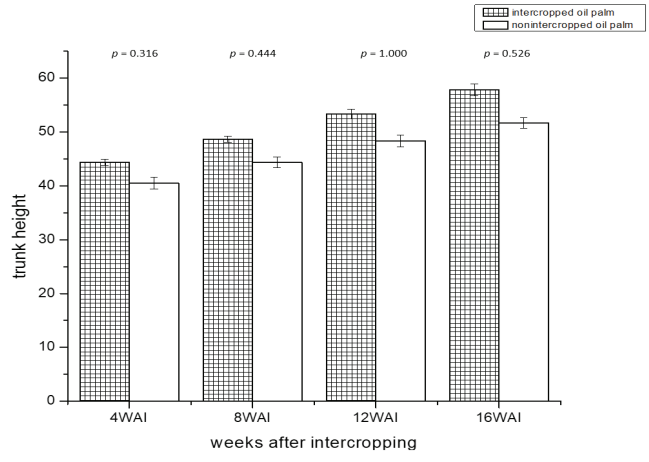

Figure 11: Effects of intercropping on trunk height of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n = 6) (2 years old)

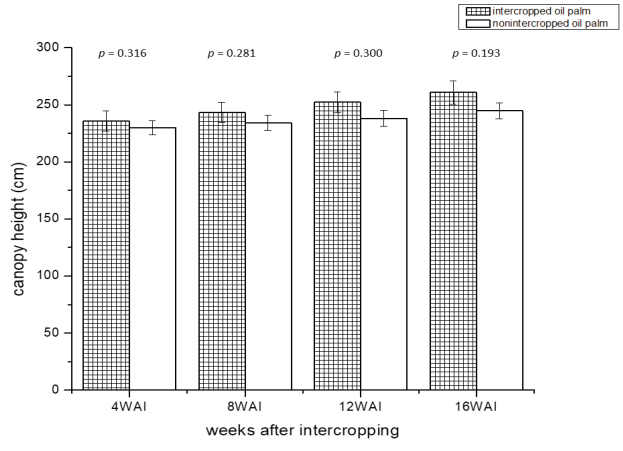

Figure 12: Effects of intercropping on canopy height of intercropped and sole juvenile oil palms at 4, 8, 12 and 16 weeks after intercropping (n = 6) (2 years old)

not statistically significant at 4 WAI through 16 WAI (Fig. 11).

Fig. 12 shows that intercropped juvenile oil palms recorded higher canopy spread at 4 WAI through 16 WAI; however, they were not statistically significant.

4 DISCUSSION

The results of the particle size indicated that all the experimental plots within the plantation were sandloamy and by rating of FAO, the soil is medium textured. This quality indicated that the soil is appropriate for oil palm cultivation and this is in harmony with earlier work by Ukaegbu et al. (2015).Following the rating of Chude et al.(2011), not any of the experimental plots is deficient in nitrate before and after intercropping. The nitrate concentration ranged between 1.23 mgl^{-1} and 2.78 mgl^{-1}. The high concentration of nitrate is also associated with the high organic matter recorded in the experimental plots. The increase in available P observed soil of the intercropped juvenile oil palm plots was also reported by earlier work of Erhabor and Filson (2008) that available P level increased throughout the experimental periods in intercropped young oil palm plot, and the soil was rated to be low to medium in P level. There was moderate changes in the Ca and Mg, and there were no significant change in soil pH except in pepper (NGB 01312)-oil palm intercrop plot. Changes in soil organic matter and total organic matter fluctuated but did not follow definite trend.

The intercropped juvenile oil palms recorded higher canopy spread number of fronds, length of fronds, number of leaflets, trunk length and canopy height. However, at the end of 16 weeks after intercropping, there was no statistical significant in the mean values of the parameter examined except canopy height. This observation indicates that intercropping juvenile oil palm with fruit vegetables does not have detrimental effect on the oil palm; rather it helped to improve the growth.

This better performance recorded in the intercropped juvenile oil palms may be attributed to more regular weeding carried out in the juvenile oil palm-vegetable intercrop plots. Furthermore, the initial minimum of 1 m space left fallow before planting the fruit vegetables reduced the competition between the juvenile oil palm and the fruit vegetables for nutrients in the soil and other growth resources. Similar result were obtained for the period of the second trial, there was no significant difference between the intercropped and sole juvenile oil palms latter at 16 weeks after intercropping. However, the sole juvenile oil palm recorded better performance in number and length of fronds than the intercropped ones probably because of no competition for resources by the vegetables intercropped.

Ogwuche et al. (2012) worked on the economies of intercropping natural rubber with arable crops as a panacea for poverty alleviation of rubber farmers. He reported that a higher annual increase in girth of rubber intercropped compared to the sole rubber plantation. He examined the influence of intercropping pattern on the girth of rubber plants and opined that at the end of every year during the intercrop, the girth of the rubber plants on both intercropped and non-intercropped plots increased; and was higher in the intercropped tree crops than the non-intercropped tree crops. This is in conformity with this study that intercropping of tree crop and arable crop such as vegetable crop would set in motion an increase in growth of the juvenile oil palm. They attributed this to an increase in organic matter content as a product of residues from the intercropped plants after harvest which promotes soil aeration and possible boost in soil nutrients required for the rubber growth and development. Decline in weed competition resulting from the intercrop may also be responsible.

More so, it could also be due to complementary of species interaction as opined by Esekhade (2003). He also indicated that intercropping tree crop with arable crops is profitable and can serve as a means of poverty alleviation among the rubber farmers if practiced. Similar report was also submitted by Giroh et al. (2010) who worked on efficiency and cost of production among gum arabic farmers; that intercropping of food crops and tree crops is profitable. This report is in agreement with findings of this study that intercropping of vegetable crop and juvenile oil palms was profitable and simultaneously improved growth of the tree crop.

According to Oladokun (1990), among many tree crops that could be intercropped with food crops, most farmers prefer oil palm.

Udosen et al. (2006) investigated the performance of oil palm and different food crops combinations in four-year sequential intercropping in a rain forest/derived savanna transition zone of Nigeria. They observed that the different crop combinations intercropped with oil palm did not have any significant negative effect on the oil palm growth, number of bunches and fresh fruit bunch yield over the period. This observation is in agreement with findings of this study that intercropped juvenile oil palm did not show negative impact arising from the intercrop; rather it performed even better than the non-intercropped juvenile oil palm. They attributed this observation to the ability of the juvenile oil palm to maximize the available resources in the environment and did not have to compete for sunlight with the food crop which consequently resulted in good performance.

According to Okyereet al. (2014) who studied the lingering effect of intercropping on the yield and output of oil palm, reported that intercropping oil palm and food crop has no significant undesirable effect on the growth, development and yielding of the oil palm. He opined that intercropping does not absorb excessive nutrients from the field that would affect the nutrient requirements of the oil palms. He further attributed these findings to decomposition of crops residues after harvesting. More so, he suggested that the regular weeding of intercropped field and its eventual decomposition of weeds might have had added advantage to the nutrient availability for the growth of the oil palm even though that was not significant. Higher canopy height, number and length of frond, number of leaflets, trunk and canopy heights recorded in the intercropped than in the non-intercropped are in agreement with their observations as there was improvement in growth of the intercropped juvenile oil palms than the non-intercropped juvenile oil palm even though it was not significant.

This is also in accordance with an earlier work by Nuertey et al. (2000) who studied the economies of intercropping annual crops in oil palm plantation for small scale farmers that it is profitable to intercrop oil palm with food crops especially for the first two to four years when the oil palms are not fruiting as compared to sole cropping.

Famaye et al. (2012) investigated the effects of intercropping of coffee with rice and plantain at early stage of field establishment in Nigeria. They observed high growth performance in the intercrops and concluded that there were no harmful effects of intercropping of coffee with rice and food crops at early stage of field establishment. This is in agreement with findings of this study as intercropping of vegetable crops with juvenile oil palm has not shown negative effects on the growth of the intercropped and non-intercropped juvenile oil palm.

This finding is also corroborated by earlier works on the beneficial effect of intercropping some food crops with coffee (Okelana, 1982; Famaye, 2000 and 2005), cocoa, oil palm and kola (Adenikinju, 1980; Ofoli and Lucas, 1988; Okpala-Jose and Lucas, 1989; and CTA, 1993).

Famaye et al. (2012) reported that the yield obtained for food crops during intercrop of tree crops and food crops were as high as their sole crops. This further affirmed the beneficial impact on food crop production other than better morphological growth due to intercrop earlier pointed out as an advantage in intercropping (Herera and Harwood 1973; Okigbo and Greenland, 1975; Okigbo, 1977).

Putra et al. (2012) who probed the effect of intercropping style on growth of one year-old oil palms reported that the food crops grown as intercrop with the juvenile oil palms did not inhibit the growth rate and performance of the juvenile oil palms which were the main crop. This is in concurrence with the findings of this study in which the intercropped juvenile oils were found to produce a better performance than the non-intercropped juvenile oil palms by recording higher canopy and trunk heights, canopy spread, and number and length of fronds. Similarly, this earlier report is also in harmony with the results of this study that though the intercropped juvenile oil palms had better growth performances than the non-intercropped palm during the second- and third-year of field establishment, the differences were largely not significant.

Contrary to the findings of this study, Rafflegeau et al. (2010) reported negative impact of annual crop intercrop with immature oil palms. They pointed out that the presence of food crops on oil palm plantations at the immature stage resulted in nitrogen and potassium deficiencies which persisted even when the plantation reach the production stage especially without appropriate annual fertilization.

5 CONCLUSION

Intercropping of fruit vegetables at a minimum of 1 m away from the row juvenile oil palms did not result in deleterious effects in the young oil palms; rather, it encouraged their growth and development.

This study established that fruit vegetables namely tomato, pepper, okra, and eggplant can successfully be intercropped with juvenile oil palm intercrops during the second and third year of field establishment of the oil palm. This success in intercrop was achieved by planting the fruit vegetables at a minimum of 2 m away from the juvenile oil palm.

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Influence of plant extracts, storage containers and storage duration on the physiological quality of watermelon (*Citrullus lanatus* (Thunb.) Mansf.) seeds stored under ambient conditions

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Influence of plant extracts, storage containers and storage duration on the physiological quality of watermelon (*Citrullus lanatus* (Thunb.) Mansf.) seeds stored under ambient conditions

Abstract: Watermelon seed being an oily seed is prone to rapid deterioration of its quality, hence maintaining quality during storage is germane. The study therefore investigated the effect of crude plant powder, storage containers and storage duration on the seed quality of two varieties of watermelon seeds. Seed lots each of 100 g of each variety were treated with 10 g of four crude plant powders of clove basil leaf, red chilli pepper fruit, garlic, neem leaf and a synthetic fungicide. All samples were stored in three storage containers (envelope, tin and glass bottle). Treated seeds were stored under ambient conditions for 180 days. Germination and seedling vigour were determined at 180 day time course after storage. Data were subjected to mean separation using Duncan's Multiple Range Test. Significant differences were observed in the quality traits examined in water melon due to differences in variety, seed treatment, storage container and storage period. 'Kaolak' was the best in storage in terms of seedling vigour and viability. Glass bottle was identified as the best storage container in maintaining seed quality of water melon throughout the storage period. The use of organic materials especially neem and clove basil leaf powder proved much better for maintaining germinability and seedling vigour and extension of seed longevity than inorganic material.

Key words: seed longevity; seed treatment; storage medium; probit modelling; seed storage life

Vpliv rastlinskih pripravkov, načinov in trajanja shranjevanja na fiziološke lastnosti semen lubenice (*Citrullus lanatus* (Thunb.) Mansf.) shranjenih v ambientalnih razmerah

Izvleček: Semena lubenice vsebujejo veliko olja in so zato podvržena hitremu propadu, zaradi česar je njihovo shranjevanje oteženo. V raziskavi so bili preučevani učinki zmletih izbranih rastlin, načina in trajanja shrambe na kakovost semen dveh sort lubenice. Vzorci semen, vsake od obeh sort lubenice po 100 g, ki so bili tretirani s po 10 g grobega prahu zmletih listov afriške bazilike, plodov rdečega čilija, strokov česna, listov azadirahta in sintetičnega fungicida so bili nato shranjeni v treh vrstah shranjevalnikov in sicer v papirnatih vrečkah, v kositrnih posodah in steklenicah. Tretirana semena so bila shranjena 180 dni v ambientalnih razmerah. Podatki meritev so bili obdelani z Duncanovim multiplim testom. Ugotovljene so bile značilne razlike v kakovosti semen lubenice glede na sorto, obravnavanje semen, način in trajanje shrambe. Sorta Kaolak je bila po shranjevanju najboljša glede na vitalnost in vigor pridobljenih sejank. Za vzdrževanje kakovosti semen so bile v celotnem obdobju shrambe najboljše steklenice. Uporaba rastlinskih pripravkov, še posebej prah iz listov azadirahta in afriške bazilike, se je za shranjevanje in vzdrževanje kalivosti semen lubenice kot za vigor sejank izkazala boljše kot sintetični fungicid.

Ključne besede: dolgoživost semen; tretiranje semen; način shranjevanja; probit model; dolgoživost shranjenih semen

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1 INTRODUCTION

Water melon belongs to the genus *Citrullus* and family Cucurbitaceae (Huxley, 1992). The Cucurbitaceae is a family of medium sized plants, primarily found in the warmer regions of the world. It is recognizable by its pinnatifid leaves and prolific fruition, up to 100 melons on a single vine. The water melon fruit, loosely considered a type of melon, possesses a smooth exterior rind (green and yellow) and a juicy, sweet, usually red, yellow or orange interior flesh (Jeffrey, 2005). Moreover, they are used as a domestic remedy for urinary tract infection, hepatic congestion, catarrh, worm remedy, abnormal blood pressure (Deible and Swanson, 2001; Amadi et al., 2003). Watermelon contains about 6 % sugar and 92 % water by mass. As with many other fruits, it is a source of vitamin C.

Seeds of watermelon are considered to have a long-life span in storage as to its quality (Priestley, 1986). In subtropical regions where watermelon seeds are intensively produced and where high relative humidity (> 70 %) is often accompanied with high temperature (30-35 °C), the quality or vigour of the seed can decline drastically within 12-18 months (Demir et al., 2011).

Seed longevity is greatly influenced by the relative humidity and storage temperature. The indiscriminate use of chemicals and their residual toxicity adversely affects the non-target animals and human beings besides affecting the seed quality. Many of the synthetic chemicals are effective but they are not readily degradable physically or biologically and yield more toxic residues. However, the use of chemicals is still in use. Hence, a safe and feasible approach is the treatment of seeds with botanicals which are safe, economical, eco-friendly and non-harmful to seed, animal and human beings (Mahesh and Hunje, 2008).

Watermelon seed being an oily seed is prone to rapid deterioration of its quality and since seed treatment with botanicals have been found to be more suitable and safe in maintaining viability of seeds (Adebisi, 2012), hence this study therefore will investigate the effects of crude plant powders, storage containers, storage duration on the maintenance of the quality of watermelon seeds.

2. MATERIALS AND METHOD

2.1. SEED MATERIALS

Two varieties of watermelon seeds ('Kaolak' and 'Sugar Baby') were sourced from the Department of Plant Breeding and Seed Technology, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. Seeds were scooped out from freshly harvested mature fruits and air-dried under ambient temperature for 4 weeks. Seed moisture content was thereafter determined using the oven drying method (ISTA, 1995).

2.2. EXPERIMENTAL DESIGN

The experiment was factorial laid out in completely randomized design with three replications. There were four factors to be considered; variety, seed treatment, storage containers and storage time.

2.3. METHOD OF SEED TREATMENT

The dried seed lots were treated with crude plant powdered treatments following the methods of Adebisi et al. (2013). Seeds were dry-dressed with four organic material which include finely powdered leaves of clove basil (*Ocimum gratissimum* L.) leaf (10 g 100 g of seed^{-1}), red chilli (*Capsicum frutescens* L.) fruit (10 g /100 g of seed^{-1}), garlic (*Allium sativum* L.) fruit (10 g 100 g of seed$^{-‡}$), neem (*Azardirachta indica* A. Juss.) leaf (10 g 100 g^{-1}) of seed and one inorganic material (Apron plus) while one with no treatment served as the control.

Seeds were dressed with recommended doses in sealed plastic containers at room temperature. After treatment, the storage containers were shaken twice daily up to seven days. Thereafter, the seed were stored in three different moisture pervasive materials (galvanized iron tin, glass bottle, and envelope).

2.4. SEED STORAGE

The containers with the treated seeds were kept in seed store under ambient conditions (temp 30 °C, RH 75 %) for 180 days to evaluate the treatment effects on the seed viability and seedling vigour traits.

2.5. QUALITY ASSESSMENT

Viability Test: Seed samples were taken from each treatment at 0, 30, 60, 120, 150 and 180 days of storage and was tested for the following seed quality traits. Viability test was carried out in the laboratory. Fifty seeds in three replications were placed inside petri dishes in an incubator and were maintained at 20 ml of distilled water and germination count was taken at 7 days (ISTA, 1995).

$$\text{Seed viability} = \frac{\text{germination count at 7 days}}{\text{number of seeds sown}} \times 100$$

Seedling Vigour Index: Seedling vigour index (SVI) was calculated by multiplying percentage (%) viability by the average of seedling length on the 7th day of germination (ISTA, 1995) and divided by 100.

$$SVI = \frac{\text{seed viability (\%) at 7 days x seedling length(cm)}}{100}$$

Seedling Length: Shoot length of 10 randomly selected seedlings were measured using a ruler in centimetre (cm).

2.6. DATA ANALYSIS

All data collected were subjected to analysis of variance using Statistical Analysis Software (SAS™, 2002). Significant means were separated using Duncan's Multiple Range Test at 5 % probability level (Duncan, 1955).

The seed viability data were subjected to probit analysis using the PROC statements of SAS in order to predict the storage life of the seeds. Seed longevity parameters were values of *Ki* (an estimate of the probit value of initial seed viability at the time of storage), slope ($1/\sigma$), an estimate of rate of seed physiological deterioration, sigma (σ), the standard deviation of seed survival curve and an estimate of time taken to lose 1 probit seed viability, and P_{50}, a measure of time taken for a seed lot to lose 50 % viability and estimate of absolute seed longevity (Ellis and Roberts, 1980).

3. RESULTS

Table 1 presents the effect of plant extract on seed viability of two water melon varieties stored for 180 days under natural ageing condition. From the result, seeds of 'Kaolak' treated with neem leaf powder had the highest viability value though statistically similar to values obtained in some other treatments at 0 days of storage. At 30 days of storage, 'Kaolak' seeds treated with chilli pepper powder gave the highest viability value (94.44 %) though statistically similar to those obtained in some other treatments. Treated seeds of 'sugar baby' had comparable values among the treatments. A progressive decline was observed among all the seeds as storage duration increases with 'Kaolak' seeds treated with neem powder still having the highest value among other treatments.

The effect of plant extract on seedling vigour of two water melon varieties is shown in Table 2. Seeds of 'Kaolak' irrespective of the treatments had seedling vigour above sugar baby at 0 day of storage. At 30 days of storage, seeds of 'Kaolak' treated with basil leaf powder gave the highest seedling vigour values though statistically similar to values obtained in some other treatments. It was observed that 'Kaolak' seed treated with neem powder maintained the highest value throughout the storage duration.

Table 3 shows the effect of container and storage time on seed viability of two water melon varieties. Seeds of 'Kaolak' stored in envelope had the highest (98.89 %) statistically similar viability value with other containers

Table 1: Effect of seed treatment on viability (%) of seeds of watermelon stored under ambient conditions at different storage duration

		Storage Duration (days)					
Variety	Treatment	0	30	60	120	150	180
Kaolak	Neem	100a	93.33ab	88.89a	88.89a	73.33a	56.67a
	Clove basil	97.78a	87.78ab	73.33a-e	55.56cd	45.56bc	34.44cde
	Pepper	97.78a	94.44a	83.33abc	71.11bc	57.78b	41.11bc
	Garlic	93.33ab	86.67abc	75.56a-d	65.56bc	53.33b	43.33b
	Apron plus	88.89ab	82.22a-d	71.11b-e	61.11bc	47.78bc	34.44cde
	Control	97.52a	92.23ab	86.67ab	77.78ab	75.56a	55.66a
Sugar Baby	Neem	81.11bc	77.78b-e	66.67cde	54.44d	46.67bc	32.22cde
	Scent leaf	82.22bc	77.78b-e	71.11b-e	60.00cd	44.44bc	27.78cde
	Pepper	74.44cd	67.78de	60.00de	44.44d	32.33c	20.00e
	Garlic	81.11bc	77.78b-e	66.67cde	58.89cd	42.22bc	25.56de
	Apron plus	66.67cd	64.44e	56.67e	44.44d	34.44c	22.22de
	Control	75.56cd	71.11bc	62.22de	53.33cd	42.22bc	27.78cde

Means followed by the same letters in each column do not differ significantly at 5 % level of probability according to Duncan's Multiple Range Test (DMRT)

Table 2: Effect of seed treatment and storage duration on seedling vigour of watermelon varieties

		Storage Duration (days)					
Variety	Treatment	0	30	60	120	150	180
Kaolak	Neem	17.16a	19.30ab	16.97ab	13.84a	12.83a	10.74a
	Clove basil	14.83abc	20.62a	14.86a-d	8.43bc	8.72b	6.77bc
	Pepper	15.55abcd	20.17ab	17.54a	10.86ab	8.83b	6.13bcd
	Garlic	15.85a	16.24bcd	12.33cde	10.33abc	8.11bc	6.39bc
	Apron plus	15.19ab	17.37a-d	12.80b-e	8.12bc	7.14bc	4.98bc
	Control	15.67a	19.23ab	15.87abc	13.61a	12.43a	9.84a
Sugar Baby	Neem	11.65cde	15.25cde	12.16cde	10.04abc	7.34bc	4.92b-e
	Clove basil	11.52cde	16.51bcd	12.14cde	9.73bc	7.85bc	4.92b-e
	Pepper	11.21de	13.73de	11.08de	7.99bc	5.56bc	3.28e
	Garlic	12.11b-e	16.24bcd	12.33cde	10.04abc	6.23bc	3.87cde
	Apron plus	9.57e	11.94e	8.71e	6.56c	5.07c	3.44de
	Control	11.39cde	13.72de	11.65cde	8.64bc	8.20bc	5.61c-e

Means followed by the same letters in each column do not differ significantly at 5 % level of probability according to Duncan's Multiple Range Test (DMRT)

Table 3: Effect of storage container and storage duration on viability (%) of watermelon varieties

		Storage Duration (days)					
Variety	Container	0	30	60	120	150	180
Kaolak	Envelope	98.89a	88.89a	80.56a	71.11ab	58.33ab	41.11b
	Glass Bottle	95.56a	90.56a	83.89a	76.11a	66.67a	55.56a
	Tin	94.44a	88.89a	75.00ab	62.78bc	51.67bc	39.44b
Sugar Baby	Envelope	76.67bc	74.44b	66.11bc	55.56cd	41.67cd	27.22c
	Glass Bottle	71.11c	67.22b	60.00c	48.89d	37.22d	24.44c
	Tin	82.78b	76.67b	65.56bc	53.33cd	42.22cd	26.11c

Means followed by the same letters in each column do not differ significantly at 5 % level of probability according to Duncan's Multiple Range Test (DMRT)

(glass bottle 95.56 % and tin 94.44 %). The same trend was observed at 30 and 60 days of storage. At 120 days of storage, seeds of 'Kaolak' stored in glass bottle gave the highest viability value though similar to that of seeds of the same variety stored in envelope. Also, it was observed that, seeds of 'Kaolak' stored in glass bottle maintained its viability when compared with other containers throughout the storage duration.

In Table 4, the effect of container and storage duration on seedling vigour of two water melon varieties. Treated 'Kaolak' seeds stored in envelope, glass bottle and tin recorded statistically similar higher values compared with 'Sugar Baby' seeds irrespective of the storage container at 0 day of storage. The same trend was observed at 30 days of storage while at 60 days, treated 'Kaolak' seeds stored in glass bottle recorded the highest value. Also, 'Kaolak' seeds in glass bottle maintained higher vigour as storage period increased compared with other treatments.

Data in Table 5 presents the probit parameters of seed longevity data after storage for 180 days in two water melon varieties. The intercept values (estimates of initial probit germination and a measure of seed germination before storage) indicate that there were higher values in neem and pepper in both varieties. Intercept vales were generally higher in 'Kaolak' for all the treatments than for 'Sugar Baby'. The value of slope indicates the speed of deterioration. Differences were exhibited in most cases in the slope values among the varieties, treatments and containers used. The lowest reduction in speed of deterioration (seed longevity) occurred in 'Kaolak' with scent leaf treatment in glass bottle (4.53 days), similar observation was recorded in 'Sugar Baby' with pepper treatment in

Table 4: Effect of storage container and storage duration on seedling vigour of watermelon varieties

		Storage Duration (days)					
Variety	Container	0	30	60	120	150	180
Kaolak	Envelope	16.41a	18.76a	16.15ab	11.04ab	11.03a	7.64a
	Glass Bottle	15.71a	19.19a	16.75a	12.25a	9.64ab	8.35a
	Tin	14.50a	19.35a	13.56bc	9.31bc	8.36bc	6.44ab
Sugar Baby	Envelope	11.59b	15.05bc	11.75c	9.72abc	7.26bc	4.79bc
	Glass Bottle	10.11b	12.88c	10.57c	8.64bc	6.18c	4.03c
	Tin	12.02b	15.77b	11.71c	8.13c	6.68c	4.20c

Means followed by the same letters in each column do not differ significantly at 5 % level of probability according to Duncan's Multiple Range Test (DMRT)

glass bottle (4.53 days) while control in glass bottle deteriorated faster than others (1.07 days).

There was increase in seed longevity extension in the two varieties after seed treatments by estimates of seed half-life (P_{50}) and storage life. Higher estimates of seed half-life and storage life after treatments occurred in 'Kaolak' than in 'Sugar Baby'. In 'Kaolak', estimate of seed storage life was the highest in neem in glass bottle (19.24 months) followed by garlic in glass bottle (14.02 months) and control in glass bottle (13.48 months). Apron plus in tin had the lowest storage life value of 7.94 months. All other treatments had storage life of above 8 months. With 'Sugar Baby', storage life estimate was also the highest in neem stored in envelope (11.50 months) followed by garlic in envelope (9.90 months), control in tin (9.72 months) and clove basil in glass bottle (9.50 months). Pepper in glass bottle had the lowest storage life value of 4.38 months while other treatments had storage life of above 5 months.

4. DISCUSSION

Seed treatment had been reported to be effective on the improvement of seed germinability and storability (Mandal et al., 2003). In this study, differential responses in seed germination and seedling vigour were observed among the five seed treatments examined suggesting ample opportunity for selection of seed treatment for maintenance of post-storage seed quality in watermelon. This also supports the findings of Adebisi (2012) who reported efficacy of nine seed invigoration treatment in the improvement of okra seed. Significant differences were observed in seed germination and seedling vigour after treatments at each storage time investigated, hence the need to pay close attention to seed treatments regardless of storage container and environments used. 'Kaolak' seeds dressed with neem powder gave better germination and seedling vigour at the end of 180 days storage.

Seeds of 'Kaolak' dressed with neem, clove basil and pepper were higher when compared with that of 'Sugar Baby'. Also, 'Kaolak' seed dressed with neem powder still recorded a value of seed germination above 50 % at the end of 180 days of storage. Also, at the end of the storage (180 days), seed treated with the organic invigoration treatments had maintained greater germination over the inorganic (apron plus). On the mode of action of the crude plant material treatments, various possibilities have been reported earlier (Basu, 1994, Manda et al., 2003). In the present study, crude plant extract materials were selected based on previous study (Adebisi, 2012) for the possible effectiveness in controlling free radicals reactions as antioxidants, antioxidant-synergist and radiation protective agents (Slater, 1972, Brand et al., 1994). Capsaicin which is an important constituent of chilli (*Capsicum frutescens*) fruit has been reported to be an inhibitor of lipid peroxidation (Manda et al., 2003). Linalool, allicin, and azadirachtin, the most active ingredient in clove basil, garlic and neem leaves might act as inhibitors of lipid peroxidation thereby partly responsible for the longevity maintenance of stored seeds.

The result revealed significant differences among the two varieties for seed germination and seedling vigour index when stored for 180 days. 'Kaolak' had the best seed germination values during and at the end of the storage. This could be due to differences in genetic constitution of the two varieties evaluated. Differential responses in seed germination and vigour among varieties of crop species with and without treatments in storage have been reported. (Daniel et al., 2012; Adebisi et al., 2012, Kehinde et al., 2019).

On the effect of storage time, seeds treated with neem consistently recorded better seed germination at each storage time investigated. Also, storage of clove basil and pepper powder treated seeds maintained greater seedling vigour at each of the storage time examined. Higher deterioration of seed quality irrespective of invigoration treatments have been reported earlier in dif-

Table 5: Results of probit modelling of seed longevity data in two water melon varieties after crude plant treatment and storage in different containers under ambient conditions

Variety	Treatment	Container	K_i	1/σ	σ	P_{50}	**Seed Storage Life (Months)
Kaolak	Neem	Glass Bottle	6.30	-0.24	4.21	9.62	19.24
		Tin	2.26	-0.40	2.51	5.71	11.42
		Envelope	1.90	-0.34	2.90	5.51	11.02
	Apron plus	Glass Bottle	1.46	-0.29	3.45	5.05	10.10
		Tin	1.22	-0.31	3.24	3.97	7.94
		Envelope	2.22	-0.42	2.37	5.26	10.52
	Control	Glass Bottle	2.28	-0.93	1.07	6.74	13.48
		Tin	3.59	-0.58	1.71	6.14	12.28
		Envelope	2.00	-0.32	3.12	6.24	12.48
	Garlic	Glass Bottle	1.80	-0.26	3.89	7.01	14.02
		Tin	1.60	-0.36	2.73	4.39	8.78
		Envelope	1.90	-0.37	2.68	5.09	10.18
	Pepper	Glass Bottle	1.81	-0.30	3.33	6.04	12.08
		Tin	2.40	-0.47	2.11	5.07	10.14
		Envelope	3.29	-0.61	1.61	5.31	10.62
	Clove basil	Glass Bottle	1.82	-0.22	4.53	6.18	12.36
		Tin	1.91	-0.42	2.37	5.22	10.44
		Envelope	1.48	-0.30	3.34	4.39	8.78
Sugar Baby	Neem	Glass Bottle	1.60	-0.36	2.80	4.49	8.98
		Tin	0.76	-0.24	4.24	3.12	6.24
		Envelope	1.57	-0.27	3.66	5.75	11.50
	Apron plus	Glass Bottle	0.86	-0.24	4.07	3.49	6.98
		Tin	0.77	-0.23	4.41	3.43	6.86
		Envelope	0.78	-0.26	3.81	2.97	5.94
	Control	Glass Bottle	0.69	-0.23	4.33	2.98	5.96
		Tin	1.51	-0.31	3.21	4.86	9.72
		Envelope	1.02	-0.24	4.07	4.17	8.34
	Garlic	Glass Bottle	0.95	-0.20	3.81	3.60	7.20
		Tin	1.47	-0.35	2.87	4.21	8.42
		Envelope	1.71	-0.34	2.89	4.95	9.90
	Pepper	Glass Bottle	0.48	-0.22	4.53	2.19	4.38
		Tin	1.84	-0.42	2.33	4.30	8.60
		Envelope	1.07	-0.31	3.21	3.44	6.88
	Clove basil	Glass Bottle	1.16	-0.24	4.08	4.75	9.50
		Tin	1.15	-0.28	3.57	3.51	7.02
		Envelope	1.05	-0.27	3.64	3.82	7.64

Ki – intercept, 1/σ- slope, σ- time taken for seed lot to lose 1 probit viability
*P50 – seed half-life in days
** Seed storage life estimated as P50 value multiplied by 2 then divided by the 30 days of a month

ferent crop species (Ajala and Adebisi, 2005; Adebisi and Oyekale, 2005). In the present study, gradual decline in seed germination and seedling vigour level was observed irrespective of the pre-storage seed treatment materials with advance in storage times and became pronounced at 180 days (6 months) of storage. Higher seed germination and vigour were maintained at 30 to 150 days of storage. The sharp decline could be due to the deteriorative processes which were enhanced by the higher temperature (30 °C) and relative humidity (75 %) under tropical humid conditions.

With respect to storage containers, 'Kaolak' seeds stored in glass bottle consistently gave higher germination and seedling vigour values. This finding conforms to expectation as seeds stored in air tight containers maintain seed qualities longer than non-air tight packaging materials like envelopes which absorb moisture from the surrounding atmosphere. This finding agrees with the report of Kumar and Singh (1983) that the seeds of sesame stored in glass bottles maintained satisfactory germination throughout storage period while seeds stored in gunny bags lost viability after six months of storage. Majhi and Bandopadhyay (1993) also reported that freshly harvested groundnut seeds dried to moisture content of 9 % stored in glass bottles for one to nine months had the highest seed viability, root and shoot length and seedling dry mass when compared to seed stored in paper and cloth bag.

On probit modelling, the result showed that the water melon seeds deteriorated at different rate, irrespective of the invigoration material and storage container in which it is been stored for a period of 180 days. 'Kaolak' seeds dressed with neem and stored in glass bottle had the highest estimate of storage life (19.24 months) followed by 'Kaolak' seed treated with garlic in glass bottle (14.02 months) and control in glass bottle (13.48 months) while 'Sugar Baby' treated with pepper in glass bottle had the lowest storage life value of 4.38 months. In other words, the PROBIT modelling predicted that 'Kaolak' seeds can be stored for an average of 19 months if the seeds are put under good storage conditions. Authors like Adebisi et al. (2003, 2008), Esuruoso (2010), Adebisi and Oyekale (2005), Oni (2012) and Kehinde (2018) have also utilized probit modelling to predict storage life of soybean, rice, kenaf, okra, and sesame, respectively under ambient humid storage conditions.

5. CONCLUSIONS

Differences were observed in the two seed quality traits examined in water melon due to differences in variety, seed treatment and storage period. 'Kaolak' was the best in storage in terms of seedling vigour and viability. The use of organic materials especially neem leaf and clove basil leaf powder proved much better for maintaining germinability and seedling vigour and extension of seed longevity than inorganic material.

5.1. RECOMMENDATION

The findings from this study showed that maintenance of seed quality in watermelon using crude plant powders is advantageous in the humid tropical conditions. Therefore, since these seed crude plant materials utilized for treating watermelon seeds are cheap, readily available and environmental friendly, these findings will be of benefit to small and medium scale investment involved in seed production in Nigeria, where resources for cold storage are scarce. Therefore, seeds could be stored in glass bottles and preferably with either neem leaf or clove basil leaf powder.

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The magic world of whiskey microbiota

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The magic world of whiskey microbiota

Abstract: Modern metagenomics techniques in combination with next generation sequencing are increasingly used for research of numerous environments inhabited by diverse microbiota. In the present study we focused on a rather unusual environment for their growth, a forgotten bottle of blended Scotch whiskey. Whiskey is a world-known popular spirit, traditionally produced in a series of steps comprising malting of barley, fermenting the malt to an alcoholic wort, distilling and at least 3-year long maturation in oak casks, followed by filtration. In the process, notably in the fermentation, microorganisms play a crucial role. However, we were primarily interested in potential microbiological and chemical changes that might have taken place over the years while the half-empty whiskey bottle was left open. We found that only a very low number of aerobic mesophilic bacteria survived in it while the ethanol content decreased from 40 % to approximately 30 %. Interestingly, the metagenomics analysis showed there was a large and diverse microbial community present in the forgotten whiskey. Among the most abundant microorganisms were members of human commensal microbiota, some potentially disease-causing and also food spoiling bacteria, in particular genus *Pseudomonas*. Surprisingly, we even found a non-negligible number of typically environmental bacterial species.

Key words: whiskey; metagenomics; bacteria

Čarobni svet mikrobiote viskija

Izvleček: Raziskave raznovrstnosti mikrobiote številnih okolij so vse pogosteje izvedene s kombiniranjem naslednje generacije sekvenciranja in metagenomskih metod. V naši študiji smo se osredotočili na nenavadno okolje za rast mikroorganizmov, in sicer pozabljeno steklenico Scotch viskija. Viski je svetovno poznana in priljubljena žgana pijača, ki je tradicionalno proizvedena v več korakih od pridobivanja ječmenovega slada, fermentacije v alkoholno sladico in destilacije, ki ji sledi vsaj 3 leta trajajoče staranje v hrastovih sodih in končna filtracija. Za fermentacijo so mikroorganizmi nujno potrebni. V naši raziskavi so nas predvsem zanimale potencialne mikrobiološke in kemijske spremembe, ki so se zgodite tekom let, ko je bila pozabljena, pol prazna steklenica odprta. Ugotovili smo, da je preživelo le zelo malo števila aerobnih mezofilnih bakterij. Koncentracija etanola se je zmanjšala iz začetnih 40 % na približno 30 %. Metagenomska analiza je razkrila veliko in raznovrstno mikrobno skupnost, ki je živela v pozabljenem viskiju. Med najpogostejšimi mikroorganizmi so bili člani običajne človeške mikrobiote, nekaj potencialno patogenih bakterij kot tudi kvarljivcev hrane, na primer bakterije rodu *Pseudomonas*. Presenetljivo smo potrdili prisotnost tudi nezanemarljivega število tipično okoljskih vrst.

Ključne besede: viski; metagenomika; bakterije

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1 INTRODUCTION

Microorganisms are extraordinarily diverse and well-conserved living beings that can be found in virtually any habitat on the Earth, regardless of how hostile it may be. Recent advances of biomolecular techniques such as next generation sequencing (NGS) have made it possible to gain an important insight in microbial genomes or their specific regions, especially coupled with metagenomics studies. Together with Fourier-transform infrared *spectroscopy* (FTIR), we can use them to closely inspect the diversity of bacteria and the composition of the medium they live in.

Metagenomics is an analysis of genetic information from a microbial sample of a specific environment rather than from identical cells cultured in a dish (Laudadio et al., 2019). These sorts of studies are therefore particularly advantageous for the characterisation of groups of microorganisms that cannot be isolated, yet there might be thousands of different species of bacteria in a sample, of which many potentially closely related. There are different NGS techniques on which metagenomics can rely, two primaries being amplicon based and shotgun (Tessler et al., 2017). In the former, a specific region of genome such as 16S rRNA gene sequence is targeted, whereas in the later regions of randomly digested DNA are sequenced (Laudadio et al., 2019). Each of the techniques has its own advantages and disadvantages. Recently, Tessler et al. (2017) have compared the two strategies in a large-scale study of microbial diversity and have shown that the 16S rRNA amplicon approach was superior in both phyla and family identification despite the fact that shotgun strategy produces over a hundred times more of reads. Furthermore, standard analytical FTIR *spectroscopy* method combined with Attenuated total reflection (ATR), or ATR-FTIR technique, can be used to study different organic materials from liquid to solid samples and can give us qualitative as well as quantitative data about matrices such as whiskey.

The central aim of the present study was to take advantage of such metagenomics studies to characterise a potential microbial community in a forgotten bottle of blended Scotch whiskey. Whiskey is a popular mature spirit drink with a long history dating back to the 15th century, originating from Scotland. It is traditionally produced in a lengthy process which consists of malting of barley, fermenting the malt or some other grain to an alcoholic wort and of distilling, followed by at least 3-year long maturation in oak casks and filtration. Before bottling, whiskey is diluted by water to the desired alcohol concentration and to enhance its rich aroma (Karlsson & Friedman, 2017). For the fermentation step in the whiskey production, lactic acid bacterial community is of a great importance. The fact that the wort is not boiled makes it possible for the bacteria to survive and participate in the mixed yeast-bacteria fermentation (Makanjuola et al., 1992). At first, bacterial growth is heavily suppressed by the growth of the yeast, then the number of lactic acid bacteria (LAB) rises exponentially in an intermediate phase characterised by heterofermentative *Lactobacillus* such as *Lb. fermentum* Beijerinck, 1901 (van Beek & Priest, 2003), and is finally followed by a stationary phase where lactic acid continues to accumulate due to activity of mostly homofermentative bacteria such as *Lb. casei* (Orla-Jensen, 1916) Hansen & Lessel, 1971 or *Lb. paracasei* Collins et al., 1989 (van Beek & Priest, 2002). The heterofermentative and homofermentative *Lactobacillus* flora was reported to comprise strains of *Lb. fermentum, Lb. paracasei, Lb. brevis* (Orla-Jensen, 1919) Bergey et al., 1934 and other more rarely detected *Lactobacillus* (Simpson et al., 2001). However, microorganisms can be also responsible for spoilage of an alcoholic beverage. For example, in brewing, Gram-negative bacteria such as acetic acid bacteria, *Zymomonas* and Enterobacteriaceae are some of the common beer spoilers that require monitoring in the process (Paradh, 2015). Furthermore, a recent study has shown that *Staphylococcus xylosus* Schleifer & Kloos, 1975 makes the beer turbid and produces organic acids and biogenic amines, which alter the taste of the drink (Yu et al., 2019). Nonetheless, the spoilers of whiskey have not yet been described.

Our studied sample was an abandoned half-empty 70 cl bottle of blended Scotch whiskey with 40.0 % ethanol content, which was matured for at least 12 years before put on sale. This Scotch whiskey was left open for a few years at the room temperature and a noticeable cloudy sediment had formed in it over time. From the microbiological standpoint whiskey appears to be far from a favourable medium for the growth of microorganisms, which intrigued us to take a closer look into biochemical alterations of the whiskey, in particular the microbial communities that might have sprouted in the bottle over the years. For this end, various approaches from standard analytical techniques such as ATR - FTIR spectroscopy, to microbiology and modern genetics were combined.

2 MATERIAL AND METHODS

2.1 MATERIAL

In our study the original sample was a half-empty70 cl bottle of blended Scotch whiskey. The bottle was originally marked as 12 years matured whiskey with 40 % of ethanol contents. The bottle was left open for a few

years at the room temperature. Over the time a noticeable cloudy sediment had formed at the bottom. In our study we used this turbid part of the whiskey.

2.2 METHODS

2.2.1 ATR – FTIR spectroscopy

ATR - FTIR instrument Spectrum 100 (PerkinElmer) was used for determination of ethanol concentration in the whiskey sample in the spectral region 4000-450 cm^{-1}, using 4 cm^{-1} resolution and 4 scans. First mixtures of water and absolute ethanol (purity ≥ 99.8 %, Sigma - Aldrich) with known ethanol concentrations were prepared and then IR spectra were collected. By increasing ethanol concentration in reference samples (10 %, 20 %, 30 %, 40 %, 50 %, 100 %) broad peak in the range of 3000-3500 cm^{-1} decreases and peaks between 2850 and 3000 cm^{-1}, at 1045 cm^{-1} and at 1087 cm^{-1} increase. Ethanol concentration in whiskey could be determined by comparison with other FTIR spectra or calculated using Spectrum Quant program (PerkinElmer).

2.2.2 Culturing of bacteria

Sample of the turbid part of the whiskey was analysed for aerobic mesophilic bacteria and LABwith plate count method using appropriate medium and incubation conditions. Plate Count Agar (PCA, Oxoid CM0325, Hampshire, England) was used for aerobic mesophilic bacteria and De Man, Rugosa, Sharpe medium (MRSc, Oxoid CM0361) with cycloheximide (Sigma Aldrich, 66-81-9, St. Louis, MO, USA; 100 mg l^{-1}) for LAB. Samples in aliquots of 1 ml were poured and mixed with media and agar plates were incubated for up to 10 days at 30 °C in normal atmosphere for aerobic mesophilic bacteria and in in anaerobic atmosphere in jar obtaining with anaerobic atmosphere generation bag (Sigma Aldrich, 68061) for LAB. After incubation the number of colonies was counted, and results were expressed as average number of colony forming unit (CFU) per ml of sample (CFU/ml).

2.2.3 Extraction of DNA and quantification

DNA was extracted using PrepMan Ultra Sample Preparation Reagent following the manufacturer's protocol. 1 ml of the turbid part of the whiskey was first centrifuged for 3 min (3,000 g) and the supernatant was removed from the pellet. After DNA extraction the concentration was measured with Qubit v4 and Qubit ds-DNA High-Sensitivity (HS) kit (all Thermo Fisher Scientific).

2.2.4 16S rRNA PCR amplification, Ion Torrent library preparation and sequencing

The seven hypervariable regions of 16S rRNA gene of bacteria were amplified in multiplex PCR reaction using the Ion 16S™ Metagenomics Kit. NGS library was prepared using Ion Plus Fragment Library Kit (Thermo Fisher Scientific), following the manufacturer instructions. The amount and size distribution of the prepared library fragments were determined with the Labchip GX microfluidic capillary electrophoresis (PerkinElmer). Emulsion PCR and the enrichment steps were carried out using Ion PGM Hi-Q View OT2 Kit, as described by the manufacturer. Library templates were sequenced with on PGM Hi-Q View Sequencing Kit using an Ion 318 chip with the Ion Torrent PGM instrument (all Thermo Fisher Scientific). Signal processing and base calling were performed with the Torrent Suite software version 5.12.

2.2.5 Bioinformatics analysis

Bioinformatics analysis was done with cloud-based software Ion Reporter 5.12 (Thermo Fisher Scientific). Only complete reads covering entire amplicons from primer to primer were used in the analysis. To avoid an artificial increase in sequencing diversity arising from random sequencing errors, only reads with 10 or more copies were used in the analysis. The reads which met all the criteria for the analysis were mapped to both the curated open-source Greengenes v13.5 database and the validated commercial MicroSEQ® 16S Reference Library v2013.1 (Thermo Fisher Scientific). Percentage identity cut-off values of 97 % and 99 % were used to assign genera and species, respectively.

3 RESULTS

3.1 FTIR ANALYSIS

Ethanol concentration in whiskey was determined by comparison with other FTIR spectra or calculated using Spectrum Quant software. By observing peaks from FTIR spectra, it can be concluded, that ethanol concentration in whiskey sample is around 30 % (Figure 1 and Figure 2). However, using Spectrum Quant software,

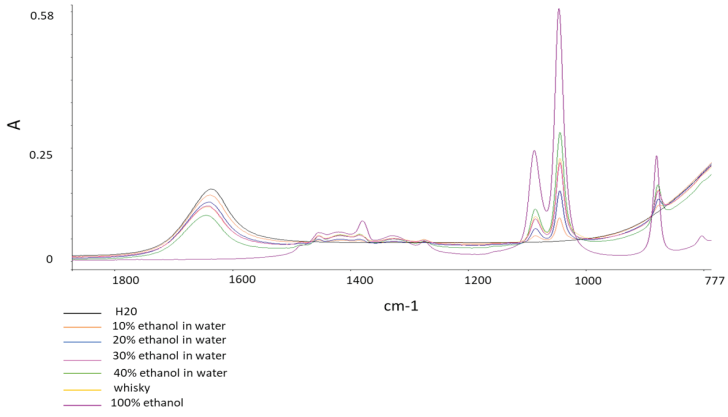

Figure 1: FTIR spectra of pure water, 100 % ethanol, mixtures of ethanol and water (10 %, 20 %, 30 % and 40 %) and whiskey sample in the range 1800-770 cm^{-1}.

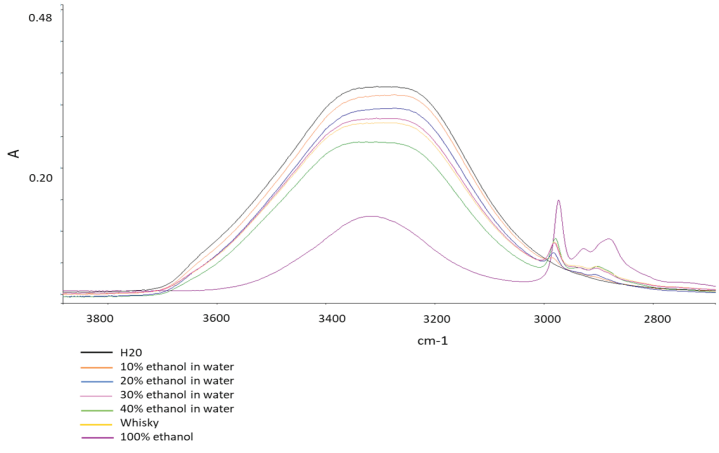

Figure 2: FTIR spectra of pure water, 100 % ethanol, mixtures of ethanol and water (10 %, 20 %, 30 % and 40 %) and whiskey sample in the range 3800-2600 cm^{-1}.

where Beer's Law algorithm was used, calculated value of ethanol concentration in tested whiskey is 30.1 %.

3.2 CULTURING OF VIABLE BACTERIA

The number of viable bacteria in whiskey was as expected very low, as the average number of aerobic mesophilic bacteria was 45 ± 10 CFU ml^{-1} (Gram-positive cocci, and Gram-negative rods), and LAB were not found (< 10 CFU ml^{-1}).

3.3 16S rRNA ANALYSIS

In total, 212,571 reads were obtained after the default filtering and quality trimming in Torrent Suite Software. Of those, 186,267 reads covered the entire amplicon and were included in further analysis. After discarding reads with less than 10 copies, 92,554 were included in the mapping pipeline. 92,101 reads mapped to at least one of the databases, with only 453 reads remaining unmapped.

Surprisingly, mapping revealed a diverse bacterial community in the sample. In total, 142 bacterial families, 149 genera and 159 species could be mapped unambiguously (Figure 3A and Figure 3B).

More precise results show that *Staphylococcus* was the most abundant genus in the sample (21.6 % of mapped reads), followed by *Anaerococcus* (6.2 %), *Methylobacterium* (5.9 %) and *Streptococcus* (5.2 %). Family distribution was more even, with Staphylococcaceae (14.1 %), Sphingomonadaceae (9.3 %), Enterobacteriaceae (9.0 %) and Comamonadaceae (6.9 %) representing the four most abundant operational taxonomic units (OTUs) at

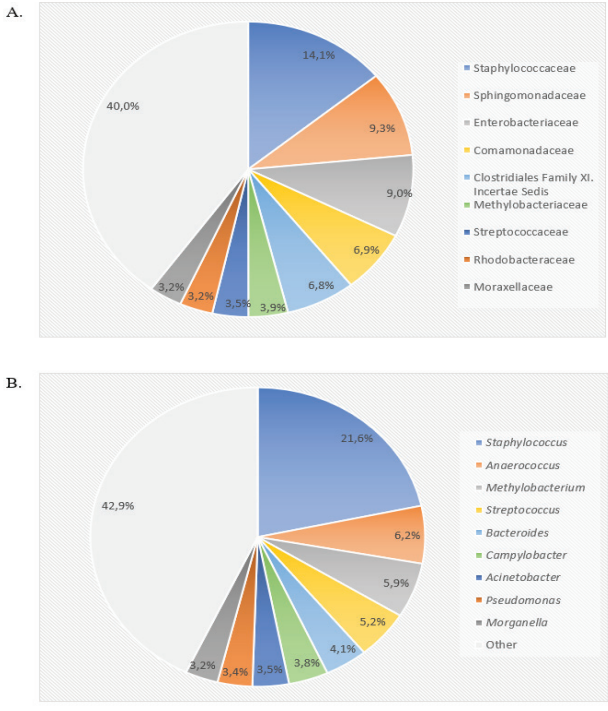

Figure 3: Distribution of (A) families a (B) genera unambiguously detected in the whiskey sample at relative abundances of 3 % or higher

this level. At genus and family level, 42.9 % and 40.0 % of reads mapped to OTUs with relative abundance lower than 3 %, which again demonstrates the high diversity of the population. *Bacteroides fragilis* (Veillon & Zuber, 1898) Castellani & Chalmers, 1919, *Campylobacter ureolyticus* (Jackson & Goodman, 1978) Vandamme et al, 2010, *Anaerococcus vaginalis* (Li et al., 1992) Ezaki et al., 2001, *Morganella morganii* (Winslow et al., 1919) Fulton, 1943, *Anaerococcus octavius* Murdoch et al., 1997) Ezaki et al., 2001and *Finegoldia magna* (Prevot, 1933) Murdoch & Shah, 2000 were the most abundant species detected. This does not match data from higher taxonomic units but is expected for 16S metagenomic analysis. In contrast to common strategies that focus only on one or two regions (V3, V4), our approach covered sequencing of 7 variable 16S regions, to ensure as much discriminatory power as possible and economically feasible. Nevertheless, for many OTUs, similarities in sequences of 16S rRNA genes for many species are still so high, that it is impossible to distinguish them at the level of species. The most abundant genus *Staphylococcus* represents such example in our analysis.

4 DISCUSSION

The abandoned bottle of Scotch whiskey might seem as a rather hostile medium for the growth of microorganisms, yet our metagenomics analysis suggests there was a large community present in it. Even though the ethanol content of the whiskey decreased from 40 % to 30.1 %, diverse species of bacteria had proliferated in it before dying off. Using the cut-off of at least 10 reads, we identified around 160 different species from roughly 150 genera in our whiskey sample. The highest number of reads was recorded for the family of Gram-positive bacteria Staphylococcocae, all of them from genus *Staphylococcus.* The most abundantly represented species was *Staph. Epidermidis* (Winslow & Winslow, 1908) Evans, 1916 which is universally present on human skin and mucosa but can also act as opportunistic pathogen (Coates et al., 2014). Taxonomic composition analysis also revealed very high number of reads for exceptionally large family of Gram-negative bacteria Enterobacteriaceae, which comprises some potentially disease-causing genera such as *Shigella, Salmonella, Klebsiella* and *Escherichia* (Brenner et al., 2005).

Amongst the most commonly detected species in the whiskey sample were predominantly commensal organisms which inhabit human alimentary tract or colonise our skin and mucous membranes, some being potentially pathogenic. For instance, the most abundantly represented species *Bacteroides fragilis* are obligate anaerobic Gram-negative bacteria that are a normal part of

human intestinal microbiota, yet become pathogenic by endotoxin secretion when the mucosal barrier is disrupted (Elsaghir & Reddivari, 2020). Similarly, a facultative-anaerobic member of Enterobactericeae family, *Morganella morganii* is a common member of human intestinal tract flora, but can also act as opportunistic pathogen causing wound and urinary tract infections (Liu et al., 2016). Another frequently detected Gram-negative anaerobic species is *Campylobacter ureolyticus,* which has been identified as gastrointestinal pathogen, causing *Campylobacter*-related gastroenteritis (O'Donovan et al., 2014). Furthermore, also aerobic Gram-positive bacteria such as those from genus *Corynebacterium* were highly represented in the whiskey sample, in particular *Corynebacterium striatum* (Chester, 1901) Eberson, 1918. This species commonly colonises skin and mucous membranes, but has also been identified as an emerging pathogen causing amongst other also bone and joints infections (Noussair et al., 2019).

On the other hand, food spoilers were not as abundantly represented in the microbial community of our abandoned bottle. Remenant et al. (2015) described LAB and genera *Clostridium*, *Serratia*, *Hafnia*, and *Pseudomonas* as the main known food spoilers. One important example highly present in our whiskey is genus *Pseudomonas*, which largely contributes to the food spoilage process. *Pseudomonas* comprise thermotolerant spoilage microorganisms that cause off-flavour release, reduction of shelf-life and quality of dairy and meat products, discoloration of dairy products and browning of vegetables, along with biofilm formation on chilled foods (Quintieri et al., 2019). Regarding LAB, only very low number of reads were recorded for *Lactococcus lactis* (Lister, 1873) Schleifer et al., 1986 and even fewer for *Lactobacillus iners* Falsen et al., 1999, but we also recorded presence of *Enterococcus faecalis* (Andrewes & Horder, 1906) Schleifer & Kilpper-Balz, 1984 which is involved in greening and spoilage of meat (Remenant et al., 2015). Taking into account these metagenomic findings, it comes as no surprise that no viable LAB were detected in the anaerobic culture.

Curiously, in the whiskey bottle there were also some species more characteristic for environmental samples such as *Rubrivivax gelatinosus* (Molisch, 1907) Willems et al., 1991, a purple nonsulfur photosynthetic bacterium found for instance in freshwater ponds and food processing wastewater (Nagashima et al., 2012). Similarly, *Diaphorobacter nitroreducens* Khan & Hiraishi, 2003, a denitrifying microorganism, is also an example of bacterium that has been previously isolated from activated sludge used in wastewater treatment (Khan & Hiairishi, 2002). Third such microorganism in our studied sample are aerobic, nitrite-oxidizing bacteria of genus *Nitrospira* which are abundant in various ecosystems such as wastewater treatment plants, freshwater, soils, groundwater and geothermal springs (Mehrani et al., 2020).

All in all, present analysis of microbial community in abandoned Scotch whiskey bottle based on multidisciplinary approach reveals a large spectrum of various bacterial species proliferating in it over the years, despite the high concentration of ethanol. The most numerously represented species in the sample differed in their metabolism, physiology and their usual habitat, some even being pathogenic or known food spoilers. Based on the composition of the microbial community we suppose that the contamination happened due to a direct oral ingestion, after which a part of bacterial families proliferated while the ethanol content dropped. However, after a while the growth ceased and bacteria died, but the remaining ethanol content contributed to the conservation of the nucleic acids.

5 ACKNOWLEDGEMENTS

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Use of gum blend in the optimization of grape molasses halva Gazi formulation with an emphasis on texture properties

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Use of gum blend in the optimization of grape molasses halva Gazi formulation with an emphasis on texture properties

Abstract: Grape molasses is a traditional sweet with high nutritional value. One factor limiting the production and storage of grape molasse halva Gazi is related to the thermal sensitivity of its texture properties and decreasing its quality during storage at ambient temperature. Therefore, this study aimed to improve the texture properties of the newly formulated halva during storage at different temperatures. Different levels of gums (0.5 %, 1 % & 1.5 %) including alginate, carrageenan, xanthan, and their blends (0.5 %-0.5 %) were used as additives in the formulation of halva to improve its texture during 48 h storage at 25 °C and 40 °C. The texture analysis tests were then performed to evaluate the firmness and fracturability of the halva. The results showed that the combination of gums, as well as using xanthan gum alone, produced an appropriate and soft texture in comparison to the control halva (0 % gum). However, the halva containing carrageenan-alginate blends showed the softest texture. The samples containing 0.5 and 1 % of alginate and xanthan as well as xanthan-alginate blend and xanthan-carrageenan blend had the best texture properties during storage at 40 °C.

Key words: grape molasses halva Gazi; gum; temperature stability; texture

Uporaba mešanice aditivov za optimizacijo molase iz grozdja pri pripravi halve Gazi s poudarkom na njeni teksturi

Izvleček: Molasa iz grozdja je osnovna sestavina tradicionalne slaščice Gazi halva, ki ima veliko hranilno vrednost. Eden izmed omejujočih dejavnikov pri pripravi in shranjevanju te slaščice je toplotna občutljivost molase iz grozdja, kar vpliva na njeno teksturo pri shranjevanju na sobni temperature.V raziskavi je predstavljen nov način priprave halve za shranjevanje na različnih temperaturah. Kot polnilo so bile pri pripravi halve dodane različne količine aditivov (0,5 %, 1 % & 1,5 %) kot so alginat, karaginan, ksantan in njihove mešanice (0,5 %-0,5 %) za izboljšanje njene teksture pri 48 urnem shranjevanju na 25 °C in 40 °C. Za oceno čvrstosti in lomljivosti halve so bili narejeni preiskusi njene teksture. Rezultati so pokazali, da je kombinacija polnil kot tudi uporaba samo ksantana dala primerno mehko teksturo v primerjavi s kontrolno pripravo halve brez aditivov (0 % aditiva). Najmehkejšo teksturo je imela halva pripravljena z mešanico karaginana in alginata. Vzorci halve, ki so vsebovali 0,5 in 1% alginata in ksantana kot tudi tisti z mešanicami alginata in ksantana ter ksantana in karaginana so imeli najboljšo teksturo za shranjevanje na 40 °C.

Ključne besede: halva Gazi z molaso iz grozdja; aditivi; teperaturna obstojnost; tekstura

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1 INTRODUCTION

Grape molasses halva (kernelled halva or halva Josie), high nutritional value and delicious product, is popular in Iran, especially in Sabzevar and Torbat-e-Heydariyeh. It containsgrape molasses (as a natural sweetener),chubak extract, sesame, and walnut kernel (Löfgren et al., 2006).

The texture is considered as an important physical aspect affecting the acceptance of the food products. Hydrocolloids are widely used as gelling agents in food systems to preserve and/or improve the texture of the products as a texture modifier (Nussinovitch, 2003). Hydrocolloids have different functional properties influencing the process parameters, evaporated water retention, freezing rate, and growth of ice crystals, as well as chemical reactions. One factor limiting the production and storage of grape molasses halva Gazi is related to the thermal sensitivity of its texture properties and decreasing its quality during storage at ambient temperature. Preserving this halva at 25 °C or higher temperatures shows an adverse effect on its texture; hence, this is usually produced during cold months of the year (from November to late April).Therefore, the use of heat-resistant gums such as carrageenan, alginate, and xanthan will be effective to increase its shelf life by preserving the gel structure and improving the texture of halva. For example, it was revealed that the xanthan gum is thermal resistant as it can preserve its viscosity at autoclave processing condition (121 °C and pressure of 15 psi) (Fallah, Motamedzadegan, 2013). Movahed et al. (2014) reported that the use of xanthan gum and potato flour increased the toast texture softening and prevented from its staling. It was found that adding guar and carrageenan affected the physical and organoleptic properties of Barbari bread; as, the firmness of bread decreased by adding guar gum especially at the level of 0.5 % (Qureshi et al., 2009).

In this study, various gums including xanthan, carrageenan, alginate gums and their blends were used to improve the texture of halva Gazi during storage at ambient temperature or higher.

2 MATERIALS AND METHODS

2.1 MATERIALS

All required ingredients for the preparation of halva Gazi including sesame, walnut and grape molasses were obtained from a local market in Sabzevar, Iran. The gums (xanthan, carrageenan, and alginate) were prepared from Sigma Aldridge Company.

2.2 PREPARATION OF HALVA GAZI

At first, the chubak extract was prepared according to the method of Keyhani et al. (2010).To prepare the halva Gazi, the chubak extract solution (10 %) was mixed with grape molasses. The mixture is then brought to the boiling temperature in a cast-iron container increased the grape molasses Brix. The boiling process was continued to achieve the desired Brix of the mixture that was determined based on observing the formed bubbles (with approximately 3 mm diameter). The temperature was then reduced. The mixture was stirred for about two hours to obtain the desired texture. To prepare the halva containing gum, different levels of heat-resistant gums including xanthan, carrageenan and alginate (0.5 %, 1 %, and 1.5 %) and their blends (0.5 %-0.5 %) were added to the mixture in the last 30 min of stirring. Finally, the sesame seeds were added and the halva was transferred into the trays or tablecloths covered with barley flour and decorated with walnut kernels before the texture hardening (Löfgren et al., 2006).The produced samples were stored at 25 °C and 40 °C for 48 h before analyzing the texture properties.

2.3 TEXTURE PROFILE ANALYSIS

As shown in Fig. 1, the texture properties of the food can be evaluated using a device by placing food under a force through deformation, disintegration, and flowing, with a combination of mass, distance, and time (Bourne, 2009). According to Table 1, the texture analyzer probe with a mass of 4.5 kg was used to evaluate the halva texture properties (firmness and factorability) according to AACC 09-74. For this purpose, a specific piece of halva

Table 1: The used levels of gums in formulation of the halva

Treatment	Gum concentration (%)
Control (traditional)	0.0
Xanthan	0.5
	1.0
	1.5
Carrageenan	0.5
	1.0
	1.5
Alginate	0.5
	1.0
	1.5
Xanthan-Carrageenan	0.5-0.5
Xanthan – Alginate	0.5-0.5
Alginate-Carrageenan	0.5-0.5

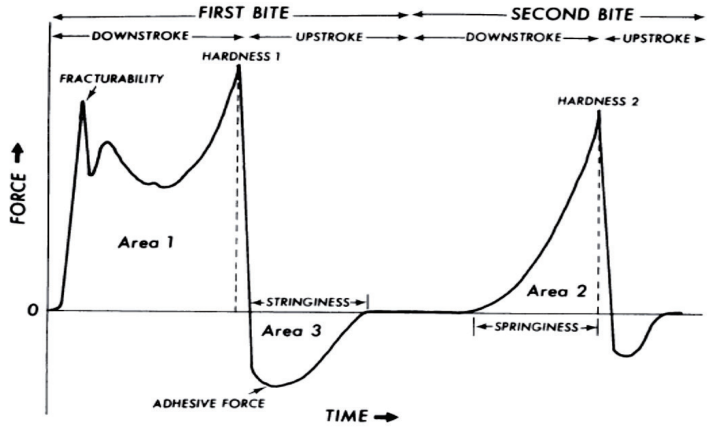


Figure 1: Texture profile analysis according to Bourn (2009)

Gazi (25 mm × 25 mm × 25 mm) was placed on the mandible of the device. The diameter and speed of the probe were 35 mm and 30 mm min^{-1}. Each sample was compressed to 40 % of initial height in two reciprocating cycles (two goings and two comings) and it was then decompressed (Takahashi et al., 2009).

2.4 THE STATISTICAL ANALYSIS

All experiments were performed in triplicate. The completely randomized factorial design was used to analyze the obtained data using SPPS 21 software. Results were submitted to analysis of variance (ANOVA) using Duncan's multiple range test with a significance level of $p < 0.05$.

3 RESULTS AND DISCUSSION

3.1 THE EFFECT OF CARRAGEENAN GUM ON FIRMNESS AND FRACTURABILITY OF HALVA GAZI

Firmness represents the resistance of the sample to applied compressive forces (Szczesniak, 2002). As shown in Fig. 2 a, the firmness of halva increased as higher levels of carrageenan gum were used ($p < 0.05$). It was also observed that the firmness decreased during storage at a higher temperature ($p < 0.05$).The interaction effect of gum concentration and storage temperature was also significant ($p < 0.05$). Using 0.5 % of carrageenan wasn't effective to improve the texture of the stored halva at 40 °C as there was no significant difference between the firmness of this sample and the control. The sample containing 1.5 % carrageenan gum had a firmer texture than the control one during storage at 40 °C. However, this sample had very firm and undesirable texture at ambient temperature (25 °C).

Movahed (2014) reported that the strength of carrageenan gel decreased at the higher temperature. At about 55 °C, the carrageenan gum molecules swell and its hydrogen bonds partially break down. During cooling, new hydrogen bonds are formed between the carrageenan chains causes the reforming of the gel structure. Heating at a higher temperature (> 80 °C) produces the stronger and firmer gel that can be stable in autoclave conditions (Phillips, 2000). During heating at a higher temperature than its melting point, the irregular loops are created in the carrageenan gel structure due to thermal perturbation allowing the formation of double helixes at the coupling point resulting in the formation of a three-dimensional network (Lofgrent, 2006).

The effects of concentrations of carrageenan gum on the fracturability index of the halva Gazi texture were shown in Fig. 2. b. The fracturability or tenderness of the halva increased as higher levels of carrageenan were applied ($p < 0.05$).This factor decreased during the storage of the samples at a higher temperature ($p < 0.05$).The interaction effect of these independent variables was also considerable ($p < 0.05$). Comparing the fracturability of the stored samples at 20 °C and 40 °C revealed that the reduction in this factor was more noticeable in the halva containing 1.5 % carrageen and gum. These results could be explained based on the structure of the gel network and the various chemical interactions between the hydrocolloid and halva ingredients. Carrageenan gels can forma connected and dense network structure causing the food ingredients place next to each other in a compact structure causing an increase in the fracturability of the food product (Löfgren et al., 2006).

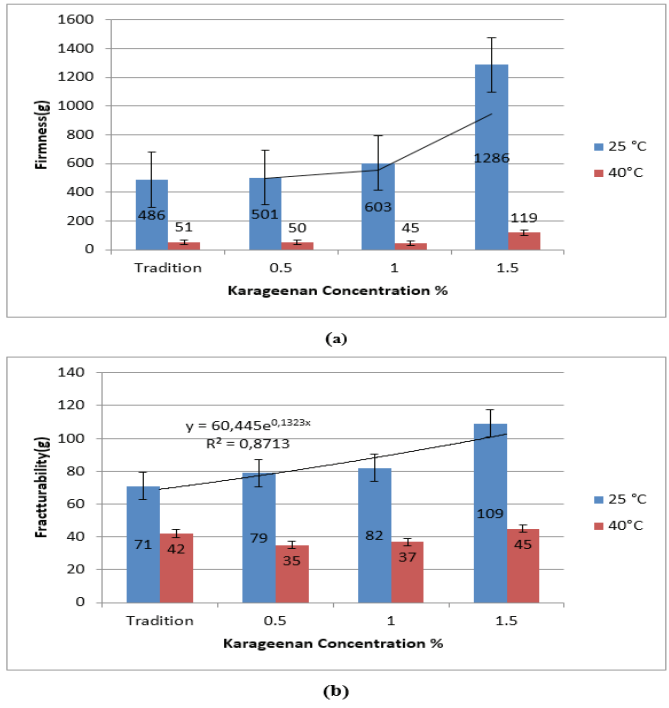


Figure 2: The effect of carrageenan gum concentration and storage temperature on the firmness (a) and fracturability (b) of halva Gazi

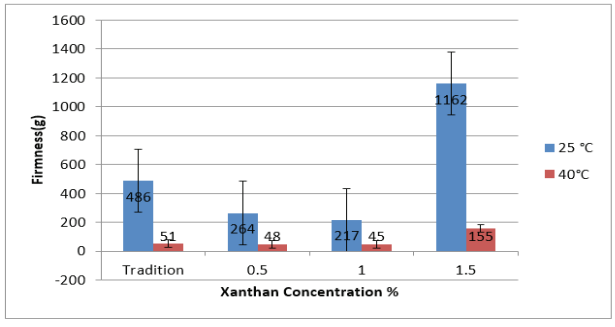
3.2 THE EFFECT OF XANTHAN GUM ON FIRMNESS AND FRACTURABILITY OF HALVA GAZI

The firmness of halva Gazi was significantly affected ($p < 0.05$) by using different levels of xanthan gum, storage temperature and their interaction effect as represented in Fig. 3 a.

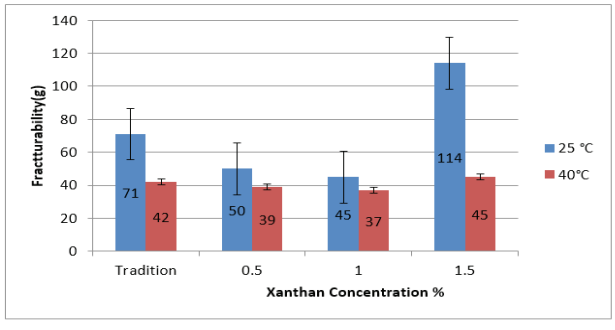
The samples containing 0.5 % and 1 % xanthan gum showed a softer texture in comparison to the control one. There was no considerable difference between the firmness of the halva containing 0.5 % and 1 % xanthan gum at 40 °C. Adding 1.5 % xanthan gum showed the preservative effect on the stored halva firmness at 40 °C; however, the texture of this sample was very firm and undesirable at ambient temperature. An increase in the xanthan gum levels up to 1 % decreased the firmness of texture because of weakening the structure of the gel network by absorbing a higher amount of water. During the heating process, it prevented from high loss in the moisture content of the Halva producing the sample with a soft texture.

Movahed et al. (2014) evaluate the texture of the toast containing potato flour (5, 10 & 15 %) and xanthan hydrocolloid (0.5 & 1 %) with wheat flour. The obtained results showed that the potato flour and xanthan gum softened the texture and prevented from the staling of toast after storage for 24, 48 and 72 h. Khalilian et al. (2011) used different levels of xanthan gum and pectin in the formulation of cantaloupe puree-based fruit pastel. It was reported that the use of xanthan in the formulation decreased the firmness of the surface of the sample. By adding the gums, the organoleptic properties of the cantaloupe pastel such as color intensity, texture firmness, and adhesiveness of surface texture were also changed. Comparing the consistency and serum reduction of ketchup containing different gums (guar, sodium alginate, acacia, xanthan, pectin, and carboxymethyl cellulose) revealed that the guar and xanthan gums produced the ketchup with the highest consistency at 0 to 50 °C (Gojral et al., 2003). Jahangir et al. (2013) reported that the using higher levels of flaxseed and xanthan gums in ketchup formulation could be effective in decreasing the amount of syneresis; as the samples containing xanthan gum showed no syneresis. Altunakar et al. (2006) found that hydrocolloids (xanthan gum and hydroxyl propyl methylcellulose) impact on the quality of chicken nugget by softening its texture.

Fig. 3b. shows the changes in the fracturability of halva Gazi influencing by the gum concentration, storage temperature and interaction effect ($p < 0.05$).The fracturability decreased ($p < 0.05$) as the higher levels of xanthan gum was used in the formulation of the halva. In the samples containing up to 1 %, the fracturability index



(a)



(b)

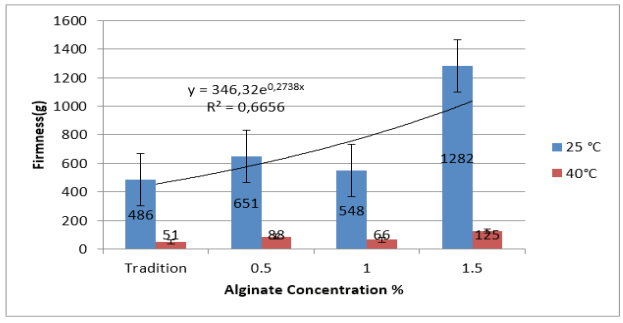
Figure 3: The firmness (a) and fracturability (b) of halva Gazi containing different levels of xanthan gum during storage at various temperature

decreased during storage at 25 °C. While a considerable increase in this factor was observed when the highest level of the gum (1.5 %) was added. During storage at 40 °C, it was found that the fracturability of the sample having 0.5 and 1 % xanthan gum was the same ($p > 0.05$) which were lower than the fracturability of control and the. Comparing the effect of storage temperate on the fracturability of halva containing 1.5 % gum determined that this gum was able to improve the texture of the sample at 40 °C, while its high firmness during storage at 25 °C was unacceptable.

3.3 THE EFFECT OF ALGINATE GUM ON FIRMNESS AND FRACTURABILITY OF HALVA GAZI

The effect of different concentrations of alginate gum on the firmness of the produced halva was significant ($p < 0.05$).In comparison to the control, the firmness of samples increased by using alginate gum (Fig. 4. a). The control and halva with 1.5 % alginate gum had the least and the most firmness values, respectively. Storage at the higher temperature resulted in the lower firmness ($p < 0.05$). Unlike the stored samples at 40 °C, the samples with 1.5 % gum stored at room temperature showed a harder texture in comparison to the control one. However, using 0.5 and 1 % alginate brought the almost similar texture quality to the control during storage at both temperatures. Moreover, the interaction effect of temperature and gum concentrations increased the firmness of halva texture($p < 0.0$ 5).The effect of alginate on the texture of the halva can be related to the linear structure of the gel network and various chemical interactions such as the calcium bonds between the hydrocolloid and ingredients of the sample. The alginate gel does not melt at the boiling point due to the presence of the strong calcium bridges at the coupling points (Draget, 2000). Besides, the firmness of the alginate gel will increase by heating for a longer duration (Gureshi et al., 2009).

As shown in Fig. 4. b., different concentrations of alginate gum showed a considerable effect on alteration in the fracturability of the halva Gazi ($p < 0.05$) as, the samples containing higher levels of the gum showed almost higher fracturability index than the control. Storing the halva at different temperatures showed a considerable effect on this property ($p < 0.05$). There was no significant difference between the fracturability of the samples with 0.5 and 1 % gum. During storage at 40 °C, the texture of the Halva with 1.5 % concentration of the alginate gum properly preserved; while, this showed very firm texture at ambient temperature that reduced the acceptance of the halva. The interaction ef-



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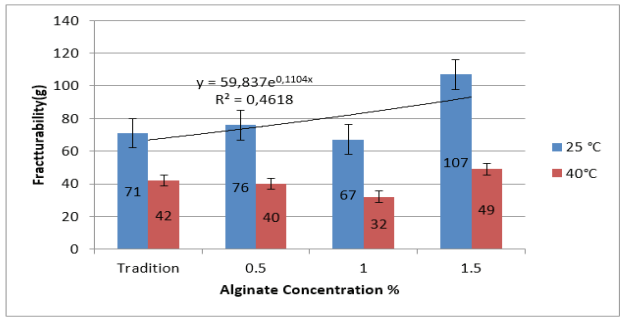


(b)

Figure 4: The firmness (a) and fracturability (b) of halva Gazi containing different levels of alginate gum during storage at various temperature

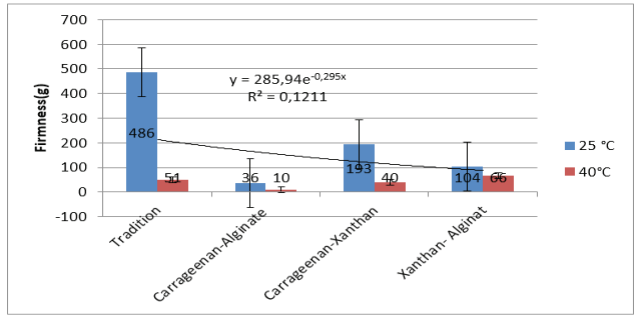
fect of temperature and different concentrations of alginate gum on the halva texture fracturability showed a reducing effect on this index ($p < 0.05$).

3.4 THE EFFECT ON USING THE BLENDS OF DIFFERENT GUMS ON THE TEXTURE OF THE HALVA GAZI

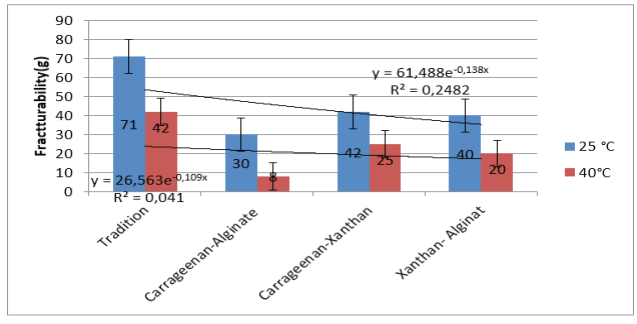
It was observed that using different blends of the gums results in producing the halva with softer texture ($p < 0.05$) in comparison to the control sample (Fig. 5. a).At 25 °C, the halva containing carrageenan-alginate gums and blends carrageenan-xanthan showed the lowest and the highest firmness. At 40 °C, the halva containing carrageenan-alginate gums blend showed the softest texture following by the samples with carrageenan-xanthan and xanthan-alginate blends. This property was also affected by the storage condition ($p < 0.05$); as the firmness of halva Gazi decreased during storage at a higher temperature. The effect of the temperature was more noticeable in the firmness of the sample containing carrageenan- xanthan gum blend. Among the gum-contained samples, the halva with the xanthan-alginate gum blend had the firmer and the more heat-stable texture. In general, the blended gum-contained samples caused softening the texture at ambient temperature and higher temperatures. In addition, the use of blends containing xanthan gum showed high effectiveness in the preservation of the texture comparison to the control samples.

Omidbakhsh et al. (2013) investigated the effect of simultaneous use of modified starch and xanthan gum on stability, organoleptic and rheological properties of tomato sauce. It was found that at lower concentrations of xanthan gum, the addition of starch preserved thestability, rheological and organoleptic properties of the sample. Formulation of low-fat meatballs with guar and carrageenan gums decreased the hardness of the sample (Ulu, 2006).

As shown in Fig. 5b, the effects of gum blend and storage temperatures on fracturability were significant ($p < 0.05$). The carrageenan-xanthan blend gives halva more tender and more brittle texture than the other ones. The fracturability of the samples decreased at a higher temperature. Various studies have shown that the combination of two or more gums improved the texture of the food products. The simultaneous use of several gums resulted in better synergistic effects (Philips et al, 2000). Dehdashtiha et al. (2015) studied the impact of the addition of xanthan and guar gums on the textural properties of hamburger. The results showed that the use of the gum blend increased the softness and also improved its organoleptic properties.



(a)



(b)

Figure 5: The effect of gum blend and storage temperature on the firmness (a) and fracturability (b) of halva Gazi

4 CONCLUSION

Halva Gazi is one of the traditional desserts with a high nutritional value which is produced in Iran. This product uses as a substitute for popular high-calorie foods such as breakfast chocolates and other fatty desserts. In this study, different levels of gums including xanthan, carrageenan, alginate gums and their blends were used to improve the texture of the halva Gazi during storage at 25 °C and 40 °C. The products containing gum blend and also xanthan up to 1% concentration produced an appropriate texture. However, the use of alginate and carrageenan gums increased the firmness of the halva in comparison to the control. The samples containing 0.5 and 1 % of alginate and xanthan as well as xanthan-alginate blend and xanthan-carrageenan blend had the best texture properties during storage at 40 °C. These samples showed the most desirable softness at room temperature and the appropriate texture stability at 40 °C.

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Effect of soil conditioner enriched with biofertilizers to improve soil fertility and maize (*Zea mays* L.) growth on andisols Sinabung area

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Effect of soil conditioner enriched with biofertilizers to improve soil fertility and maize (*Zea mays* L.) growth on andisols Sinabung area

Abstract: Andisol soil contains a lot of heavy metals Al and Fe, which results in P being unavailable to plants and can cause low soil pH, this will inhibit plant growth. One effort that can be made to increase the availability of nutrients in the soil is by utilizing soil enhancing ingredients enriched with biological fertilizers. The research design used was factorial randomized block design (RBD) consisting of: Factor I: (biological fertilizers) M0 = without application, M1 = *Talaromyces pinophilus* (Hedgecock), M2 = *Azotobacter* sp, M3 = Mycorrhizae, M4 = *Talaromyces pinophilus* + mycorrhizae + *Azotobacter* sp. Factor II Soil enhancing ingredients, namely P0 = Without Soil Improvement, K1 = Zeolite 50 g, K2 = Humic acid 50 ml, K3 = compost / manure fertilizer for agriculture 50 g. From the results the combination of microbial treatment and soil conditioner can increase nitrogen in the soil by 2-40.81 %, cation exchange capacity by 1.7-44.29 % and P available by 1.3-49.36 %. Soil conditioner combined with biological fertilizers can improve soil quality in general, the best treatment is a combination treatment of *T. pinophilus* + mycorrhizae + *Azotobacter* sp. with coffee skin (M4P4).

Key words: andisol; biological fertilizers; maize; soil conditioner; soil fertility

Dodatek izboljševalcev tal obogatenih z biognojili izboljša rodovitnost tal in rast koruze (*Zea mays* L.) na andisolih na območju Sinabung, Indonezija

Izvleček: Andisoli vsebujejo veliko težkih kovin, Al in Fe, kar povzroča nedostopnost P rastlinam in lahko zniža pH tal in s tem zavira rast rastlin. Eden od načinov za povečanje razpoložljivosti hranil v takšnih tleh je uporaba talnih izboljševalcev obogatenih z biognojili. Poskus v raziskavi je bil popolni naključni bločni poskus, ki je obsegal naslednja obravnavanja: obravnavanje I: (biognojila) M0 = brez gnojil, M1 = *Talaromyces pinophilus* (Hedgecock), M2 = *Azotobacter* sp., M3 = mycorrhiza, M4 = *Talaromyces pinophilus* + mycorrhizae + *Azotobacter* sp. Obravnavnaje II- z dodatki izboljševalcev tal: P0 = brez izboljševalcev tal, K1 = zeolite 50 g, K2 = huminska kislina 50 ml, K3 = kompost / kurji gnoj 50 g. Rezultati so pokazali, da je kombinacija obravnavanja z mikrobi in talnimi izboljševalci povečala vsebnost dušika v tleh za 2 do 40,81 %, kationska izmenjevalna kapaciteta se je povečala za 1,7-44,29 % in vsebnost dostopnega fosforja za 1,3 do 49,36 %. Izboljševalci tal v kombinaciji z biognojili lahko na splošno izboljšajo kvaliteto tal. V poskusu je bilo najboljše obravnavanje *T. pinophilus* + mycorrhizae + *Azotobacter* sp. z dodatki ostankov iz pridelave kave (M4P4).

Ključne besede: andisol; biognojila; koruza; izboljševalci tal; rodovitnost tal

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1 INTRODUCTION

The accumulation of organic matter and the occurrence of organic matter complexes with Al are characteristic of some andisol soils. The research results of Ritonga et al (2015) and Sembiring et al. (2016), stated that the pH of andisol soil affected by the eruption of Mount Sinabung ranges from 3.7 to 4.7 which is in the very acidic category. The low pH of the soil affected by the eruption of Mount Sinabung results that the availability of P in the soil can be hampered. One effort that could be made to increase nutrient availability and andisol soil fertility is by applying soil conditioner enriched with biological agents, namely organisms that are useful in increasing the availability of nutrients for plants. According to Sembiring et al. (2016; 2017a; 2017b), application of phosphate solvent microbes (*Talaromyces pinophilus*) can increase the availability of P by 9.63 to 49.78 % in andisol soil. Observations of Marbun et al. (2015), shows that application of phosphate solvent fungi and organic matter can increase P uptake and potato plant growth on andisol soil affected by the eruption of Mount Sinabung. According to Sembiring et al. (2018), Hijikata et al. (2010), Kikuchi et al. (2014) application of mycorrhizae can improve plant growth. Application of *Azotobacter* can increase plant growth (Kizilkaya, 2009; Patil et al., 2011; Ponmurugan et al., 2012).

Agricultural development in Indonesia is faced with soil quality problems which are generally classified as low, characterized by problems with poor nutrients and organic matter, high soil acidity, and soil physical properties that do not support plant growth. High soil fertility shows high soil quality (Biswas and Kole, 2017; Doran and Parkin, 1994). Soil quality is the capacity of the soil which functions to maintain crop productivity. Good soil quality will support soil function, working as a medium for plant growth, regulate and divide water flow and support a good environment (Krener, 2013; Pal, 2016).

Soil conditioner is a material that can be used to accelerate the recovery / improvement of soil quality. Organic matter, besides being able to function as a source of nutrients, s functions as a soil conditioner has also been widely proven. In addition to organic soil conditioner, there are mineral soil conditioner that can be used to improve soil quality. Zeolite is a mineral material that can be used as a soil conditioner (Suwardi and Goto, 1996; Juarsah, 2016). Application of zeolite can increase the efficiency of fertilization, cation exchange capacity (CEC), soil potassium, P availability and plant growth (Juarsah, 2016; Balqies, et al., 2018). Cover crops that produce organic materials play an important role in improving soil quality because they protect the soil from erosion, and create suitable environmental conditions for microbial habitats that play a role in nutrient cycling (Marzaioli et al., 2010; Krener, 2013). Maize is a plant that can be used as an indicator because it requires fertile soil to produce properly. This is because maize plants need nutrients, especially nitrogen (N), phosphorus (P) and potassium (K) in large quantities. In general, maize plants require loose soil, fertile and rich in organic matter. Therefore this study aims to improve soil fertility and growth of maize using a soil conditioner and biofertilizers.

2 MATERIALS AND METHODS

Research was carried out in Kuta Rayat Village, Karo Regency. Indicator plant used was maize variety Super Sweet. The research was conducted for 3 months. The andisol soil characteristics used were: pH 4.56, 4.8 % organic C (Walkey and black titration method) P availability is at 67.28 ppm (Bray II method), soil N is at 0.55 % (Kjedahl-Titrimetry Method) and CEC 19.87 %me kg^{-1}. Materials used were: *T.pinophilus* (Hedgecock) Samson et al., *Azotobacter* sp. and mycorrhiza *(Glomus* sp.*)* used were obtained from the Laboratory of Soil Biology, Faculty of Agriculture, University of Sumatera Utara.

The research design used factorial randomized block design (RBD) with two factors and three replications. Factor I was biofertilizers consisting of treatments M0 = without application, M1 = 30 g *T.pinophilus* (18 x 10^9 CFU g^{-1}), M2 = 30 g mycorrhiza (*Glomus* sp.), M3 = 30 g *Azotobacter* sp. (18 x 10^9 CFU g^{-1}) and M4 = 10 %g *T. pinophilus* + 10 g mycorrhiza + 10 g *Azotobacter* sp.. Factor II: material for soil conditioner (application 50 %g/plant, equivalent to 1t ha^{-1}), P0 = without soil conditioner, P1 = zeolite, P2 = humic acid, P3 = chicken manure and P4 = coffee shell. Plot size 0.6 x 4.20 m, the distance between plots within a block was 30 cm and spacing between blocks was 50 cm. Soil conditioner application was carried out 2 weeks before the corn plant was planted by applying it to the planting hole and then mixed evenly. Application of biofertilizers was carried out after 1 week of plant growth by applying it around the plant roots. Basis fertilizers used were urea 3 g, super phosphat 36 (SP 36) and KCl 5 g/plant application was carried out 2 day before the corn plant was planted by applying it to the planting hole and then mixed evenly.

2.1 DATA COLLECTION

Phosphorus analysis (P) availability in soil with Bray II method, soil N nutrient content, soil K nutrient content in 25 % HCl, cation exchange capacity (CEC),

soil pH, microbial population, nutrient content of N, P, K of plants and plant dry mass all parameters observed at the end of the vegetative period. Soil and plant samples were taken after plant growth for 45 days. By taking 1 sample/plot where the sample is randomly determined at the beginning of plant growth.

2.2 STATISTICAL ANALYSIS

Statistical analysis used to see the effect of treatment was carried out on F test at the level of 5 % and continued with LSD test at 5 % level (Gomez and Gomez, 1984).

3 RESULTS AND DISCUSSION

3.1 RESULTS

The results of observations and statistical analysis of soil conditioner applications and biological agents on all parameters of observation, can be seen in Table 1 and Table 2. Application of biological fertilizers had no significant effect on increasing soil pH, soil total N, soil K and soil CEC but had significant effect ($p \leq 0.05$) on available P in soil (Table 1). Application of biological agents (M) can increase the availability of phosphorus (P) in soil. Soil conditioner have a significant effect ($p \leq 0.05$) on the soil K content and plant dry mass, zeolite treatment (P1) increases soil K levels for 24.34 % when compared with no application (P0). The treatment combination of soil conditioner and biological fertilizers can increase cation exchange capacity for 44.29 % when compared with control (Figure 1), soil N content, the best treatment was *T.pinophilus* and coffee peels (M1P4) increased soil N content by 40.81 % (Figure 2) and available P for 49.36 % when compared to control (Figure 3).

Application of biofertilizers increases nutrient content of N, P, K in plants and plant dry mass (Table 2). The application of *T. pinophilu*s + mycorrhizae + A*zotobacte*r sp. (M4) increases the nutrient P content in plants by 19.81 % and plant dry mass by 25.94 % when compared with the control. Application of mycorrhiza and zeolite (M3P1) can increase potassium (K) content in soil by 19.10 % (Figure 4). The treatment combination of soil conditioner and biological fertilizers can increase nutrient K content in plants (Figure 5), Treatment of *T. pinophilus* + mycorrhizae + *Azotobacter* sp. (M4) and coffee skin increase nutrient K content in plants by 28.83 % and plant dry mass by 25 % when compared to control.

Table 1: Average of soil pH, microbial population, levels of N, P, K soil and CEC treatment of soil conditioner enriched with biofertilizers

Treatment	Soil pH	Microbial Population (10^8 CFU g^{-1})	Soil N (%)	P-Available (mg kg^{-1})	Soil K (mg kg^{-1})	CEC (mol kg^{-1})
Biofertilizers (M)						
M0	5.46±0.24	33.53±11.48a	0.58±0.05	93.69±27.64a	601.19±54.07	19.38±2.34
M1	5.41±0.13	44.87±9.80bcd	0.56±0.08	118.07±15.26ab	581.46±52.72	19.81±2.39
M2	5.53±0.05	44.13±8.35ab	0.53±0.04	84.86±8.34a	563.24±110.07	18.12±1.60
M3	5.47±0.17	38.93±8.74a	0.56±0.06	101.99±19.16a	610.20±168.77	19.87±2.72
M4	5.61±0.10	44.47±13.24abc	0.55±0.03	100.63±26.02a	611.74±71.06	19.27±1.73
Soil conditioner (P)						
P0	5.5±0.17	39.53±10.93a	0.52±0.05	101.34±19.76	562.93±59.10a	18.44±1.22a
P1	5.46±0.18	41.80±7.78a	0.57±0.05	103.83±20.48	700.74±115.48b	21.16±2.84ab
P2	5.45±0.21	39.6±11.86a	0.56±0.05	103.84±17.26	567.23±73.34a	19.96±1.86a
P3	5.59±0.07	45.07±11.70ab	0.56±0.04	88.49±24.95	568.21±77.67a	18.76±2.01a
P4	5.47±0.15	39.93±13.59a	0.57±0.08	101.72±30.37	568.74±85.46a	19.13±2.20a
M	NS	*	NS	*	NS	NS
P	NS	*	NS	NS	*	*
MxP	NS	NS	*	*	*	*
CV%	6.38	27.67	10.34	27.8	10.17	11.89

Description: *significant at $p \leq 0.05$ and NS Not significance. Means in a column followed by a common letter are not significantly different at the level 0.05 level by LSD. M0 = without application, M1 = *Talaromyces pinophilus*, M2 = *Mycorrhizae*, M3 = *Azotobacter* sp., M4 = *T. pinophilus* + Mycorrhizae + *Azotobacter* sp., P0 = without soil conditioner, P1 = zeolite, P2 = humic acid, P3 = chicken manure and P4 = coffee skin.

Table 2: Average of N, P, K nutrient content in plants and plant dry mass in soil conditioner treatment enriched with biofertilizers

Treatment	N nutrient content in plant (%)	P nutrient content in plant (%)	K nutrient content in plant (%)	Plant dry mass (g)
Biofertilizers (M)				
M0	1.32±0.11	0.222±0.06a	2.42±0.23	206.46±41.69b
M1	1.52±0.23	0.256±0.03a	2.31±0.14	211.19±37.95c
M2	1.34±0.18	0.257±0.02b	2.44±0.28	236.53±45.45d
M3	1.32±0.13	0.261±0.02ab	2.22±0.34	200.02±33.20a
M4	1.42±0.21	0.266±0.02bc	2.34±0.33	260.02±28.06e
Soil conditioner (P)				
P0	1.35±0.22a	0.241±0.03a	2.21±0.39a	207.69±43.19a
P1	1.23±0.11a	0.251±0.02a	2.36±0.25ab	205.14±36.20a
P2	1.38±0.17a	0.266±0.03a	2.45±0.21bcd	230.77±49.94d
P3	1.49±0.19bc	0.273±0.01ab	2.28±0.17a	210.53±35.29bc
P4	1.47±0.13ab	0.231±0.05a	2.43±0.27abc	260.09±27.22e
M	NS	*	NS	*
P	*	*	*	*
MxP	NS	NS	*	NS
CV	18.4	15.9	9.74	22.09

Description: *significant at $p \leq 0.05$ and NS Not significance. Means in a column followed by a common letter are not significantly different at the level 0.05 level by LSD. M0 = without application, M1 = *Talaromyces pinophilus*, M2 = *Mycorrhizae*, M3 = *Azotobacter* sp, M4 = *T. pinophilus* + *Mycorrhizae* + *Azotobacter* sp, P0 = without soil conditioner, P1 = Zeolite, P2 = Humic acid, P3 = Chicken manure and P4 = Coffee skin.

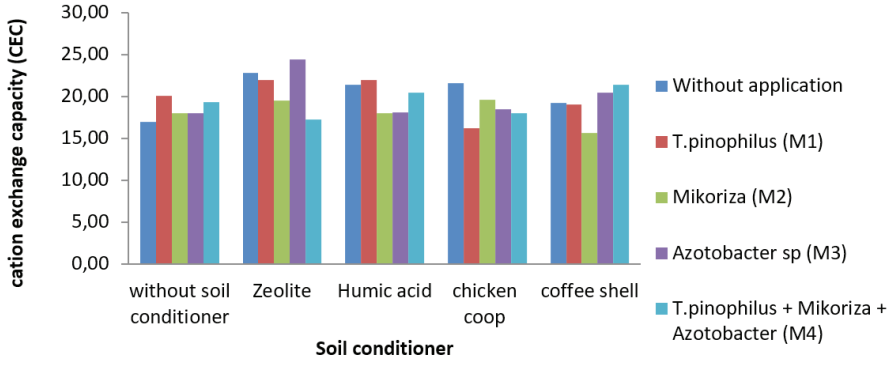

Figure 1: Interaction of soil conditioner and biofertilizers to CEC in the soil (mol kg $^{-1}$)

3.2 DISCUSSION

The application of *T. pinophilus* (M1) increases the P availability by 26.02 % when compared to without application (M0) and can increase the P available by 102.32 % when compared with the initial soil analysis (67.28 %). This is because *T.pinophilus* used is an environment-specific microbe isolated from areas affected by Mount Sinabung eruption (Sembiring and Fauzi, 2017). Rao (1999) argues that the mechanism of releasing P-bound from Al, Fe, Ca and Mg can be carried out in the presence of organic acids through chelating processes so that P becomes available and be absorbed by plants.

Soil conditioner increases soil K content and plant dry mass, zeolite treatment (P1) can increases soil K nutrient levels by 24.34 %. According to Suwardi (2009), zeolite as an enhancer given to the soil with sufficient quantities can improve soil physical, chemical and bio-

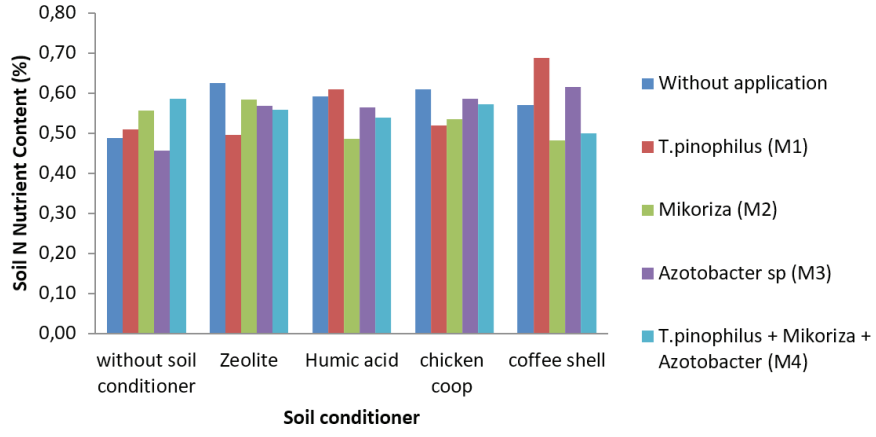

Figure 2: Interaction of soil conditioner and biofertilizers to soil N nutrient content (%)

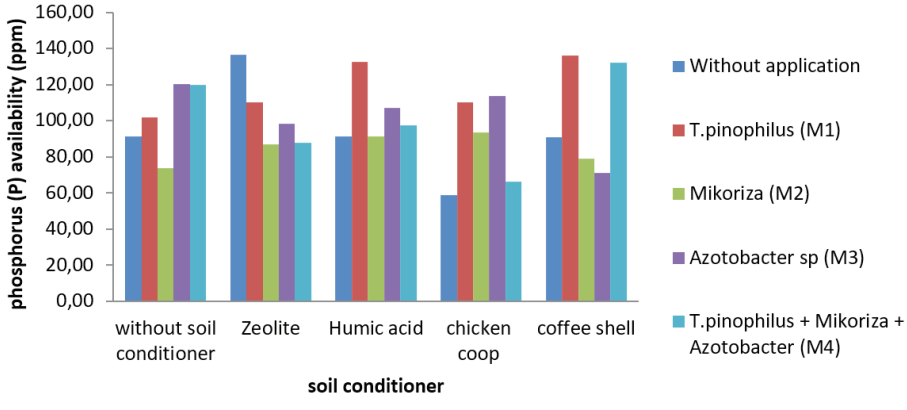

Figure 3: Interaction of soil conditioner and biofertilizers to P available in the soil (mg kg^{-1})

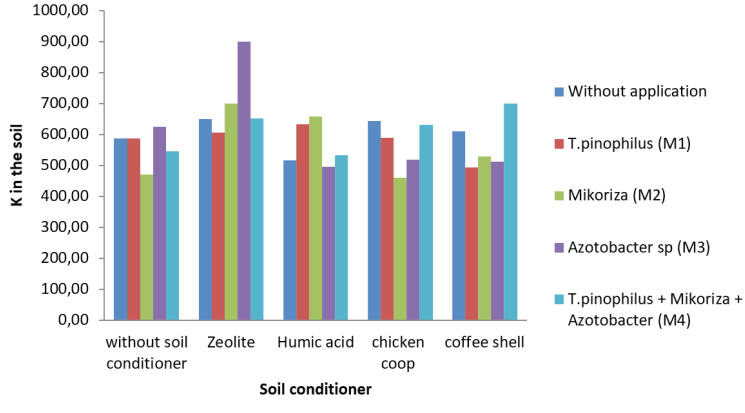

Figure 4: Interaction of soil conditioner and biofertilizers to K in the soil (mg kg^{-1})

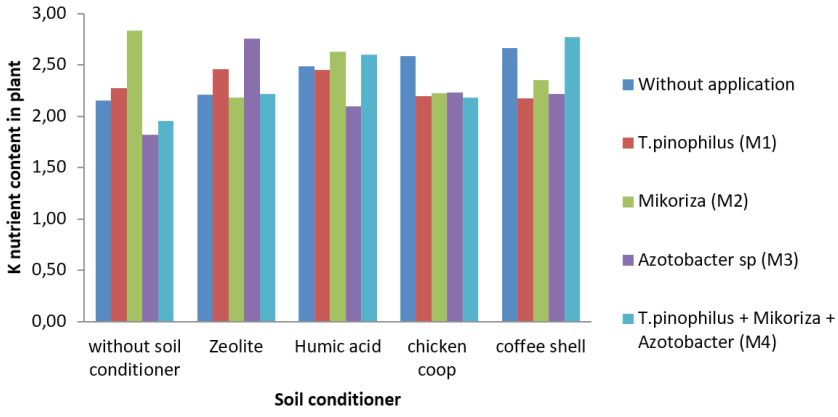

Figure 5: Interaction of soil conditioner and biofertilizers to nutrient K content in the plant (%)

logical properties so that agricultural production can be increased. According to Ahmed et al. (2010), zeolite is able to increase the availability of nutrients in the soil and soil nutrient uptake from fertilizers that are used to improve the efficiency of the availability of nutrients in the soil and reduce soil bleaching so that nutrients can be used for corn plant growth.

The treatment combination of soil conditioner and biological agents can increase soil N content for 40.81 % and P available for 49.36 %. The research results of Sembiring et al., 2017b; Masdariah et al., 2019; Siswana et al., 2019 stated that the application of phosphate solubilizing microbes and organic matter can increase the P availability in andisol. Phosphate solubilizing microbes and organic matter are able to produce organic acids so that the availability of nutrient elements in the soil increases (Richardson, 2001; Gyaneshwar et al., 2002). Application of mycorrhiza and zeolite (M3P1) can increase potassium (K) content in soil and cation exchange capacity. The ability of zeolites which can improve the physical, chemical and biological properties of soil combined with mycorrhiza applications is expected to increase the cation exchange capacity of the soil as observed from the results of Suwardi and Wijaya (2013).

The application of *T. pinophilu*s + mycorrhizae + A*zotobacte*r sp. (M4) increases the nutrient P content in plants and plant dry mass. The application of *T.* p*inophilu*s (M1) can increase the P available for 26.02 % when compared to without application (M0) and can increase the P available for 102.32 when compared with the initial soil analysis (67.28 %). This is because T.*pinophilus* used is an environment-specific microbe isolated from areas affected by Mount Sinabung eruption (Sembiring and Fauzi, 2017). According to Al-Karaki (2000; 2006); Gamper et al., 2004; Chiou et al., 2001) application of mycorrhizae can increase nutrient P uptake of plant. The research results of Sembiring et al, (2018); Farzaneh et al. (2011) stated that the application of mycorrhizae could increase nutrient P uptake for 67.83 % and plant growth for 59.45 %. According to Redman et al., 2002; Lewis 2004, the presence of mycorrhizae in plant roots can improve the ability to absorb nutrients so that plant growth becomes elevated.

Soil conditioner can increase soil nutrient content. Chicken manure treatment (P3) increases the nutrient N content in plants for 10.37 % and the nutrient P content in plants for 13.27 % when compared to control (P0). Sembiring and Fauzi (2017) stated that the application of chicken manure could increase the P uptake of plant for 6 % when compared to without application. Application of humic acid (P2) can increase the nutrient K content in plants for 28.83 % when compared to control. The treatment combination of soil conditioner and biological agents can increase nutrient K content in plants, treatment of *T. pinophilus* + mycorrhizae + *Azotobacter* sp. (M4) and coffee skin can increase nutrient K content in plants, plant dry mass. This is because the ability of each microbial is different and so if put together will expected to increase plant growth. This finding is in consistence with the findings of Sembiring et al. (2016).

Soil conditioner and biological agents can improve soil quality, namely pH 4.56 to 5.61 in this case ΔpH is 1.05. The effect of the increasing P available in the soil, the initial soil was 67.28 ppm to 118.07 ppm the increase in p available was at Δ50.76 ppm. The soil N parameters observed increased with the treatment, the initial soil analysis of soil N content was 0.55 % to 0.57 increase in soil N by ΔN 0.02 and CEC 20.87 increased to 21.16 me kg^{-1} so that ΔCEC 0.29 was obtained. From the results of this research we observe that the treatment applications improve soil quality which is in line with the findings of according to Kirener (2013); Marzioli (2010).

4 CONCLUSIONS

This study found superior soil repairers and microbes that can increase soil fertility and plant growth. This research will help researchers and farmers to overcome the low soil fertility in andisols. Thus the new theory found that the interaction of coffee skin with *T. pinophilus* + mycorrhiza + *Azotobacter* sp. was best for increasing plant growth in andisol soil which had an impact on Sinabung eruptions.

5 ACKNOWLEDGEMENT

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Antioxidant activity and acute toxicity of two *Lagenaria siceraria* (Molina) Standl. varieties from Sudan

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Antioxidant activity and acute toxicity of two *Lagenaria siceraria* (Molina) Standl. varieties from Sudan

Abstract: The present study was conducted to evaluate the antioxidant capacity and acute toxicity of the methanol extract of two *Lagenaria siceraria* (Molina) Standl. varieties of dried seeds, Sweet gourd Water Jug (WJ) and bitter gourd Basket Ball (BB). The seed extracts were tested for their total phenolic contents (TPC), total flavonoid contents (TFC), ferric reducing antioxidant power (FRAP) and scavenging activity to the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Both dried seed extracts were then tested for acute toxicity at doses 2 and 5 g kg^{-1} each. Sixty female and male rats were assigned into five groups per sex. Four groups were given low and high doses of each dried seed extract and the fifth group was given 10 % Tween 20 as a control. Kidneys and livers of all rats were assessed for biochemistry and histopathology. The seed extract of WJ revealed higher TPC, FRAP and DPPH activities compared to BB seeds, while TFC results were reversed. Toxicologically, no toxicity signs were recorded in animals. Biochemistry results were within the normal ranges with a slight increase in bilirubin and Alanine aminotransferase (ALT), and histology of kidneys and livers showed normal architecture. In conclusion, WJ and BB dried seed extracts exhibited high antioxidant activity suggesting promising therapeutic regimen against oxidative stress.

Key words: Lagenaria siceraria; antioxidant; acute toxicity

Antioksidacijska aktivnost in akutna toksičnost dveh sort vodnjače (*Lagenaria siceraria* (Molina) Standl.) iz Sudana

Izvleček: Raziskava je bila izvedena za ovrednotenje antioksidacijske sposobnosti in akutne toksičnosti metanolnih izvlečkov suhih semen dveh sort vodnjače (*Lagenaria siceraria* (Molina) Standl.)), sladke 'Water Jug' (WJ) in grenke vodnjače 'Basket Ball' (BB). V izvlečkih semen so bili analizirani celokupna vsebnost fenolov (TPC), celukupna vsebnost flavonoidov (TFC), velikost redukcije železa (FRAP) in lovilna aktivnost stabilnega prostega radikala 2,2-difenil-1-pikrilhidrazila (DPPH). Oba izvlečka sta bila potem preiskušena na akutno toksičnost z odmerkoma 2 in 5 g kg^{-1}. 60 podganjih samic in samcev je bilo razdeljeno glede na spol v pet skupin. Vsaka od štirih skupin je dobivala velike in majhne odmerke izvlečka semen, peta skupina pa je dobila 10 % Tween 20 kot kontrolo. Ledvice in jetra vseh podgan so bile ocenjene po biokemičnih in histopatoloških parametrih. Izvleček semen WJ je pokazal večjo aktivnost TPC, FRAP in DPPH v primerjavi s semeni BB, a rezultati TFC so bili obratni. Na živalih ni bilo opaziti nobenih znakov zastrupitve. Biokemični parametri so bili znotraj normalih meja z rahlim povečanjem vsebnosti bilirubina in aktivnosti alanin aminotransferaze (ALT). Tudi histološki pregled ledvic in jeter je pokazal normalno zgradbo. Zaključimo lahko, da imajo izvlečki suhih semen WJ in BB veliko antioksidacijsko aktivnost, kar obeta njihovo uporabo pri blaženju oksidacijskega stresa.

Ključne besede: *Lagenaria siceraria;* antioksidant; akutna toksičnost

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1 INTRODUCTION

Medicinal plants are still considered as cheap and safe, natural resource of drugs with less toxicity all over the world. Sudanese locals and people of other developing countries have relied on traditional herbs to treat their diseases (Elhadi et al., 2013). Therefore, it is useful to explore more about medicinal plants (Koko et al., 2008). Family Cucurbitaceae is a very large family composed of 118 genera and 825 species. One of the most important genera of this family is *Lagenaria* (Molina) which is also known as bottle gourd, calabash gourd or white flowered gourd plant (Fader et al., 2013). In addition to *Lagenaria siceraria,* genus *Lagenaria* consists of five other wild species, namely *L. brevifilora* (Benth) Roberty, *L. rufa* (Gilg) C Jeffrey, *L sphaerica* E Mey, *L. abyssinia* (Hook. F.) C Jeffrey and *L. guineensis* (G Den) C Jeffrey. *L. siceraria* is an indigenous species native to India (Shah et al., 2010) and became widely spread in Africa and the most cultivable species in Asia and America (Erickson et al., 2005). Shah et al. (2010) reported that *Lagenaria* has two distinguished varieties, one is bitter bottle gourd and sweet variety useful bottle gourd. The bitter variety is wild plant and used for the pharmacological application, while sweet variety is used as a vegetable and medicinal. In Sudan, varieties of *L. siceraria* ,the sorcerer is broadly diffused in different areas of the South and West, while some of them are edible such as snake gourd. Recently, researchers focused their study on *L. siceraria* varieties exploring more about their chemical composition. Studies reported that they can be used as nutritious source, in cosmetics and medicine (Mariod et al., 2015). Literature review reveals that *L. siceraria* showed a broad spectrum of pharmacological activities such as antioxidant (Mayakrishnan et al., 2013), antimutagenic (Thakkar, 2013), antiulcer (Srivastava et al., 2011), anti-inflammatory (Ghule et al., 2006), antidiabetic, hepatoprotective (Deshpande et al., 2008) and cardioprotective (Upaganlawar and Balaraman, 2011). Due to the presence of high percentage of alkaloids, phenolic glycosides, carbohydrates, proteins and minerals, *L. siceraria* can be suggested to replace allopathic medicines (Mariod et al., 2015; Sivannarayana et al., 2013). In folk medicine, different parts (leaves, stem, flower, root, and seeds, fresh and dry fruits) of *L. siceraria* have been used in the ointment for ailment of various diseases (Kumar et al., 2015).

Traditionally, *L. siceraria* seeds can be cooked, dried and served as snacks in some countries such as Egypt. Also can be ground and fermented for use as a flavor enhancer in gravies and soups in West Africa (Achigan-Dako et al., 2008). At the phytochemistry level, studies proved the presence of various phytochemicals like cardiac glycosides, terpenoides, carbohydrates, resins, saponins and phytosterols in *L. siceraria* seeds (Sood et al., 2012). Singh et al. (2012) suggested that the ethanol extract of *L. siceraria* seeds exhibits cardioprotective potential in experimental animals against cardiac toxicity induced by doxorubicin. Another study revealed that the ethanol extract of *L. siceraria* seeds showed excellent free radical scavenging activity (Gill et al., 2012). As an extension to our previous study on the eight Sudanese *Lagenaria* varieties (Mariod et al., 2015), the present study was conducted to determine the total phenol and total flavonoid contents, and acute oral toxicity of the methanol extract of sweet and bitter varieties of *L. siceraria* seeds from Sudan.

2 MATERIAL AND METHODS

2.1 IN VITRO ANTIOXIDANT CAPACITY STUDY OF WJ AND BB METHANOL EXTRACTS OF DRIED SEEDS

2.1.1 Plant material

Two varieties of *Lagenaria siceraria* dry fruits {Sweet gourd 'Water Jug' (WJ) and bitter gourd 'Basket Ball' (BB)} were obtained from the market of Omdurman, State of Khartoum, Sudan.

2.1.2 Preparation of seeds extracts

By the aid of 4.8 mm knife, the seeds were removed from the collected dried fruits. The removed seeds were properly washed and left dried at 50 °C in drying oven (FD 115; Fisher Scientific, Loughborough, Leicestershire, UK). The dried seeds were then ground to a powder using home blender and stored in polyethylene bags in the refrigerator at 4 °C till the onset of the experiment as previously done (Mariod et al., 2015). For seed extraction, the seed powder was firstly defatted using n-hexane at 50–60 °C in a Soxhlet apparatus for six hours following the AOCS method (Firestone, 2009). The dried seeds (seedcake) were extracted for 72 h in methanol 95 % at room temperature with occasional stirring. The solvent was filtered off using filter paper (Whatman No.1, Fitchburg, WI, USA) and the filtrate obtained was concentrated under reduced pressure on a rotary evaporator at 40 °C and finally freeze dried using a freeze-drying machine (LabConco, Kansas City, MO, USA). The dried seed extracts obtained from both water jug seeds (WJ) and basketball seeds (BB) were then stored at 4 °C for further use for various investigations.

2.1.3 Total phenol content (TPC) determination

Total phenolic content was determined using Folin-Ciocalteu reagent following the method of Singleton and Rossi, (1965) and using gallic acid as a standard. An amount of 10 µl of each WJ and BB dried seed extract solution (1 mg ml^{-1}) was added in a test tube followed by the addition of 0.5 ml of 1:10 Folin-Ciocalteu reagent. The mixture was incubated at room temperature for 5 minutes. Following incubation, 0.35 ml of 115 mg ml^{-1} natrium carbonate (Na_2CO_3) was added and mixed thoroughly. The mixture was then allowed to stand at room temperature for 2 hours. Absorbance reading taken spectrophotometrically at 765 nm and all determinations were done in triplicates. The total phenolic content of WJ and BB were expressed as milligrams of gallic acid equivalent to grams of dried seed extract.

2.1.4 Total flavonoid content (TFC) determination

Total flavonoid content of WJ and BB seed extracts were determined by aluminum chloride colorimetric method using quercetin as a standard (Chang et al., 2002). Briefly, 0.5 ml of each seed extract solution (1 mg ml^{-1}) was sampled in a test tube and mixed with 1.5 ml ethanol 95 %, 0.1 ml aluminum chloride and 2.8 ml distilled water. The mixtures were left to incubate for 30 minutes at room temperature followed by reading the absorbance at 415 nm. The total flavonoid content of WJ and BB were expressed as milligrams of quercetin equivalent to grams of dried plant material.

2.1.5 Ferric reducing antioxidant power (FRAP) determination

Ferric reducing activity of the WJ and BB seed extracts was estimated using the method developed by Benzie and Strain (1996), while vitamin C (Vit. C) was taken as reference. The principle of the method depends on the chelating power of substances to ferric tripyridyltriazine complex. Briefly, the working FRAP reagent was freshly prepared by mixing 300 mmol l^{-1} acetate buffer, 10 mmol l^{-1} TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mmol l^{-1} of HCL and 20 mmol l^{-1} of $FeCl_3.6H_2O$. The prepared mixture was then incubated at 35 °C in water bath for five minutes and then a blank reading was taken spectrophotometrically at 593 nm. A quantity of 10 µl of each extract/reference was sampled separately and mixed with 290 µl of the working FRAP reagent followed by vortexing and reading the absorbance immediately at 593 nm to get the record of zero minutes. Thereafter, the absorbance reading was taken every four minutes for a period of 2 hours. All the results were expressed as mmol ferric reducing activity of the extract per gram of dried weight based on three experiments.

2.1.6 DPPH free radical scavenging activity determination

In the DPPH radical scavenging test, the scavenging of DPPH is followed by monitoring the decrease in absorbance at 515 nm that occurs due to reduction by the antioxidant or reaction with a radical species. DPPH is widely used to test for the ability of compounds to act as hydrogen donors or free radical scavengers and to evaluate antioxidant activity of foods (Kadhim et al., 2019). Scavenging activity of the WJ and BB dried seed extracts to the stable 2, 2 diphenyl-1-picryl dyhydrazyl (DPPH) free radical was determined according to the method of Gorinstein et al. (2005) with slight modification. Briefly, the dried seed extracts and the reference standard (ascorbic acid) were prepared (1 mg ml^{-1}) as separate stock solutions and then serial dilution with 8 varying concentrations were prepared (0.37, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 µl) from each stock solution. Five microliters of each sample was loaded in 96-well plate followed by the addition of 195 µl of DPPH reagent; the mixtures were then incubated in the dark at room temperature for 2 hours. At the end of the incubation period, the absorbance was measured spectrophotometrically at 515 nm with a Hitachi spectrophotometer (Hitachi, LTD.Tokyo, Japan), the DPPH percentage inhibition was calculated using the following formula:

DPPH (%) = [(Abs of blank-Abs of sample)/Abs of blank] ×100.

The results were expressed as IC_{50} value which is the extract concentration required to reduce 50 % of the DPPH free radical.

2.2 IN VIVO ACUTE TOXICITY STUDY OF WJ AND BB METHANOL EXTRACTS OF DRIED SEEDS

2.2.1 Experimental animals

Sixty adult and healthy Sprague Dawley (SD) rats (6–8 weeks old) were obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur (Ethic no.PM/30/05/2012/NSIAW (R). The body

mass of SD rats was between 195–210 g. The animals were fed standard rat pellets and tap water.

2.2.2 Acute toxicity test

The acute toxicity study was performed to determine the safe dosage of the dried seed extracts. Sixty SD rats (30 males and 30 females) were randomly and equally divided into 5 groups per sex. The groups were categorized as control (10 % Tween 20, 5 ml kg^{-1}), high dose WJ extract 5 g kg (HD-WJ), low dose WJ extract 2 g kg^{-1} (LD-WJ), high dose BB extract 5 g kg^{-1} (HD-BB), and low dose BB extract 2 g kg^{-1} (LD-BB) (Co-operation and Development, 2002). To prepare the animals for dosing, they were allowed to fast for 14 hours without accessing food but with free access to water. All the rats were weighed recording the body mass on day 0. After dosing, food was prevented for an additional four hours and the rats were observed for 30 min and 2, 4, 8, 24 and 48 hours, post dosing for the appearance of any clinical or toxicological signs such as respiration, salivation, diarrhoea, tremors, eyes and mucus membrane, skin and fur, sleep pattern and any signs associated with the nervous system. The presence of any morbidity, mortality or behavioural changes was recorded. At the end of the experimental period, the body mass of all the rats was recorded. On the 15th day, the animals were given over-dose of anaesthesia (xylazine with ketamine) then sacrificed to collect their blood for biochemistry study and kidneys and livers for histology study. Serum biochemical parameters were investigated at University of Malaya Medical Centre using a Hitachi Autoanalyzer, Japan, after optimizing the machine as previously performed (Al Batran et al., 2013). Liver function parameters were assayed for total protein, albumin, globulin and conjugated bilirubin. In addition aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl tansferase (GGT) were measured as markers of liver injury (Fujii 1997; Young et al., 2008).

2.3 STATISTICAL ANALYSIS

Statistical analysis of the data was carried out by using the statistical program for the social sciences (SPSS) version 16 while applying the ANOVA test for comparing means, followed by Post-Hoc Tukey test. All the data were expressed as mean ± standard deviation (SD) of triplicates of the *in vitro* study and n = 6 for the *in vivo* study. The statistical value $p \leq 0.05$ was considered significant.

3 RESULTS AND DISCUSSION

3.1 IN VITRO ANTIOXIDANT RESULTS

3.1.1 TPC and TFC of WJ and BB methanol extracts of dried seeds

Antioxidants are the compounds that when added to food products, especially to lipids and lipid-containing foods, can increase the shelf life retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products during processing and storage. Synthetic antioxidants, such as butylatedhydroxyanisole (BHA) and butylatedhydroxytolune (BHT), have restricted use in foods as they are suspected to be carcinogenic *via* their binding ability to DNA and cellu-

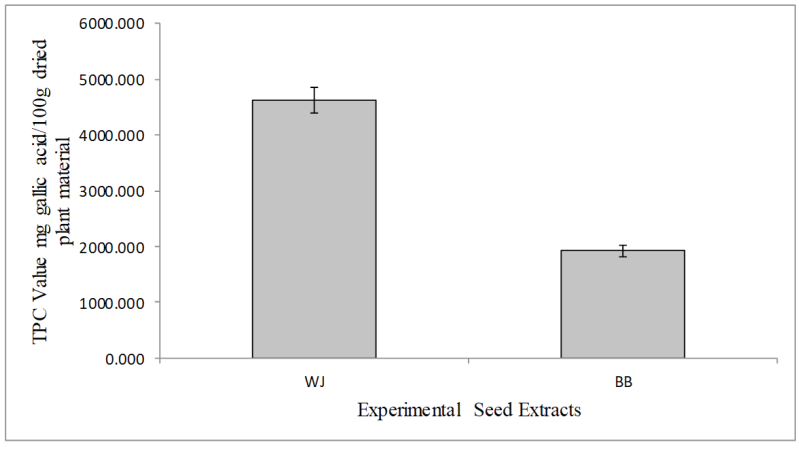

Figure 1: Total phenol content (TPC) of the methanol extracts of sweet gourd 'Water Jug' (WJ) and bitter gourd 'Basket Ball' (BB) dried seed extract

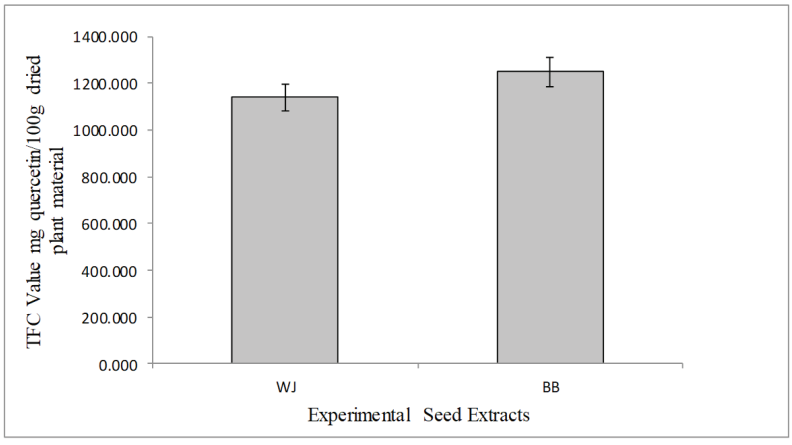

Figure 2: Total flavonoid content (TFC) of the methanol extracts of sweet gourd 'Water Jug' (WJ) and bitter gourd 'Basket Ball' (BB) dried seed extract

lar components (Dolatabadi and Kashanian, 2010; Mirza, Asema, and Kasim, 2017). Therefore, the exploitation of natural antioxidants, especially those of plant origin became necessary and attracted the attention of researchers in recent years (Moayedi et al., 2017). Aras et al. (2017) defined antioxidants as the substances that are capable of quenching or stabilizing free radicals.

In the present study, the seeds of dry fruits of two *Lagenaria siceraria* varieties, (sweet gourd Water Jug (WJ) and bitter gourd Basket Ball (BB) were ground, defatted and extracted in methanol and then tested for their total phenol and total flavonoid contents. The results of total phenol content (TPC) measured from the methanol extract of WJ and BB dried seeds are displayed in Fig.1. Results revealed that WJ seeds measured approximately two and half folds more than BB seeds indicating higher TPC of WJ seeds compared to BB seeds. The results of total flavonoid content (TFC) determined from the methanol extract of WJ and BB dried seeds are illustrated in Fig.2. Presented data recorded that the total flavonoid content of BB seeds was higher compared to that of WJ seeds.

3.1.2 FRAP and DPPH results of WJ and BB methanol extracts of dried seed

Free radicals, often called reactive oxygen species (ROS) and they are the core of any biochemical process representing a vital part in metabolism (Sangeetha and Venkatalakshmi, 2017). On the other hand, free radicals are highly generated in many pathological conditions and involved in the development and progress of common chronic degenerative diseases such as cardiovascular and neurodegenerative diseases, diabetes and cancer (Umeno et al., 2017). Disturbance in the balance between the formation of free radicals in the cells and their antioxidants load leads to oxidative stress that damages cellular components such as proteins, lipids and nucleic acids, and eventually leads to cell death (Shaker and Mnaa, 2017).

The FRAP values of WJ and BB seed extracts were displayed in Fig. 3 compared to vitamin C standard. The scavenging abilities of WJ and BB seed extracts to the stable DPPH free radical is diagrammed in Fig. 4 in comparison with ascorbic acid standard (vitamin C). Results recorded that IC_{50} of both dried seed extracts were significantly higher than that of ascorbic acid indicating less DPPH scavenging activity compared to ascorbic acid. However, the WJ seed extract showed higher DPPH scavenging activity as indicated by the lower IC_{50} value compared to BB seed extract.

Studying the chemical composition of natural products plays a crucial role in screening their biological activities. Natural products having a sufficient antioxidant capacity contain mainly phenolic and flavonoid compounds which exhibit a broad range of biological activities such as anticancer, anti-inflammation, anti-aging and anti-atherosclerosis (Rahman et al., 2013). Antia et al. (2015) studied the antioxidant activity of short-hybrid variety of *Lagenaria siceraria* seeds reporting that the methanol seed extract of that variety acquires significant antioxidant activity and the seeds can be used as excellent natural antioxidant. Later, another study conducted on the seed oil of short-hybrid variety and revealed significant DPPH scavenging activity (Antia et al. 2016).

In the present study, the seeds of dry fruits of two *Lagenaria siceraria* varieties, (sweet gourd 'Water Jug' (WJ) and bitter gourd 'Basket Ball' (BB) were ground, defatted and extracted in methanol and then tested for their total phenol and total flavonoid contents, their reducing power to Fe (III) and their ability to scavenge the stable DPPH free radical. The methanol extract of both seeds exposed high

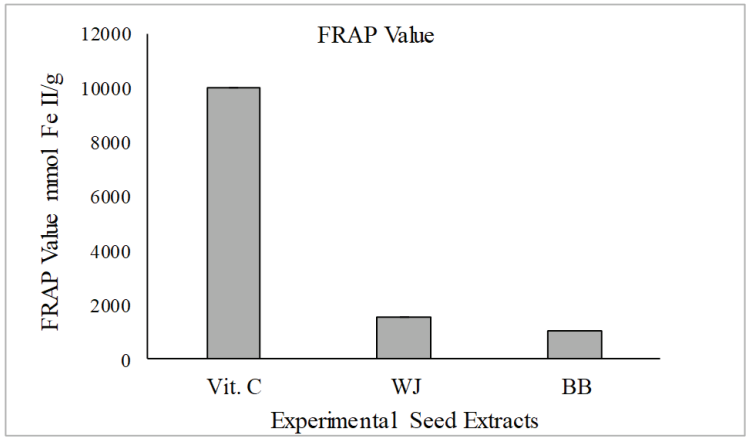

Figure 3: Ferric reducing antioxidant power (FRAP) of the methanol extracts of sweet gourd 'Water Jug' (WJ) and bitter gourd 'Basket Ball' (BB) dried seeds

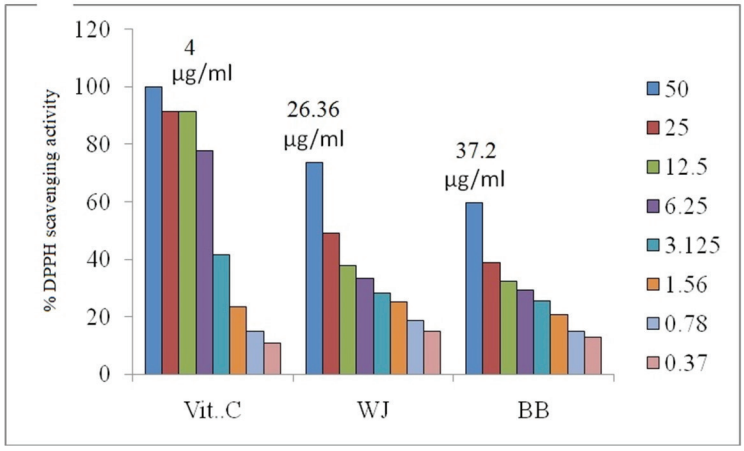

Figure 4: DPPH scavenging activities of the methanol extracts of sweet gourd 'Water Jug' (WJ) and bitter gourd 'Basket Ball' (BB) dried seeds compared to ascorbic acid standard (vitamin C). IC_{50} values are indicated on the bars.

antioxidant activity. Although WJ seeds acquired higher phenolic contents, FRAP and DPPH activity more than BB seeds extract, but BB seeds showed higher TFC compared to WJ seeds. Our study is consistent with a previous study on the remarkable antioxidant capacity of *L. siceraria* seeds (Satvir et al., 2012). Based on the results of DPPH of ascorbic acid, plant extracts WJ and BB, the R-squared (R^2) values of the standard curve equations generated were 0.9516, 0.9478 and 0.9765, respectively. All values were close to 1.0 indicating acceptable standard curves for all the tested samples ascorbic acid, WJ and BB (Salama et al., 2013). Recent studies on the antioxidant power of natural products manifested that they are very well recognized antioxidants, playing vital role in protecting biological cells and tissues from the detrimental effects of free radicals (Zeb and Mehmood, 2004). Therefore, they can be promising pharmaceutical compounds in preventing cancer and degenerative diseases as well (Olszowy, 2019).

3.2 IN VIVO ACUTE TOXICITY RESULTS

Acute toxicity studies in animals are usually necessary for any pharmaceutical substance intended for human use. The information obtained from these studies is useful in selecting doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity. Acute toxicity studies may also aid in the selection of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans (CDER and FDA, 1996). Past studies on acute toxicity of petroleum ether, ethanol and chloroform extracts of *L. siceraria* fruits in

rats recommended safe dose up to 2 g kg^{-1} (Deshpande et al., 2008). Additionally, previous study on the acute toxicity and sub-chronic toxicity of the methanol extract of *L. siceraria* aerial parts showed that it is quite safe and can be used in the treatment of chronic diseases like diabetes (Saha et al., 2011). In the present study, the methanol extracts of WJ and BB dried seeds of *L. siceraria* revealed safe dose up to 5 g kg^{-1}.

The findings from the acute toxicity study did not indicate any morbidity or mortality from treating male and female SD rats with the methanol extracts of WJ and BB dried seeds at doses 2 g kg^{-1} and 5 g kg^{-1} throughout the 14 days study period. Physical observation of the treated rats throughout the study didn't show any changes on skin, fur, eyes, mucus membrane, behaviour pattern, tremors, salivation or sleep. In addition no diarrhea or coma observed on any of the treated rats from both sexes.

Table 1: Effects of WJ and BB dried seed extracts on the body mass of the rats

Groups	Body mass (g) day 0	Body mass (g) day 14
10 % Tween 20, male	202.50±4.4	208.16±3.7
10 % Tween 20, female	194.16±8.5	196.83±8.9
HD-WJ, male	205.16±4.5	207.66±5.3
HD-WJ, female	195.33±7.5	198.00±7.4
LD-WJ, male	205.16±4.8	207.66±5.1
LD-WJ, female	194.16±8.4	196.00±7.0
HD-BB, male	205.83±3.6	207.33±3.6
HD-BB, female	194.50±7.9	196.00±7.5
LD-BB, male	205.83±9.3	208.00±8.1
LD-BB, female	193.16±10.5	196.00±10.0

Values expressed as mean ± SD, the significant value was set at $p \leq 0.05$.

Table 2: Effects of WJ and BB dried seed extracts on renal function parameters

Groups	Sodium mmol l^{-1}	Potassium mmol l^{-1}	Chloride mmol l^{-1}	Carbon Dioxide mmol l^{-1}	Anion Gap mmol l^{-1}	Urea mmol l^{-1}	Creatinine umol l^{-1}
10 % Tween20 Male	146.5±2.3	5.66±1.20	105.00±1.2	26.06±1.10	23.70±0.89	5.00±1.90	36.30±2.14
10 % Tween20 Female	147.3±0.8	5.66±1.20	102.00±1.5	24.07±1.23	22.00±0.76	5.40±2.16	36.70±2.31
HD-WJ, Male	145.3±1.3	4.95±1.20	103.66±1.0	25.96±0.80	20.83±0.75	4.25±0.20	35.50±2`94
HD-WJ Female	146.3±1.2	4.66±.019	104.16±0.9	23.88±2.00	22.66±1.90	6.10±0.95	37.00±1.78
LD-WJ Male	144.3 ±1.3	5.85±0.72	103.58±1.6	24.25±1.70	23.00±1.30	5.81±0.33	33.33±2.64
LD-WJ Female	144.5±2.0	5.03±0.08	103.83±1.1	23.33±1.80	21.50±1.10	5.80±0.54	37.16±1.66
HD-BB Male	144.50±0.8	4.80±0.90	102.83±1.1	26.00±0.57	20.33±0.50	5.45±0.53	38.33±2.73
HD-BB Female	141.83±1.7	4.68±0.50	100.50±0.8	24.13±1.71	22.00±1.10	5.66±0.71	37.66±7.45
LD-BB Male	144.16±1.6	4.60±0.30	101.16±2.1	25.78±1.58	22.00±1.00	5.70±0.90	37.33±4.90
LD-BB Female	141.66±1.5	4.33±0.50	100.00±1.2	24.15±2.70	21.83±1.80	6.26±0.38	38.16±9.92

Values expressed as mean ± SD, the significant value was set at $p \leq 0.05$.

3.2.1 Effect of WJ and BB dried seed extracts on body mass of rats

The body mass measurements of the treated and control rats on day 0 and day 14 are shown in Table 1. Data showed reasonable increase in the body mass of the treated and control rats. In addition, the body mass of the treated rats were insignificantly different as compared to control.

3.2.2 Effect of WJ and BB dried seed extracts on renal function test

Renal function parameters of both WJ-treated and BB-treated rats are presented in Table 2. From the results, the concentration of all measured parameters (sodium, potassium, chloride, carbon dioxide, anion gap, urea, and creatinine) in the animals treated with the low dose (2 g kg^{-1}) and high dose (5 g kg^{-1}) of both seed extracts were insignificant compared to control rats. The concentration of all measured parameters (sodium, potassium, chloride, carbon dioxide, anion gap, urea, and creatinine) is lying within the normal range, however there was slight increase in the concentration of anion gap and decrease in creatinine level, but they were alike in both control and treated groups indicating safe treatment. This showed that the seed extract at different levels tested did not produce considerable change in the levels of the different parameters studied.

3.2.3 Effect of WJ and BB seed extracts on liver function test

Table 3 reorded the effects of the low dose (2 g kg^{-1}) and high dose (5 g kg^{-1}) of both WJ and BB methanol extracts of dried seeds on liver function parameters. No significant changes were observed in the values of the parameters studied [(total protein, albumin, globulin, conjugated bilirubin, aspartate aminotransferase (AST) and gama glutamyl transferase (GGT),] in comparison with the control animals and the values obtained were within the normal biological and laboratory limit. On the other hand, the total bilirubin recorded slight decrease than normal ranges and showed significant difference in the low and high doses of BB-treated rats of both sexes compared to control. Additionally, alanine aminotransferase (ALT) revealed slight elevation than normal ranges.

Table 3: Effects of WJ and BB dried seed extracts on liver function parameters

Groups	Total protein g l^{-1}	Albumin g l^{-1}	Globulin g l^{-1}	Total bilirubin mmol l^{-1}	ALT IU l^{-1}	AST IU l^{-1}	GGT IU l
10% Tween 20 Male	69.33±2.9	40.33±0.80	28.67±1.20	3.50±1.00	46.83±2.00	47.12±9.1	<3
10% Tween 20 Female	72.66±4.3	37.83±0.70	34.83±1.90	3.50±0.54	45.16±2.30	46.66±8.3	<3
HD-WJ Male	68.16±3.3	36.83±0.40	31.33±0.51	3.00±0.00	49.16±300	44.92±5.6	<3
HD-WJ Female	70.60±40	35.00±0.80	35.60±2.00	2.83±0,40	41.50±2.10	48.33±60	<3
LD-WJ Male	68.83±0.88	39.00±0.63	29.83±2.10	2.83±0.46	48.66±2.70	49.45±7.2	<3
LD-WJ Female	66.66±1.4	43.16±1.30	23.50±1.80	3.33±1.80	46.00±1.90	46.66±11	<3
HD-BB Male	63.66±2.2	37.33±0.80	26.33±5.30	2.33±0.50*	41.83±8.70	49.66±10	<3
HD-BB Female	69.16±2.1	35.00±0.90	34.16±7.10	1.50±1.70*	38.33±6.20	45.00±13	<3
LD-BB Male	62.50±1.9	36.00±0.60	26.50±5.50	1.83±1.90*	43.83±5.10	42.83±16	<3
LD-BB Female	68.83±3.9	38.50±2.20	30.33±4.10	1.33±2.50*	40.16±3.40	47.50±9.5	4

Values expressed as mean ± SD, the significant value was set at $p \leq 0.05$. *p <0.05 compared to normal control

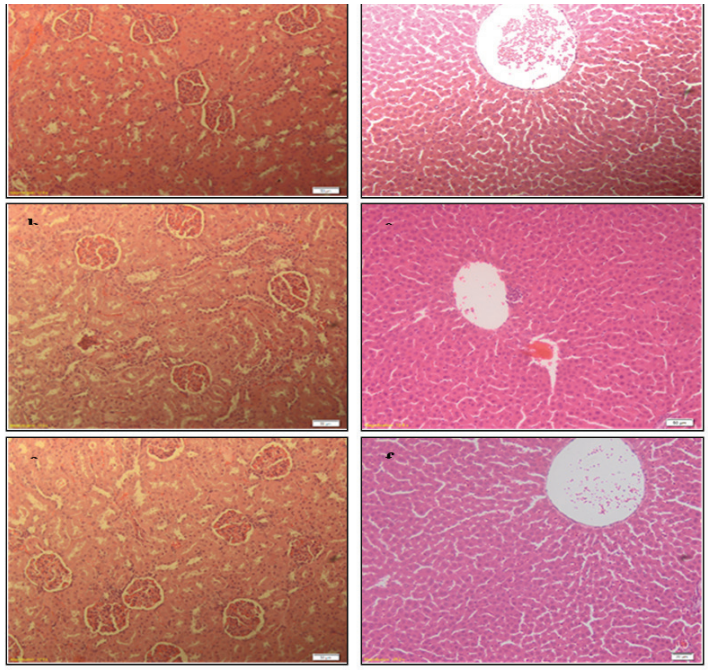

Figure 5: Histological sections (20 x magnifications) of kidneys and livers from the acute toxicity test on the methanol extract of WJ and BB dried seeds. (a & d) Vehicle kidney and liver respectively treated with 5 ml kg^{-1} (10 % Tween 20). (b & e) Kidney and liver respectively treated with WJ seed extracts (5 g kg^{-1}). (c & f) Kidney and liver respectively treated with BB seed extract.

3.2.4 Effect of WJ and BB dried seed extracts on the histopathology of liver and kidney of rats

The qualitative data from the histological sections of kidneys and livers collected from the control and rats treated with WJ and BB seed extracts at the highest dose tested (5 g kg^{-1}) are illustrated on Fig. 5. No significant difference was observed between the architecture of livers or kidneys collected from the WJ-treated and BB-treated animals compared to control group.

4 CONCLUSION

In conclusion, the current study showed that the methanol extracts of both WJ and BB dried seeds of *L. siceraria* possesses high antioxidant power. The WJ seeds variety reported higher TPC, FRAP and DPPH values than BB variety, while the TFC results of BB seeds variety recorded higher value than that of WJ. The acute toxicity test of the methanol extract of both seed varieties reported safe dose up to 5 g kg^{-1} in both sexes of rats.

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Cytogenetic and molecular studies on two faba bean cultivars revealed their difference in their aluminum tolerance

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Cytogenetic and molecular studies on two faba bean cultivars revealed their difference in their aluminum tolerance

Abstract: Two cultivars of faba bean (*Vicia faba* 'Giza 843' and 'Nobaria 3') that differ in aluminum (Al) tolerance were used to study cytogenetic and genomic alterations under the influence of Al Cl_3 (5, 15, and 25 mmol $AlCl_3$) for different periods (6, 12 and 24 h). Under Al treatments, mitotic index in both cultivars decreased and total chromosomal abnormalities increased. The frequencies of micronuclei and chromosomal abnormalities (C-anaphase, metaphase-star chromosomes, breaks, sticky and disturbed chromosomes during metaphase or anaphase) in 'Giza 843' were lower than in 'Nabaria 3'. Increase of the registered cytogenetic events under the influence of Al stress led to increase the detected polymorphism using RAPD and ISSR markers. Application of RAPD primers gave the same value of polymorphism in both faba bean cultivars under Al stress. Polymorphism average of nine ISSR primers of 'Giza 843' (65.36 %) was lower than that of 'Nobaria 3' (71.59 %). Molecular markers, cytogenetic characteristics and seedling growth data indicate that Al tolerance of 'Giza 843' was higher than of 'Nobaria 3'. This work shows that cytogenetic and ISSR techniques could be used efficiently to distinguish between the ability of two faba bean cultivars to tolerate toxic effects of Al.

Key words: aluminum tolerance; *Vicia faba* L.; chromosomal abnormalities; cytogenetics; RAPD, ISSR

Citogenetske in molekularne raziskave dveh sort boba so odkrile njuno različno toleranco na aluminij

Izvleček: Dve sorti boba (*Vicia faba* 'Giza 843' in 'Nobaria 3'), ki sta se razlikovali v toleranci na aluminij (Al) sta bili uporabljeni v raziskavi citogentskih in genomskih sprememb, ki so jih povzročile različne koncentracije Al Cl_3 (5, 15, in 25 mmol $AlCl_3$) v različnih časovnih obdobjih (6, 12 in 24 h). Pri obravnavanjih z Al se je mitotski indeks obeh sort zmanjšal, celokupne kromosomske aberacije pa so se povečale. Pogostost malih jeder (micronuclei) in kromosomskih aberacij (C-anafaze, zvezdasti kromosomi v metafazi, lomi kromosomov, zlepljeni in nenormalni kromosomi v metafazi in anafazi) sta bili pri 'Giza 843' manjši kot pri 'Nabaria 3'. Povečanje ugotovljenih citogenetskih dogodkov zaradi vpliva Al stresa je povečalo ugotovljeni polimorfizem z RAPD in ISSR označevalci. Uporaba RAPD začetnih oligonukleotidov je dala enako vrednost polimorfizma pri obeh sortah izpostavljenih aluminijevemu stresu. Poprečje polimorfizma devetih ISSR začetnih oligonukleotidov je bilo pri'Giza 843' (65,36 %) manjše kot pri'Nobaria 3' (71,59 %). Molekulski označevalvi, citogenetske značilnosti in parametri rasti sejank so pokazali, da je tolerance na aluminij pri 'Giza 843' večja kot pri 'Nobaria 3'. Raziskava kaže, da bi se citogenetske metode in ISSR tehnike lahko učinkovito uporabljale za razlikovanje sposobnosti tolerance različnih sort na toksične učinke Al.

Ključne besede: toleranca na aluminij; *Vicia faba* L.; kromosomske aberacije; citogenetika; RAPD; ISSR

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1 INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the most important legume plants where it is cultivated for humans as well as animals. Faba bean seeds contain high proteins and other important components. It is a diploid species with 2n = 12. It belongs to *Viceae* tribe of Papilionoideae subfamily of Fabaceae family. In general, legume plants are used to improve the fertility of soil through nitrogen fixation. Around the world, 37 collections including about 38,360 faba bean germplasm have been conserved; the largest collection belongs to International Center for Agricultural Research in the Dry Areas - ICARDA in Syria (Duc et al., 2010).

Genetic variation in faba bean ranges from 10 to 60 % depending on plant genotype and the environmental conditions (Suso et al., 2006). Faba bean is cultivated under several conditions, but water deficiency, increased soil salinity and pollution with toxic metals such as aluminum (Al) limit the faba bean production (Abdelhamid et al., 2010). Consequently, faba bean growth and yield retardants should be investigated.

Aluminum abundance in the earth's crust is very high. In nature, the highest amount of Al is present in unsoluble form (aluminum silicate), but very small amount of Al is present in soluble form; this enters and affects biological systems (May and Nordstrom, 1991). The detected negative effects of Al on plant root tips were attributed to increase of cell wall rigidity, decrease of cell respiration and DNA replication, and interfere with cell division (Llugany et al., 1995). Some authors confirmed that root tips play a major role in Al toxicity/protection (Bennet and Breen, 1991), but others refuse this hypothesis (Ryan et al., 1993). In Al-sensitive maize cultivar, Al was accumulated in the distal transition zone of the root apex (1-2 mm) and inhibited root elongation (Sivaguru and Horst, 1998).

In plants, Al tolerance varies between species or cultivars of each one and it depends on detoxification of accumulated Al or prevention of Al uptake (Rengel, 1996). Aluminum toxicity is usually associated with chromosomal changes, which can be detected by karyologic analysis of Al-treated root tips by estimating mitotic indices (MI), micronucleus (MN) formation and chromosomal abnormalities (CA) (Yi et al., 2010; Kotelnikova et al., 2019). Faba bean could be used as a test species for monitoring the genotoxicity of Al because of its small number of chromosomes and their length make it easy to detect the damage occurring under the influence of the studied factor (Gopalan, 1999; Yi et al., 2010; Altwaty et al., 2016).

Induction of reactive oxygen species (ROS) formation under Al toxicity was reported (Rout et al., 2001) causing single and double strand DNA breaks. Consequently, the frequency of CA and MN is increased (Yi et al., 2010). In faba bean root tips, high concentrations of Al resulted in decreased MI, reduced mitotic activity and blocked DNA synthesis (Mohanty et al., 2004), and DNA recombination (Achary and Panda, 2010). Yi et al. (2010) reported that some mitotic cells could enter and continue mitotic cell division with abnormal DNA.

Chromosomal abnormalities due to abiotic stress agent could be detected not only by cytogenetic approach but also with molecular testing based on PCR. Chromosomal abnormalities as well as point mutations may result in alterations either at or between the RAPD or ISSR primer binding sites leading to alterations of the PCR products. These products can be used to calculate polymorphism which gives clear indication about the stability of the studied genome under the influence of the studied agent (Hassanein et al., 2018).

The data available so far on the toxic effects of high concentrations of Al on plant species are few. While relatively high concentrations of $AlCl_3$ was used in our study (5- 25 mmol for 24 h), they were lower than what used by others (Karimai and Poozesh, 2016). Under relatively high concentrations of $AlCl_3$, studies linking results of cytogenetical and molecular techniques are scarce. Consequently, the aim of this study was to investigate genome stability of two faba bean cultivars under the influence of relatively high dose of $AlCl_3$ using cytogenetical and molecular techniques. Also, to know which of the easiest and cheapest molecular biology techniques, such as RAPD or ISSR, confirm the obtained cytogenetic data.

2 MATERIAL AND METHODS

In preliminary experiment, seeds of ten faba bean cultivars were obtained from Agriculture Research and Seeds Center in Qena, Egypt. Faba bean seeds were surface sterilized with 5 % (v/v) commercial sodium hypochlorite for 5 min followed by 0.2 % (w/v) mercuric chloride for 5 min. Seeds were rinsed in sterilized deionized water 3 times, 10 min each. For seed germination, they were subjected to a series of $AlCl_3$ concentrations (5, 10, 15, 20, 25, 30, 35 and 40 mmol) for different exposure times (6, 12 and 24 hr). After ten days, seed germination and seedling growth parameters were estimated. Consequently, three $AlCl_3$ concentrations (5, 15 and 25 mmol) as well as the most tolerant ('Giza 843') and sensitive ('Nobaria 3') cultivars were used for further studies.

Seeds of 'Giza 843' and 'Nobaria 3' were selected to be homogeneous in size and color, and without wrinkles. Ten sterilized seeds were grown in sterilized petri dish contains filter paper and a thin layer of sterilized distilled

water (15 ml) for 2 to 3 days. Seedlings with root length of 2-3 cm were immersed in solution of $AlCl_3$ of three concentrations (5, 15 and 25 mmol) for three time periods (6, 12 and 24 h). Faba bean seeds treated with distilled water were used as control. In each treatment, three replicates were used, each with 10 seeds/Petri dish. Seeds were germinated under lab condition at 28 ± 2 °C. For each treatment, ten root tips were cut and fixed overnight in ethanol:acetic acid 3:1 (v/v) at 4 °C. Then, root tips were hydrolyzed with 1 N HCl, stained microsopic slides were prepared by at 60 °C for 8-10 minutes. Then, roots were washed and transferred into the Feulgen stain for one hour. Root tips were teased out on clean slide, coated with egg-albumen, in a drop of 45 % glacial acetic acid and they squashed between cover and slide. To separate covers, slides were turned face down in a Petri-dish containing distilled water. Plant materials were dehydrated using a series of ethyl alcohol (50 %, 70 %, 96 %) for 10 minutes in each alcohol concentration. The slides were immersed in a solution of absolute ethanol and xylol (1:1). Slides were cleared in pure xylol for 10 minutes, mounted in Canada balsam and transferred to dry in a hot air oven at 35-40 °C for 2-3 days (Darlington and La Cour, 1976; Kanaya et al., 1994).

The percentage of MI, phase indexes, total abnormalities and the percentage of the aberrations were determined according to the following formulas:

Mitotic index (M.I.) = (No. of dividing cells x 100)/ (No. of non dividing cells + No.of dividing cells)

Total abnormalities = (No. of abnormal dividing cells x 100)/(No. of dividing cells)

% of the phase = (No. of dividing cells in phase x 100)/(No. of dividing cells)

The percentage of the aberrations = (No. of aberrant cells x 100)/(No. of dividing cells)

To determine the genetic variation and genome stability of root tips treated with 5 mM $AlCl_3$ for different periods, the RAPD and ISSR techniques were used. DNA extraction was carried out three times of each treatment and according to the method of Porebski et al. (1997). Hexadecyl trimethyl ammonium bromide (CTAB) procedure based on the protocol of Porebski et al. (1997) was used. Frozen root tissues (200 mg) were ground using 1000 µl of CTAB buffer. The obtained homogenate was mixed gently with 2 µl of RNAse, incubated at 65 °C for 90 min and centrifuged at 10000 rpm for 5 min. Supernatant was transferred to a new tube, where 500 µl of 24:1 chloroform-isoamyl alcohol were add and mixed well by shaking. The mixture was centrifuged at 10000 rpm for 5 min, and the aqueous phase (top) was quickly measured and isolated. Then, one volume of cold isopropanol was added. Samples were let to sit at – 20 °C for 45-60 min. Samples were centrifuged for 10 min at 12000 and supernatant was carefully removed without disturbing the resultant DNA pellet. Then, 700 µl of cold 70 % ethanol were added, inverted once to mix and centrifuged for 1 min at 12000 rpm. The DNA pellet was dried at 55 °C, resuspended in 100 µl of TE buffer and allowed to resuspend for 1hr at 55 °C before use.

A total of ten RAPD and nine ISSR primers (Table 1) were used to analyze genome stability of Al treated roots. The obtained genomic DNA was amplified using Thermal Cycler (Biometra TPersonal Combi, Biometra GmbH, Germany). The RAPD and ISSR reactions were performed in a 25 µl volume containing 12.5 µl of Go Taq® Green Master Mix (Promega, Madison, USA), 3 µl of primer 10 pmol, 6.5 µl of free nuclease water and 3 µl of 100 ng genomic DNA templates.

Table 1: The applied RAPD and ISSR primers

RAPD Primer	Primer Sequence (5'---------3')	ISSR Primer	Primer Sequence (5'---------3')
OPA-02	TGCCGAGCTG	ISSR1	ACACACACACACACACCTG
OPA-05	AGGGGTCTTG	ISSR2	CACACACACACACACAAAGCT
OPA-07	GAAACGGGTG	ISSR3	ACACACACACACACACAAG
OPA-17	GACCGCTTGT	ISSR4	GAGAGAGAGAGAGAGACTG
OPat-08	TCCTCGTGGG	ISSR5	GAGAGAGAGAGAGAGACTC
OPaw-10	GGTGTTTGCC	ISSR7	CTCTCTCTCTCTA (CT)6A
OPD-1	ACCGCGAAGG	ISSR8	TCTTCTTCTTCTG
OPD-18	GAGAGCCAAC	ISSR9	TGTTGTTGTGC
OPJ-15	TGTAGCAGGG	ISSR10	GTGGTGGTGGC
OPP-13	GGAGTGCCTC		

The program of PCR amplification was applied with initial denaturation cycle at 94 °C for five minutes. The next 40 cycles were carried out using denaturation step at 94 °C for 45 sec, annealing step (optimized for each primer), and elongation step at 72 °C for 1 min. Finally, extension step was performed at 72 °C for 7 min. The obtained PCR products were subjected to electrophoresis using 1.5 % or 2 % agarose gel with ethidium bromide (0.5 µg ml^{-1}). Run was carried out in 1X TBE buffer at 70 volts. The amplified PCR products were visualized and photographed. Polymorphic, monomorphic, unique, total and molecular weight of each band were determined.

Reproducibility was taken in consideration to minimize personal errors. In this concern, each primer was used three times under the same PCR conditions. The DNA banding patterns obtained from amplification of the studied genome using RAPD and ISSR primers were analyzed by a computer program; Gene Profiler (version 4.03). Microsoft excel file was prepared for scoring the data as '1' for matched and '0' for the unmatched of DNA band. Monomorphic, polymorphic and unique bands were taken into consideration to calculate polymorphism of each primer to determine the number of events leading to alteration of primer binding sites on the genome under the toxic effect of Al compared to control plants. Dendrograms were generated for cluster analysis according to Legendre and Legendre (1983) using the Community Analysis Package Software Program (CAP) Version 4.0 (Richard and Peter, 2007).

3 RESULTS

Depending on data of preliminary seed germination and seedling growth, 'Giza 843' was found to be the most tolerant but 'Nobaria 3' was the most sensitive faba bean cultivar to $AlCl_3$. Cultivar 'Nobaria 3' recorded the highest decrease of root growth parameters, where they were 5.71 %, 74.29 % and 77.14 % for root lengths, 53.41 %, 80.68 % and 87.50 % for fresh mass, and 50 %, 75 % and 75 % for dry mass under the treatment with 5, 15 and 25 mmol $AlCl_3$, respectively. Under these conditions, 'Giza 843' recorded increased fresh (6 %) and dry mass (22 %) but reduced shoot length (3.3 %) when 5 mmol $AlCl_3$ was used. In addition, 'Giza 843' showed reduced root growth parameters where they were 68.85 % and 72.13 % for root length, 53.76 % and 68.82 % for fresh mass, and 44.44 % and 66.67 % for dry mass of 15 and 25 mmol $AlCl_3$, respectively.

Aluminum chloride caused a significant decrease in the mitotic activities in treated root tips of 'Giza 843' or 'Nobaria 3' cultivars (Tables 2 and 4) when they were treated with different concentrations of $AlCl_3$ for different time periods. Data of MI indicate that 'Giza 843' was the least affected when root tips were subjected to different concentrations of $AlCl_3$ for 6 hr. For example, under the influence of the lowest concentration of $AlCl_3$ (5 mmol) and the shortest exposure time (6 h), MI of 'Giza 843' (2.70) was higher than that of 'Nobaria 3' (1.87). The same was observed when the highest concentration of $AlCl_3$ (25 mmol) was used for 6 hr. MI of 'Giza 843' (3.10) was higher than that of 'Nobaria 3' (2.60). The lowest MI values were detected when root tips of both cultivars were subjected to different Al concentrations for 24 hr. In 'Giza 843', complete inhibition of mitotic cell activities was detected when the highest concentration of Al (25 mmol $AlCl_3$) for the longest time of exposure (24 hr) was used.

In comparison to that of control ($AlCl_3$-untreated plants), the increase in $AlCl_3$ concentration or exposure time led to significant increase in the values of interphase and it associated with significant increase in total chromosomal abnormalities in both cultivars (Table 2 and 4). In addition, while the highest values of interphase were detected under 25 mmol Al for 24 hr, prophase stage was inhibited completely in both cultivars. In 'Giza 843', when the $AlCl_3$ exposure time was 6 hr, total abnormalities increased with the increase of Al concentrations. In additions, when the concentration of $AlCl_3$ was 5 mmol, total abnormalities increased with the increase of exposure time. Under the influence of all $AlCl_3$ concentrations for 6 or 12 h, total abnormalities of 'Giza 843' were lower than those of 'Nobaria 3'. While total mitosis and MI drastically decreased under $AlCl_3$ treatments for 24 h, high total chromosomal abnormalities were detected in both cultivars. The frequency of abnormalities in all mitotic phases increased under $AlCl_3$ treatments in both cultivars.

In both cultivars, the frequency of MN increased under the influence of all $AlCl_3$ treatments, this increase was lower in 'Giza 843' than 'Nobaria 3' (Tables 3 and 5). Cells with one, two or more micronuclei appeared in both cultivars (Fig. 1).

All $AlCl_3$ concentrations induced chromosomal abnormalities at all stages of mitosis; depended on the used cultivar. While the frequency of C-metaphase in 'Giza 843' was higher than 'Nobaria 3', vice versa was detected in case of C-anaphase (Table 3 and Table 5). On the other side, the frequency star chromosomes in metaphase of 'Giza 843' (Table 3) was lower than that of 'Nobaria 3', vice versa was detected during ana-telophase. In addition, under the influence of different $AlCl_3$ treatments, the frequency of different abnormalities was higher in 'Nobaria 3' than 'Giza 843'. For example, the frequencies of chromosomal breaks and sticky and disturbed chromosomes during metaphase or ana-telophase in 'No-

Table 2: Cytogenetic analysis of *Vicia faba* 'Giza 843' root tip treated with three concentrations of $AlCl_3$ for three exposure times. Abbreviations: Mitotic index (MI), total abnormalities, interphase and mitotic phases (prophase, metaphase and ana-telophase), include normal (Total) and abnormal (Abn.) mitotic phases.

Treatment					% Interphase		% Prophase		% Metaphase		% Ana-telophase	
Exposure time (h)	$AlCl_3$ conc.	Total mitosis	MI (%)	% Total abnormal	Total	Abn.	Total	Abn.	Total	Abn.	Total	Abn.
6	Control	355	7.10±0.36	4.09±0.42	92.9	0.4	35.9	0.4	23.6	1.4	40.5	0
	5 mmol	138	2.70±0.40*	59.10±14.40*	97.2	0.6	44.6	11.8	29.7	25.7	25.7	15.8
	15 mmol	115	2.30±0.10*	74.50±9.50*	97.6	0.6	63.1	16.7	17.1	17.1	19.8	12.6
	25 mmol	157	3.10±0.30*	76.47±0.55*	96.4	0.7	69.1	17.6	11.5	9.4	19.4	9.4
12	5 mmol	53	1.07±0.25*	76.90±14.00*	99.0	0.5	32.6	28.5	28.6	24.5	38.8	22.4
	15 mmol	63	1.27±0.15*	67.73±1.05*	98.7	0.6	40.2	18.2	35.1	32.5	24.7	20.8
	25 mmol	182	3.60±0.10*	79.47±3.55*	98.4	0.07	43.4	32.4	27.5	28.0	29.1	17.6
24	5 mmol	73	1.47±0.15*	93.37±1.05*	98.5	1.4	38.7	35.5	54.8	54.8	6.5	3.2
	15 mmol	75	0.10±0.00*	100.00±0.00*	99.9	3.9	0	0	100	100	0	0
	25 mmol	0	0	0	100	0.9	0	0	0	0	0	0

* The mean difference is significant at the 0.05 level.

Table 3: Types and percentage (%) of chromosomal abnormalities in *Vicia faba* 'Giza 843' root tip treated with three concentrations of $AlCl_3$ for three exposure times. Abbreviations: C-metaphase (C-m), sticky (Stick), star, break, disturbed (Dist), and diagonal (Diag), bridge, free, C-anaphase (C-ana), micronuclei (MN) and bi-nucleated cell (BN).

Treatment		Interphase abnormalities		Metaphase abnormalities					Ana-telophase abnormalities							
Exposure time (h)	$AlCl_3$ conc.	MN	BN	C-m	Stick	Star	Break	Dist	Stick	Bridge	Dist	Diag	Free	Break	Star	C-ana
	Control	0	0	0	1.3	0	0	0	0	0	0	0	0	0	0	0
6	5 mmol	0.16	0.5	19.8	1.9	2.9	0.9	1.9	1.9	0.9	1.9	5.9	0	0.9	4.9	0.9
	15 mmol	0.36	0.04	15.3	0	0	0.9	0	2.7	0	0.9	0.9	0.9	1.8	5.4	0
	25 mmol	1.46	0.4	7.2	0	0	0.7	1.4	2.2	0	0	5.7	0	1.4	0	0
12	5 mmol	0.17	0.2	20.4	4.1	0	0	0	8.2	2.1	0	4.1	0	0	6.1	2.1
	15 mmol	0.24	0.1	28.6	0	0	2.6	0	2.6	1.3	0	2.6	0	6.5	1.3	2.6
	25 mmol	1.22	0	28.0	0	0	0	0	3.8	0	5.5	0	1.6	0	0.5	6.1
24	5 mmol	0.32	0	54.8	0	0	0	0	0	0	0	0	3.2	0	0	0
	15 mmol	0.07	0.7	100	0	0	0	0	0	0	0	0	0	0	0	0
	25 mmol	0.67	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4: Cytogenetic analysis of *Vicia faba* 'Nobaria 3' root tip treated with three concentrations of $AlCl_3$ for three exposure times. Abbreviations: Mitotic index (MI), total abnormalities, interphase and mitotic phases (prophase, metaphase and ana-telophase), include normal (Total) and abnormal (Abn.) mitotic phases.

Treatment					% Interphase		% Prophase		% Metaphase		% Ana-telophase	
Exposure time (h)	$AlCl_3$ conc.	Total mitosis	MI (%)	% Total abnormal	Total	Abn.	Total	Abn.	Total	Abn.	Total	Abn.
6	Control	355	7.10±0.36	4.09±0.42	92.9	0.4	35.9	0.4	23.6	1.4	40.5	0
	5 mmol	91	1.87±0.30*	87.25±5.68*	97.6	2.4	65.7	50.6	27.4	27.3	6.9	4.10
	15 mmol	49	1.98±0.12*	78.30±5.85*	96.7	1.4	67.9	20.7	11.3	9.4	20.8	13.32
	25 mmol	130	2.60±0.30*	86.00±2.00*	97.4	1.6	41.9	20.3	23.2	18.4	34.9	17.7
12	5 mmol	160	3.20±0.15*	92.77±2.15*	97.6	0.8	52.2	21.7	13.1	8.6	34.7	21.7
	15 mmol	110	2.19±0.46*	86.87±0.95*	96.2	2.6	25.9	22.9	22.2	22.2	51.9	24.07
	25 mmol	22	0.44±0.03*	95.70±3.55*	97.8	1.3	30.2	27.9	46.5	45.3	23.3	16.3
24	5 mmol	30	0.60±0.20*	93.64±1.70*	98.3	0.7	17.4	17.4	43.5	39.1	39.1	30.4
	15 mmol	27	0.54±0.04*	87.00±3.00*	97.5	1.8	42.9	28.5	32.1	28.5	25	34.3
	25 mmol	16	0.33±0.08*	97.27±1.80*	99.5	1.7	0	0	3.1	5.2	96.9	63.2

* The mean difference is significant at the 0.05 level.

Table 5: Types and percentage (%) of chromosomal abnormalities in *Vicia faba* 'Nobaria 3' root tip treated with three concentrations of $AlCl_3$ for three exposure times. Abbreviations: C-metaphase (C-m), sticky (Stick), star, break, disturbed (Dist), and diagonal (Diag), bridge, free, C-anaphase (C-ana), micronuclei (MN) and bi-nucleated cell (BN).

Treatment		Interphase abnormalities		Metaphase abnormalities					Ana-telophase abnormalities							
Exposure time (h)	$AlCl_3$ conc.	MN	BN	C-m	Stick	Star	Break	Dist	Stick	Bridge	Dist	Diag	Free	Break	Star	C-ana
	Control	0	0	0	1.3	0	0	0	0	0	0	0	0	0	0	0
6	5 mmol	2.44	0.23	6.8	1.4	0	1.4	17.8	1.4	1.4	1.4	0	0	0	0	0
	15 mmol	0.91	0.05	0	0	1.9	0	7.5	1.9	0	5.7	1.9	0	5.7	0	0
	25 mmol	0.80	0.09	6.1	1.3	0	2.6	7.7	1.3	4	11.3	0.6	0	1.3	0.3	0.6
12	5 mmol	0.71	0.1	0	4.3	0	4.3	0	4.3	0	0	0	4.3	4.3	8.7	0
	15 mmol	1.38	0.1	0.9	0.9	0	1.9	18.5	10.2	0	7.4	0.9	0	3.7	1.8	0.9
	25 mmol	1.88	0.1	40.6	1.2	0	1.2	2.3	4.7	0	2.3	2.3	1.2	1.2	4.7	0
24	5 mmol	2.40	0.1	30.4	0	4.3	0	4.3	8.7	0	17.4	0	4.3	0	0	0
	15 mmol	1.46	0	10.7	0	3.6	7.1	7.1	3.6	0	10.7	0	0	0	0	0
	25 mmol	1.45	0.2	5.3	0	0	0	0	63.2	0	0	0	0	0	0	0

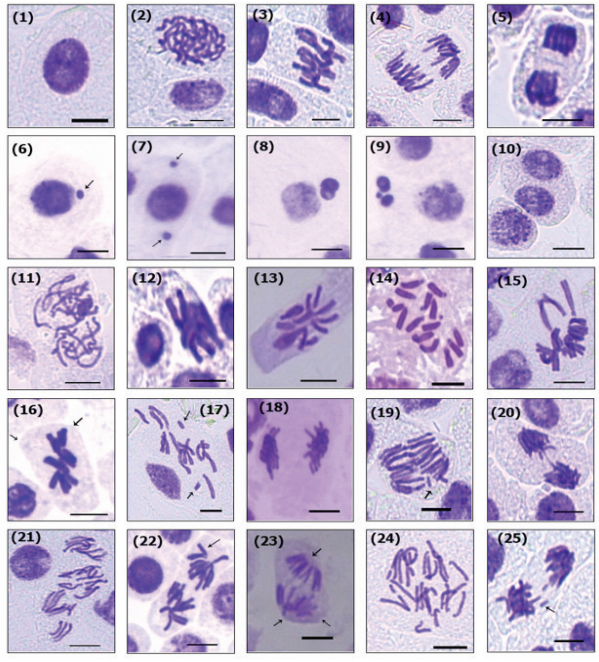

Figure 1: Chromosomal aberrations in *Vicia faba* 'Giza 843 and Nobaria 3' root tips under the influence of $AlCl_3$: interphase (1), prophase (2), metaphase (3), anaphase (4), telophase (5), micronuclei with different sizes and different number (6, 7, 8, 9), binucleated cell (10), irregular prophase (11), sticky metaphase (12), star metaphase (13), C-metaphase (14), dis 5rturbed metaphase (15), diagonal metaphase (16), metaphase break (17), sticky anaphase (18), anaphase break (19), anaphase bridge (20), C-anaphase (21), anaphase with free chromosome (22), diagonal anaphase (23), disturbed anaphase (24) and sticky anaphase break (25). The scale bar equals 10 µm.

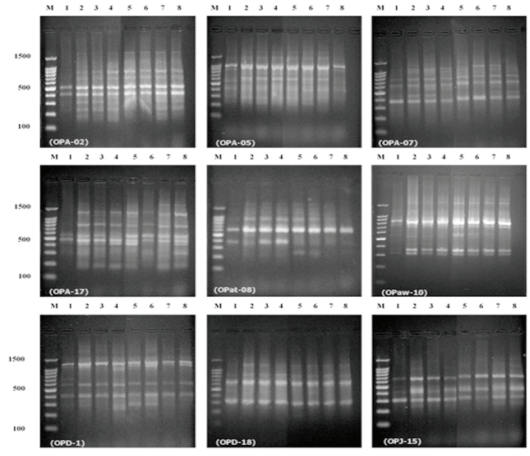

Figure 2: RAPD profile generated by 9 primers using roots of two *Vicia faba* cultivars treated with 5 mmol $AlCl_3$ for different periods. Lanes 1, 2, 3 and 4: 'Giza 843' for 0 (control), 6, 12 and 24 h, respectively. Lanes 5, 6, 7 and 8: 'Nobaria 3' for 0 (control) 6, 12 and 24 h, respectively. M: DNA ladder.

baria 3' cells were higher those of 'Giza 843'. In concern to ana-telophase abnormalities, the frequency of diagonal chromosomes under different $AlCl_3$ treatments was higher in 'Giza 843' than in 'Nobaria 3' (Tables 3 and 5). In comparison to other chromosomal abnormalities, free chromosomes appeared in low frequency in both cultivars (Table 3 and Table 5); it never appeared when low concentration of $AlCl_3$ in combination with short exposure time was applied.

Root tips treated with 5 mmol $AlCl_3$ for different periods were subjected to molecular analysis using RAPD (Table 6) and ISSR primers (Table 7). When ten RAPD

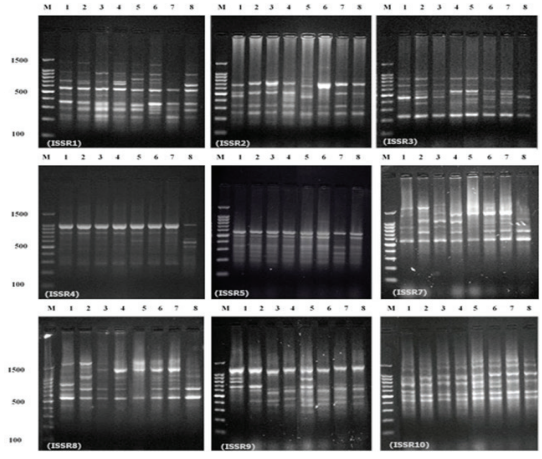

Figure 3: ISSR profile generated by 9 primers using roots of two *Vicia faba* cultivars treated with 5 mmol $AlCl_3$ for different periods. Lanes 1, 2, 3 and 4: 'Giza' 843 for 0 (control), 6, 12 and 24 h, respectively. Lanes 5, 6, 7 and 8: 'Nobaria 3' for 0 (control) 6, 12 and 24 h, respectively. M: DNA ladder.

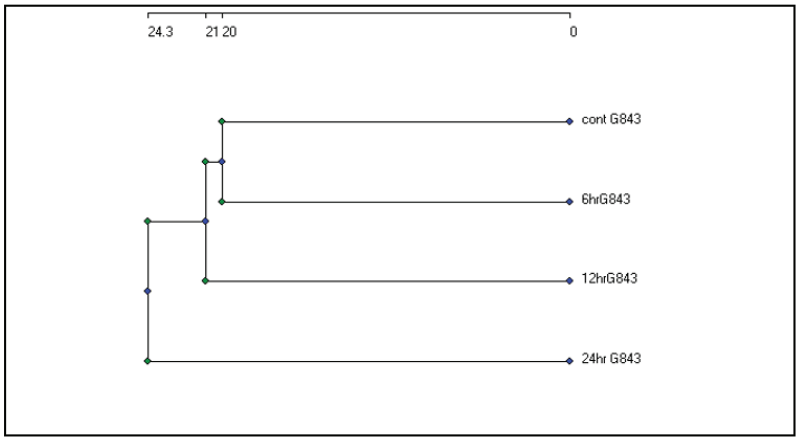

Figure 4: UPGMA based cluster tree of *Vicia faba* 'Giza 843' exposed to 5 mmol $AlCl_3$ for 6, 12 and 24 h based on ten RAPD and nine ISSR primers.

primers were used for amplification of genomic DNA of two faba bean cultivars ('Giza 843' and 'Nobaria 3'), different profiles were obtained (Table 6; Fig. 2). They expressed 27 and 26 polymorphic, 20 and 20 monomorphic, 8 and 10 unique out of total 55 and 56 bands when genomes of 'Giza 843' and 'Nobaria 3' were amplified, respectively. Consequently, while percentage of polymorphism ranged from 0 % (OPD-18) to 85.72 % (OPA-17) with an average of 61.74 % in 'Giza 843', they ranged from 20 % (OPD-18) to 90.91 % (OPA-17) with an average of 61.24 % in 'Nobaria 3'.

The applied ISSR primers on 'Giza 843' genome resulted in amplification of 38 polymorphic out of 69 fragments (55.07 %), they included 18 monomorphic, 9 unique and 38 polymorphic bands (Table 7; Fig. 3). Consequently, the percentage of polymorphism in 'Giza 843' PCR products ranged from 25 % (ISSR5 or ISSR10) to 100 % (ISSR9) with an average of 65.36 %; it was higher than that of RAPD primers. When root tips of 'Nobaria 3' were subjected to $AlCl_3$ for different periods using ISSR primers, 72 fragments were amplified including 22 monomorphic, 7 unique and 43 polymorphic bands. Percentage of polymorphism ranged from 25 % (ISSR5) to 100 % (ISSR9) with an average of 71.59 %; it was higher than that of RAPD primers.

Cluster tree based on ten RAPD and nine ISSR primers of 'Giza 843' cultivar resulted in grouping of control plant with others subjected to 5 mmol Al for 6 or 12 h (Fig. 4). On the other side, the dendrogram of 'Nobaria 3' included two main clusters; the first cluster contained control plants and others subjected to $AlCl_3$ for 6 h. The

Table 6: Ten RAPD primers, annealing temperature, size of amplified fragments (bp), total number of amplified fragments, number of polymorphic bands and unique bands identified per primer used to access genome stability of two *Vicia faba* cultivars (Giza 843 and Nobaria 3) under the influence of Al stress.

Primer	Annealing temperature (°C)	Polymorphic bands		Monomorphic bands		Unique bands		Total bands		Size range (bp)	Polymorphism (%)	
		Giza 843	Nobaria 3	Giza 843	Nobaria 3	Giza 843	Nobaria 3	Giza 843	Nobaria 3		Giza 843	Nobaria 3
OPA-02	32	4	3	2	2	0	0	6	5	141-753	66.67	60.00
OPA-05	30	4	3	3	3	1	0	8	6	225-1102	62.50	50.00
OPA-07	30	3	2	2	2	1	3	6	7	497-1428	66.67	71.43
OPA-17	30	5	7	1	1	1	3	7	11	162-1811	85.72	90.91
OPat-08	32	2	1	1	1	0	1	3	3	218-958	66.67	55.67
OPaw-10	30	3	4	1	1	1	0	5	5	313-912	80.00	80.00
OPD-1	32	2	2	3	3	3	0	8	5	236-1364	62.5	40.00
OPD-18	30	0	0	4	4	0	1	4	5	292-1278	00.00	20.00
OPJ-15	30	2	2	1	1	0	0	3	3	229-737	66.67	66.67
OPP-13	32	2	2	2	2	1	2	5	6	345-876	60.00	66.67
Total		27	26	20	20	8	10	55	56		61.74	61.24

Table 7: Nine ISSR primers, annealing temperature, size of amplified fragments (bp), total number of amplified fragments, number of polymorphic bands and unique bands identified per primer used to access genome stability of two *Vicia faba* cultivars (Giza 843 and Nobaria 3) under the influence of Al stress.

Primer	Annealing temperature (°C)	Polymorphic bands		Monomorphic bands		Unique bands		Total bands		Size range (bp)	Polymorphism (%)	
		Giza 843	Nobaria 3	Giza 843	Nobaria 3	Giza 843	Nobaria 3	Giza 843	Nobaria 3		Giza843	Nobaria3
ISSR1	56	6	7	3	3	1	1	10	11	175-1287	70.00	72.73
ISSR2	60	4	5	1	1	2	0	7	6	239-703	85.71	83.33
ISSR3	58	6	6	1	1	0	1	7	8	206-870	85.71	87.50
ISSR4	50	1	3	2	2	0	2	3	7	167-985	33.33	71.43
ISSR5	50	2	2	6	6	0	0	8	8	188-565	25.00	25.00
ISSR7	38	5	6	1	1	1	1	7	8	804-1376	85.71	87.50
ISSR8	36	5	5	2	2	2	0	9	7	295-1489	77.78	71.42
ISSR9	32	7	6	0	0	3	0	10	6	169-828	100	100
ISSR10	38	2	3	6	6	0	2	8	11	457-1557	25.00	45.45
Total		38	43	18	22	9	7	69	72		65.36	71.59

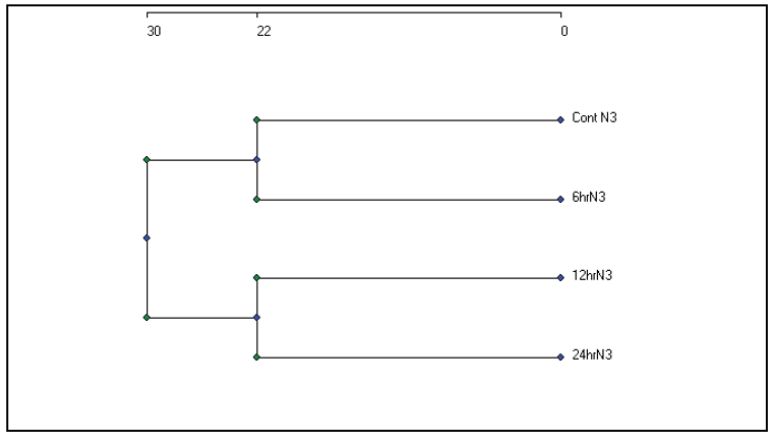

Figure 5: UPGMA based cluster tree of *Vicia faba* 'Nobaria 3' exposed to 5 mmol $AlCl_3$ for 6, 12 and 24 h based on ten RAPD and nine ISSR primers.

second cluster included plants subjected to $AlCl_3$ for 12 or 24 h (Fig. 5).

4 DISCUSSION

When seed were germinated and subjected to $AlCl_3$ concentrations, they showed retardation of seed germination and seedling growth, which were lower in 'Giza 843' than in 'Nobaria 3'. Retardation in plant growth under the influence of Al toxicity could be attributed to DNA damage (Achary and Panda, 2010), inhibition of cell division and elongation (De Campos and Viccini (2003), stimulation of oxidative stress (Rout et al., 2001), inhibition of water movement through roots, and retardation of root development and the ability of plants to develop symbiosis (Aroca et al., 2007; Belachew and Stoddard, 2017). Consequently, aluminum toxicity exacerbates the effects of other abiotic stresses (Muktadir et al., 2020). Seedlings were used in this study because they are more susceptible to $AlCl_3$ than older plants (Thawornwong and van Diest, 1974) especially when relatively high concentration of $AlCl_3$ was used. Aluminum toxicity on faba bean cultivars as well as other plant species was reported (Rout et al., 2001; Yi et al., 2010; Altwaty et al., 2016), but its effect varies within plant species and genotypes (Rengel, 1996). To mimic many unfertile acid soils around the globe, 50 mmol $AlCl_3$ for 72 h was used to test germplasm sensitivity to Al toxicity (Domingues et al., 2013). In our study, high concentrations of Al Cl_3 up to 25 mmol were used for cytogenetic studies. Under these conditions, seedling growth of 'Giza 843' was less influenced by $AlCl_3$ than 'Nobaria 3'.

Aluminum chloride caused a significant decrease in mitotic activities of root tip cells of 'Giza 843' and 'Nobaria 3'. The reduction in MI of 'Giza 843' was lower than that of 'Nobaria 3' indicating that 'Giza 843' was more Al-tolerant than 'Nobaria 3'. The obtained data indicate that Al Cl_3 induced cell cycle defects in faba bean root cells (Zhang et al., 2009; Yi et al., 2010). The lowest values of MI were detected when the highest concentration of $AlCl_3$ (25 mmol was used for 24 h leading to the complete inhibition of cell division in 'Giza 843'. Inhibition of mitotic cell cycle was attributed to the retardation of cell cycle during interphase (Mohandes and Grant, 1972), decline of energy resources (Rost and Morrison, 1984), inhibition of transition from G1 to S and G2 (Van' t Hoff,1985) and binding of Al on DNA molecules (Matsumoto et al., 1976).

While total mitosis and MI drastically decreased, total abnormalities increased in both cultivars when they were subjected to $AlCl_3$ for 6 or 12 h. Under these conditions, total abnormalities in 'Giza 843' were lower than those of 'Nobaria 3', which may be due to lower abnormalities in prophase and ana-telophase stages, especially under the influence of the lowest exposure period. Increased exposure time to $AlCl_3$ for more than 6 h resulted in irregular trends of the obtained data. Under Al stress, cell divisions in faba bean root tips was often associated with reduction in genomic template stability and an increase in RAPD band frequencies (Rout et al., 2001; Taspinar et al., 2018). Hartig and Beck (2006) confirmed the survival of the stressed cells if they tolerated the occurred mutation (Hartig and Beck, 2006). In both cultivars, while frequency of interphase was very high under the highest Al treatment (25 mmol for 24 h), the prophase was inhibited completely in this study and others (Yi et al., 2010).

The frequency of MN increased under the influence of all $AlCl_3$ treatments, this increase was lower in 'Giza

843‘ than ‘Nobaria 3’. Micronuclei formation was used as an effective tool to measure of chromosomal DNA damages and analyzes the mutagenic effect of chemicals (Fenech, 2008). These studies indicated that some of the cells enter the mitotic cell division with damaged DNA (Hartig and Beck, 2006). Under stress conditions, positive correlation was detected between chromosomal abnormalities and MN, and antioxidant enzymes and lipid peroxidation (Souguir et al., 2011). Since ‘Giza 843’ had less micronuclei and chromosomal abnormalities than ‘Nobaria 3’ under $AlCl_3$ stress, ‘Giza ‘843‘ was considered more tolerant to Al than ‘Nobaria 3’ cultivar.

While several types of chromosomal abnormalities at all stages of mitosis were detected under $AlCl_3$ treatments, they depended on the used cultivar. The most common types of these abnormalities were bridges, laggards, fragments and micronuclei as was reported by Yi et al. (2010). Under the influence of different $AlCl_3$ treatments, frequencies of appearance of different abnormalities including C-anaphase, star metaphase chromosomes, and breaks, sticky and disturbed chromosomes during metaphase or anaphase were higher in ‘Nobaria 3’ than those of ‘Giza 843‘. Our data confirmed that Al is a genotoxic and cytotoxic reagent in plant cells leading to chromosomal abnormalities in several forms (Yi et al., 2010).

Under the toxic effect of $AlCl_3$, changing the frequency of primers binding loci increased as cytogenetical events increased; which could be identified by the values of the obtained polymorphism. When ten of RAPD primers were used for amplification of genomic DNA of ‘Giza 843‘ and ‘Nobaria 3’, the polymorphism average 61.74 % in ‘Giza 843‘ and 61.24 % in ‘Nobaria 3’. Link et al. (1995) confirmed that RAPD was useful molecular markers to study genetic variation in faba beans. In our work, application of RAPD technique to detect genetic variation due to Al treatments in faba bean was not recommended.

Application of ISSR primers on ‘Giza 843‘ and ‘Nobaria 3’ genomes resulted in amplification of 69 and 72 fragments, respectively. ISSR technique was efficiently used for classifying faba bean and other plant species (Abdel-Razzak et al., 2012; Salem and Hassanein, 2017; Hassanein et al., 2018). The calculated polymorphism average of ISSR primers of ‘Giza 843‘ was lower than that of ‘Nobaria 3’ indicating that ‘Giza 843‘ genome was more stable than that of Nobaria 3 under Al stress. The detected DNA polymorphism may be due to mismatching at the primer site, appearance of a new primer site and/or change the distance between two opposite primers. These events may result from point, and chromosomal mutations/abnormalities (Kumar and Rai, 2006). Consequently, these events could be detected by cytogenetical techniques (through micronuclei formation and chromosomal abnormalities) or molecular techniques such as ISSR (through polymorphism average). Data obtained from both techniques confirmed that Al tolerance of ‘Giza 843‘ was higher than that of ‘Nobaria 3’. ISSR was efficiently used to detect variation in faba bean (Abdel-Razzak et al., 2012). Combination of cytogenetical and molecular techniques for efficient evaluation of genetic variation in plants was recommended in this study and others (Begum and Alam, 2017; Hossain et al., 2017).

Construction of dendrograms depending on the data of molecular techniques and study of their details supplemented us with another evidence confirmed the harmony between cytogenetical and molecular biology techniques as well as seedling growth parameters. The obtained cluster tree of ‘Giza 843‘ grouped of control plant with others subjected to 5 mmol Al for 6 or 12 hr. On the other side, the cluster tree of ‘Nobaria 3’ grouped control plants with only others subjected to 5 mmol $AlCl_3$ for 6 hr. Comparison between the two obtained dendrograms, polymorphism and cytogenetical data indicated that ‘Giza 843‘ tolerated Al more than ‘Nobaria 3’.

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Analyzing cereal and grain legumes (pulses) yields patterns in the forest and forest-steppe zones of Ukraine using geographically weighted principal components analysis

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Analyzing cereal and grain legumes (pulses) yields patterns in the forest and forest-steppe zones of Ukraine using geographically weighted principal components analysis

Abstract: This paper aims to explore spatial heterogeneity present in the crop yields data collected from 170 administrative districts in the forest and forest-steppe zones of Ukraine for 27 years using the PCA and GWPCA methods. As a result of the principal component analysis of cereal and grain legumes (pulses) yields variability seven principal components were determined which together explain 66.8 % of the overall yields variability. The global PCA revealed the presence of dynamic processes of the cereal and grain legumes yields variation which have the oscillatory nature with different frequencies. We associate oscillatory processes of the varying frequency with causes of a different nature. The oscillating processes with a period of ten years or more may be of climatic origin. The oscillatory process with the longest period (13 years) is characteristic for the principal component 1, which explains the largest part of cereal and grain legumes yields variability (22.6 %). It is possible to assume that among agroecological factors climate change mostly affects crop productivity. The cluster analysis of administrative districts was conducted based on the cereal and leguminous yield dynamics. The clusters are geographically defined administrative districts that together forming spatially connected areas, which we identified as agroecological zones.

Key words: yield; cereals; leguminous crops; spatial and temporal variability; geographically weighted principal components analysis

Analiza vzorcev pridelkov žit in zrnatih stročnic na območju gozda in lesostepe Ukrajine z geografsko tehtano analizo glavnih komponent

Izvleček: Namen prispevka je bil preučiti prostorsko heterogenost pridelkov poljščin iz podatkov zbranih iz 170 administrativnih okrožij na območju gozda in lesostepe Ukrajine v obdobju zadnjih 27 let z uporabo PCA in GWPCA metod. Rezultat analize spremenljivosti pridelkov žit in zrnatih stročnic z analizo glavnih komponent je bila določitev sedmih glavnih component, ki so skupno razložile 66,8 % celokupne variabilnosti pridelkov. Globalna analiza glavnih component je odkrila prisotnost dinamičnih procesov v spremenljivosti pridelkov žit in zrnatih stročnic, ki nihajo z različnimi frekvencami. Oscilatorne procese z različnimi frekvencami povezujemo z različnimi vzroki. Nihajoči procesi s periodo desetih ali več let so lahko povezanimi s podnebjem. Oscilatorni proces z najdalšo period (13 let) je značilen za prvo glavno komponento, ki razloži največji delež nihanja pridelkov žit in zrnatih stročnic (22,6 %). Mogoče je zaključiti, da med agroekološkimi dejavniki sprememba podnebja najbolj vpliva na pridelek poljščin. Klasterska analiza administrativnih območij je bila izvedena na osnovi dinamike spremeljivosti pridelkov žit in zrnatih stročnic. Grozdi so zemljepisno omejena administrativna območja, ki tvorijo skupaj prostorsko povezana območja, ki so označena kot agroekološke cone.

Ključne besede: pridelek; žita; zrnate stročnice; prostorska in časovna spremenljivost; analiza geografsko tehtanih glavnih komponent

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1 INTRODUCTION

Agricultural producers have known for many years that temporal and spatial variations in crop yields are a reality of farming. Year-to-year fluctuations in crop performance can be caused by seasonal differences in factors such as growing season conditions, differences in weed, insect and disease pressures and possibly the appropriateness of management decisions (Lauzon et al., 2005; Frieler et al., 2017).

Crop yields are frequently heterogeneous across space and time. Spatiotemporal variation can be broken down into its spatial and temporal components (Hammond & Kolasa, 2014). Synchrony and persistence are important components of spatiotemporal variability. When the same crop rises or declines in the same year in each of the two regions of the country, they are in synchrony. Persistence on the other hand refers to consistent differences in mean yield between two regions or other spatial units. Spatial patterns are diagnostic when they are used to uncover hidden mechanisms in the landscape, and predictive when they indicate the likely future behavior of a process (Hammond & Kolasa, 2014; Kong et al., 2018).

There have been several approaches to explore long-term trends in historical crop yield data based on process-based or statistical models (Lobell et al., 2013; Frieler et al., 2017). In this study, we use a collection of local (non-stationary) statistical models, termed geographically weighted principal components analysis (GWPCA).

GWPCA is a localized version of PCA that is an exploratory tool for investigating spatial heterogeneity in the structure of multivariate data. It not only provides a useful investigative tool, but also lends itself to many uses of PCA at a localised scale (Harris et al., 2011).

Principle component analysis (PCA) is a statistical method widely used in exploratory data analysis (Pearson, 1901). This non-parametric method compresses the dimension of a dataset and thus can reveal some simplified structures hidden in the dataset (Liu et al., 2012). Principal component analysis has been applied by various research area with the aim of exploring and characterizing the relationships between regionalized variables and related environmental factors, and to quantify the spatial variability pattern of these variables (Kumar et al., 2012; Li & Huang, 2008). In an ecological setting, common applications of PCA are to environmental data sets e.g., the soils biogeochemistry data (Kaspari & Yanoviak, 2009), although via a suitable transform, PCA can also be applied to species abundance data (Legendre & Gallagher, 2001; Harris et al., 2015).

PCA analysis can be easily expanded using geographically weighted correlation coefficients where the weights are determined with respect to each observation location allowing a separate PCA (geographically weighted PCA, GWPCA) to be conducted for each sampling location (Kumar et al., 2012). Hence, a GWPCA investigates how outputs from a PCA vary spatially (Comber et al., 2016). Spatial changes in data dimensionality and multivariate structure can be explored via maps of the GWPCA outputs (Fotheringham et al., 2002). GWPCA can also be used to detect multivariate spatial anomalies (Harris et al., 2015).

In the published literature, GWPCA has been extensively applied for analyzing multivariate population characteristics (Lloyd, 2010), social structure (Harris et al., 2011), soil characteristics (Kumar et al., 2012) and freshwater chemistry data (Harris et al., 2015; Li et al., 2015). However, GWPCA has not been applied to assess the spatial variability of crop yield in agricultural landscapes inherently with spatially heterogeneous. To fill in the gap, this paper aims to explore such spatial heterogeneity present in the crop yields data collected from 170 administrative districts in forest and forest-prairie zone of Ukraine for 27 years using the GWPCA method.

The maps produced from GWPCA provide quantitative evidence and spatial details for supporting spatial land management and regional development strategy and help identify the spatial differentiation status of the regional agricultural development.

2 MATERIALS AND METHODS

Crop data were obtained from the State Statistics Service of Ukraine. Specifically, the organized data set included the average per year yields of the cereal and grain legumes (pulses) for 7 regions of Ukraine, which include 170 administrative districts over 27 years (1991– 2017). The cereal crops includes wheat (winter and spring), rye (winter and spring), barley (winter and spring), spring oats, buckwheat and millet. Grain legumes are beans and peas. The State Statistics Service of Ukraine provides information on the yield of cereals and grain legumes in one category. We considered the yield of cereal and grain legumes crops as a marker of the productive potential of the agrolandscape.

The time series of crop yields for each administrative district was divided into two components: total trend and trend residual. The total trend was explained by the dependence of the yield from time. As an analytic form of the trend we chose the fourth-degree polynomial (Zymaroieva et al., 2019b; Zymaroieva et al., 2020a). The residuals of the corresponding regression models that describe the trends consist of the random component (noise) and, probably, the regular one that cannot be explained by the selected trend model. These two components are distinguished by their properties: the random component

Table 1: Summary of global PCA

Principal components	Adjusted eigenvalues*	Unadjusted eigenvalues	Estimated bias	Proportion of variance	Standard deviation
1	5.28	6.10	0.82	22.60	2.46
2	2.46	3.15	0.69	11.68	1.77
3	2.07	2.67	0.59	9.88	1.63
4	1.23	1.74	0.51	6.45	1.32
5	1.14	1.58	0.44	5.84	1.25
6	1.06	1.43	0.37	5.31	1.19
7	1.04	1.35	0.31	4.99	1.16

Legend: * – by Horn's parallel analysis

is independent for different points of space, and the regular component must be correlated to all or some points in space (administrative districts). We used the principal components analysis for the residuals in order to isolate the regular component of trend models. The presence of the principal components, whose eigenvalues more than one unit, indicates the existence of correlation in crop yields variation (Zymaroieva et al., 2019a).

How data is suited for the principal component analysis was estimated by Kaiser-Meyer-Olkin (KMO) test (Kaiser, 1974). Calculations were performed using library REdaS (Hatzinger et al., 2014) in the environment for statistical computing R (R Core Team, 2017). PCA was performed using library stats (R Core Team, 2017). The GWPCA method is implemented using the GWmodel R package (http://cran.rstudio.com/) (Zhukov & Ponomarenko, 2018). The spatial database was created in ArcGIS 10.0. The spatial autocorrelation, *I*-Moran's statistics (Moran, 1950), was used to calculate the global coefficient. *I*-Moran's is a measure of autocorrelation similar to the Pearson's correlation statistics, and both statistics range from +1.0 meaning strong positive spatial autocorrelation, to 0 meaning a random pattern, to –1.0 indicating strong negative spatial autocorrelation (Iqbal et al., 2005). Heteroscedastic testing becomes very challenging for high-dimensional regressions. Heteroscedasticity implies that the variance of the disturbance term is not constant over the data range. Koenker-Bassett test evaluates heteroscedasticity by comparing different quantile or expectile estimates (Koenker & Bassett, 1978). The global Moran's statistics were calculated using Geoda095i (http://www.geoda.uiuc.edu/) (Anselin et al., 2005).

3 RESULTS

The residuals of the corresponding regression models have a complex nature. Definitely, there is a random noise associated with objective errors in the source data. Also, in the regression residuals, we can expect a component that is associated with a regular variation that may have an ecological nature (Zymaroieva et al., 2019a).

The dissimilar magnitude between these residuals for administrative areas may lead to biased results from PCA as the variables with the highest sample variances tend to be emphasized in the first few principal components. Hence, all the selected variables need to be standardized by subtracting its mean from that variable and dividing it by its standard deviation. Such data standardization makes each transformed variable have equal importance in the subsequent analysis (Li et al., 2015).

There is another question to be answered before implementing a PCA analysis: is the sample size large enough for the statistical analysis? Is there a certain redundancy between the variables? We studied crop yields in 170 administrative districts for 27 variables (years). The Kaiser-Meyer-Olkin (KMO) index is run for the overall data set to detect sampling adequacy. As the KMO value is 0.65, according to the Kaiser empirical rule (Kaiser, 1974), the study data should be considered mediocre for the PCA.

The PCA of the residuals of the regression model allowed establishing that the number of statistically probable principal components is 7 according to the Horn procedure (Horn, 1965). The first seven components with eigenvalues larger than unity totally explain up to 66.8 % of variation in the regional cereal yield.

The variables used in the PCA are the ordinal quantities – the years, so the loadings of the principal components on the variables can be represented as dynamic changes in time (Fig. 1). This form of presentation allows interpreting meaningfully the installed principal components as oscillation processes with the different frequency. Thus, principal component 1 explains 22,60 % of the total variability of the grain and grain legumes yields. It is characterized by a predominant oscillation process within the period of 13 years. The variation of

the principal component 1 is clearly spatially determined (*I*-Morana 0.29, $p = 0.001$). The zones with higher values of the principal component 1 form clusters in some areas of the east of the studied region, as well as in the south, southwest and west. The zone with the lower values of the principal component 1 forms a clear cluster in the southwestern direction from the center of the region (Fig. 2).

Principal component 2 explains 11.68 % of the variability of the space of signs and its fluctuation has characteristic period of 6-7 years. This component demonstrates spatially regular patterns of variation (*I*-Moran 0.48, $p = 0.001$). Clusters with higher values of the principal component 2 are located on the north and northeast, and with the lower ones – the east and south (Fig. 2).

Principal component 3 explains 9.88 % of the total variability of the cereal and grain legumes yields and it has characteristic fluctuations with a period of 8-9 years. This component has a high level of spatial variability (*I*-Moran 0.51, $p = 0.001$). Clusters with higher values of the principal component 3 are common for the northwest and southeast, and with lower values for the east and southwest (Fig.2).

Principal component 4 explains 6.45 % of the variability of the space of signs. For its fluctuations in time, the period of 8-9 years is also the most characteristic (phase shift between components 3 and 4 is 4 years) (Fig. 1). The spatial patterns of this component are statistically significant (*I*-Morana 0.29, $p = 0.001$). The cluster with the higher values of the principal component 4 is characteristic for the center and north of the region, and with lowered values – for the east (Fig.2).

Principal component 5 describes 5.84 % of the variability of the feature space and is characterized by fluctuations with a period of 4 years. The *I*-Moran's index value for the PC5 is 0.39 (p = 0.001), which reveals a statistically positive spatial autocorrelation and as such demonstrates a highly clustering spatial pattern. Clusters with higher values of the principal component 5 are concentrated in the west, and with the lower ones in the central part of the region (Fig.2).

Principal component 6 explains 5.31 % of variability and represents the most high-frequency component of the grain yield dynamics with the most typical fluctuation period of 3 years. The variability of this component is characterized by the spatial component (*I*-Moran 0.19, $p = 0.001$). The zone with the higher scores of the main component 6 forms a clearly defined cluster in the south of the region (Fig. 2).

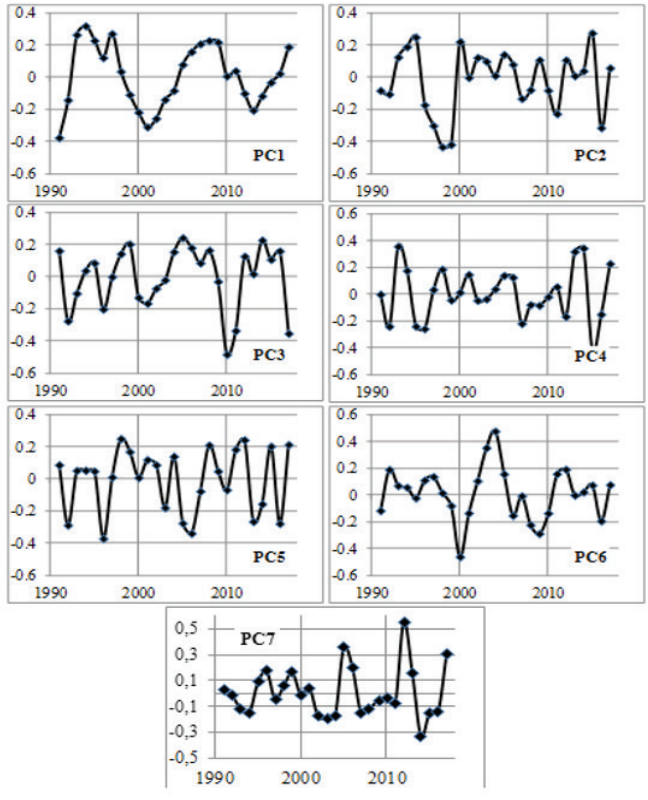

Figure 1: The principal components loadings to the variables 1 – 7

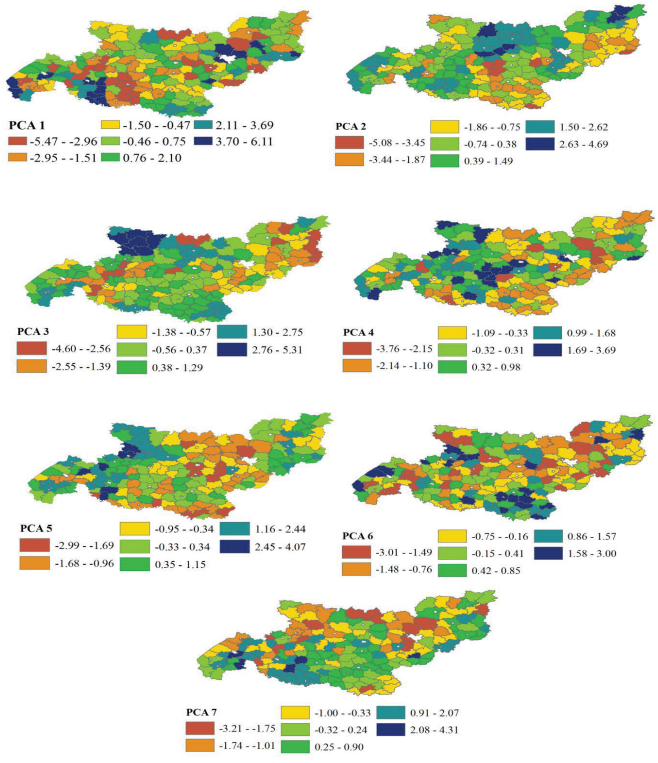

Figure 2: Spatial variation of the principal components 1-7

Principal component 7 describes 4.99 % of the variability of the feature space and varies in time within a period of about 5 years. The *I*-Moran statistic value (0.27, $p = 0.001$) indicates statistically positive autocorrelation and, thus, demonstrates the presence of a significantly clustered spatial pattern. Clusters with higher scores of the principal component 7 are concentrated in the southwest, and with the lower ones, in two clusters in the north of the region (Fig.2).

3.1 GEOGRAPHICALLY WEIGHTED PRINCIPAL COMPONENT ANALYSIS

Monte Carlo test was conducted to examine whether data matrix eigenvalues are spatially varying. As shown in Figure 3, the *p*-value for testing the local eigenvalues of standard deviations from GWPCA is 0.05. This value demonstrates that the spatial invariant hypothesis of local eigenvalues is significantly rejected at the 95 % level; or rather, there is a certain degree of spatial non-stationarity present in the data of regional cereal yield.

A key feature in GWPCA is finding the scale at which each localized PCA should operate; that is, choosing the kernel bandwidth. Before searching for an optimal bandwidth, it is necessary to decide a prior upon the number of components to retain (Harris et al., 2015; Gollini et al., 2015; Li et al., 2015). The previous global PCA results indicate that the first seven components can collectively explain 66.8 % of the variance in data structure. Consequently, it is reasonable to retain seven components for further GWPCA analysis.

For this study, we are guided by an automatic routine for bandwidth selection. Through an adaptive bandwidth selection procedure, an optimal bandwidth of 441 km has been reached, which is chosen to run the GWPCA analysis.

The GWPCA outputs can be visualized and interpreted, focusing on: (1) how the data dimensionality varies spatially and (2) how the original variables influence the components (Li et al., 2015). The spatial distribution of local PTV of first seven components can be mapped. The percentage of the spatial variation of the general variation shows a clearly pronounced variability, with the formation of spatially homogeneous clusters in the meridional direction. Compared with the outputs from global PCA, the GWPCA has exhibited its power and strength in analyzing spatial patterns of regional cereal yields by mapping spatial variations of local principal components. Further, the local variance at each admin-

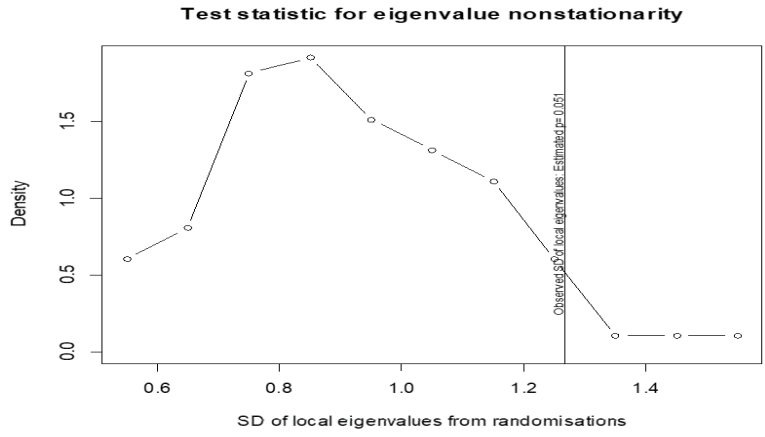

Figure 3: Monte Carlo test for GWPCA

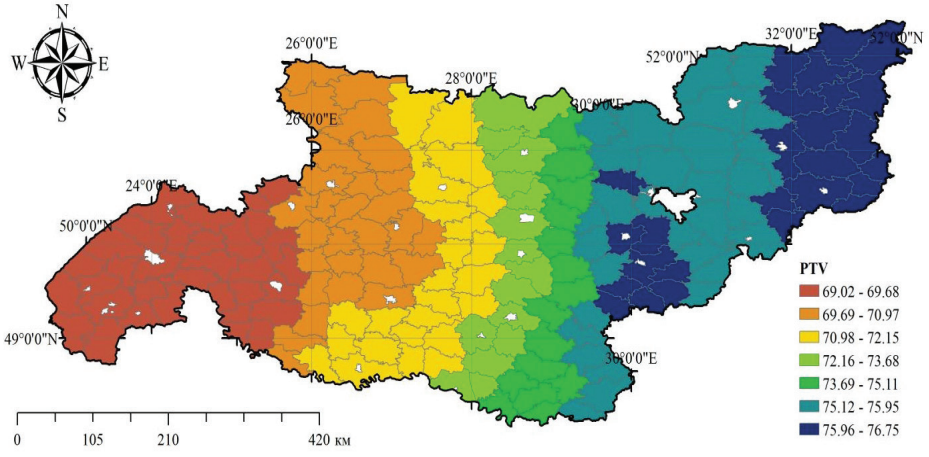

Figure 4: Spatial variation of the percentage of the total variation of the first seven principal components (percentage of total variance – PTV)

istrative district explained by the calculated GWPCA 1–7 can be visualized by mapping as well (fig. 4), which shows a clear east-west trend with the highest percentage variances distributed in the east, intermediate level in the central areas and the lowest values in the west. The obvious spatial clustering trend identified from the variance values in Figure 4 suggests that the interactions among these variables converge spatially.

It was suggested that the variables with the highest loading values and their impact intensity values can be mapped locally (Lloyd, 2010). We can next visualize how each of the seven variables locally influences a given component, by mapping the 'winning variable' with the highest absolute loading. Figure 5 shows the spatial distribution of variables with the absolute highest loading from GWPC 1–7 respectively.

The largest absolute loadings of a variable, which are the peculiarities of the spatial distribution of the grain yields in a given year, can be interpreted as a marker of the greatest sensitivity to oscillatory dynamics over time, as was shown for the global PCA. The local solutions can either largely correspond to the global outcome or vary in the significance of the oscillatory processes at the regional level, which may cause a change in the order of the components, or may be manifested in gaining statistical significance for processes that are not statistically significant at the global level.

The traditional representation of the "winning" variables for the principal components cannot fully reveal the nature of the spatially dependent relationship between the indicators estimated by the PCA. The overweigh of the factor loading is one of the aspects that reflects in the crop yields dynamics. Due to the oscillating nature of this dynamics, overweighs are the random outlier of the indicator at a certain moment of time in comparison with the general repetitive dynamics. Therefore, for each

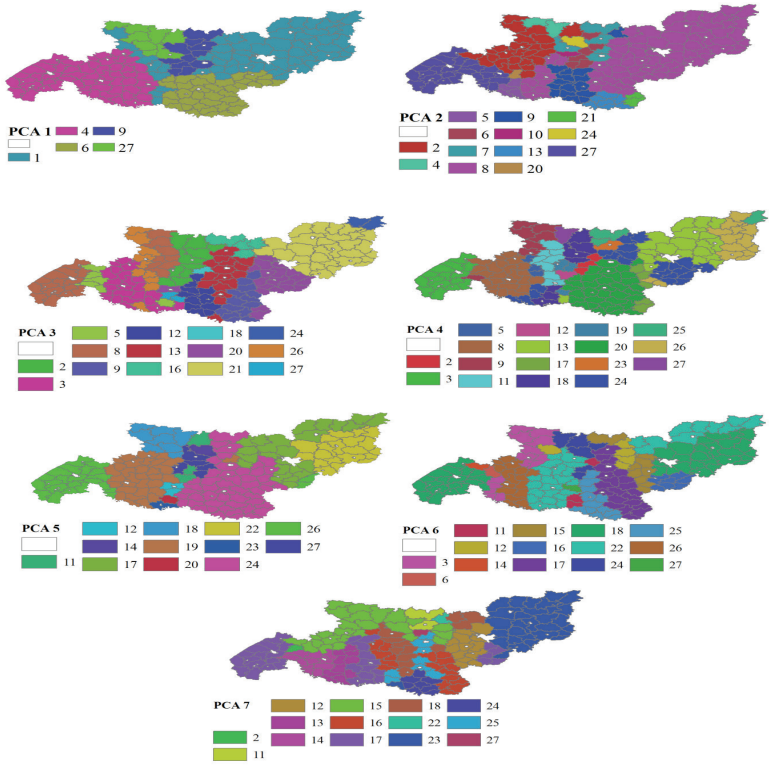

Figure 5: Spatial location of variables with the largest loading for the principal components 1-7

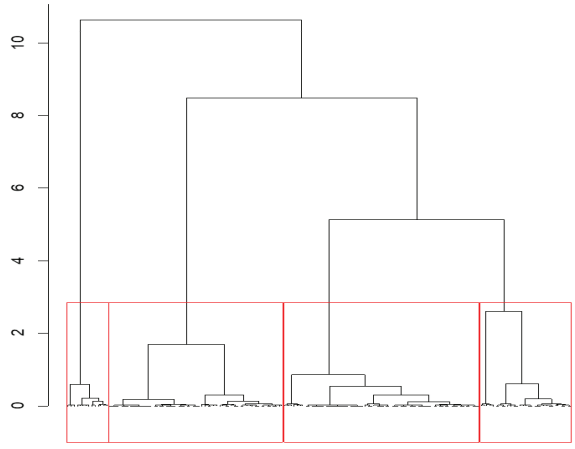

Figure 6: Cluster analysis of administrative districts by factor loadings values GWPC

of the statistically significant principal components, we conducted the classification of administrative districts by cluster analysis based on distance, which is opposite to the Pearson correlation coefficient. Such a distance is sensitive to the form of comparable indicators, and not to their absolute values. This approach allows identifying groups of administrative districts with the similar time dynamics of cereal and grain legumes yields in the aspect of the corresponding principal component. It can be assumed that the aggregate of the administrative districts with similar yields dynamics are also geographically close and form the homogeneous ecological regions.

To be consistent with the global PCA analysis, only the first component GWPC 1 from GWPCA will be interpreted in details, because it explains 22.60 % of the total variability.

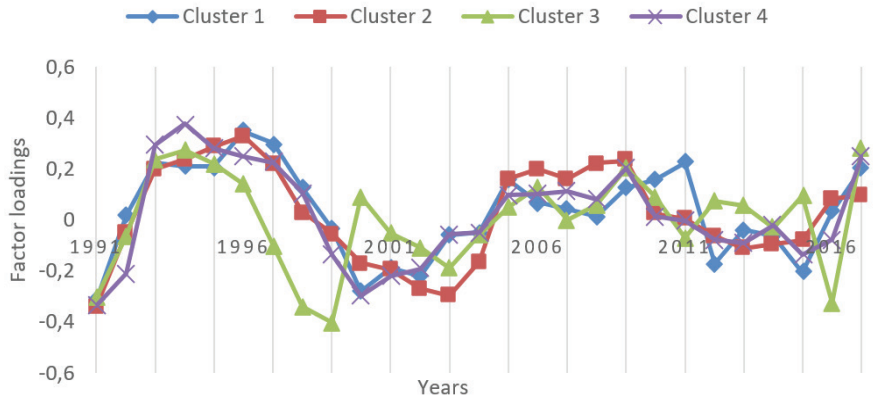

Figure 7: The average values of factor loadings of GWPC 1 for clusters 1-4

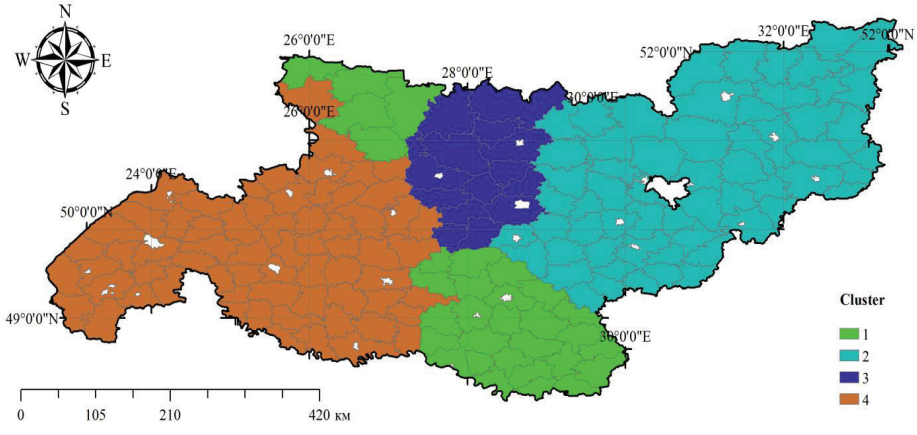

Figure 8: Spatial location of clusters obtained on the basis of factor loadings of GWPC 1

Cluster analysis of administrative districts by factor loading values GWPC 1 allowed the establishment of four homogeneous clusters (Fig. 6).

The average factor loadings for each cluster were calculated and the specifics of the corresponding clusters can be appreciated (Fig. 5, 7). For cluster 4, the greatest loading is characteristic for the variable 4 (yield in 1994), which corresponds to the so-called "winning" variable. But the information of the "winning variable" does not indicate anything about the characteristics of the overall yield dynamics within the corresponding cluster. For the cluster 3, the "winning" variable is the smallest (the largest module) variable 9, which is obviously an outlier, if the factor loading by this variable would be smaller, then the bidder for the "winning" would be the variable 27, which is also by its origin most likely an outlier.

Established clusters and their factor loadings distributions explaining the process progress that are characteristic for the corresponding cluster. Apparently, the general trend of the principal component 1 is the damping of the amplitude of oscillations during the research period and the predominance of higher frequency components of oscillatory dynamics corresponding to the heterogeneity of observations overtime or the heteroscedasticity. So, the Koenker-Bassett test for cluster 1 indicates the heteroscedasticity of the time dynamics of factor loadings (3.54, $p = 0.06$) (Koenker & Bassett, 1982). The heteroscedasticity level is even higher for cluster 2 (6.89, $p = 0.008$) and for cluster 4 (10.71, $p = 0.001$). The variation of factor loadings in cluster 3 is homogeneous over time (Koenker -Bassett test 1.62, $p = 0.20$). Thus, the qualitative feature of the grain and grain legumes yields dynamics in the corresponding clusters is the different level of damping of the oscillations of the principal component 1 over time.

Spatial arrangement of administrative areas included in the corresponding clusters is spatially regular (fig. 8).

Cluster 2 occupies the east and the center of the studied area. Cluster 3 is located northwest of cluster 2. Cluster 1 is disruptive and is located in the north and south of the region. Cluster 4 covers the west of the region. The central part of the region is characterized by a steady-state oscillation regime during the research period (cluster 3) or a relatively low level of heteroscedasticity (cluster 1). For the east and the west, the damping amplitude of oscillation of factor 1 is characteristic.

4 DISCUSSION

One of the most recent approaches to the quantifying spatial variations for specific land management is based on the division of the field into land management zones according to yield level (Khosla et al., 2002). This analysis of yield maps is a fundamental tool in the investigation and understanding of the causes of yield and crop quality variations and may become the decision procedure for land management (Filho et al., 2010).

In the course of our research, we made emphasis on the evaluation of the correlative relationship between time series of cereal and grain legumes (pulses) yield within the administrative districts of the forest and forest-prairie zone of Ukraine (1991-2017). The obtained results indicate that productivity as a result of agroecosystems functioning has a complex nature and is affected by the influence of different factors. The impact of these factors can be identified through the research of synchronous dynamics characteristics. The synchronous dynamics expresses itself through the forming of the correlation relationship. The correlation matrix is the basis for the PCA and cluster analysis. PCA allows to discover the main variability trends of agricultural crops productivity. Cluster analysis led to the establishment of the homogeneous ecological area (Zhukov et al., 2018; Zymaroieva et al., 2020b).

As such, PCA enables to identify the main statistical characteristics of the regional agricultural development and reveal the intrinsic complicate interactions among the selected variables (Li et al., 2015). Thus, the global PCA revealed the presence of dynamic processes of cereal and grain legumes yields which have the oscillatory nature with different frequencies. We associate oscillatory processes of the varying frequency with causes of different nature. The oscillating processes within a period of 10 years or more may be of climatic origin. So, the oscillatory process within the longest period (13 years – larger periods are hypothetical due to relatively limited time series) is characteristic for the principal component 1 (PCA 1), which explains the largest part of grain and grain legumes yields variability (22.6 %). It is possible to assume that among agro-ecological factors climate changes mostly influence crop productivity. For all other principal components, oscillation processes are more frequent (from 3 to 9 years). High frequency yield components may have the character of noise and may have an environmental origin as a consequence of such phenomena as the impact of diseases and pests, or the impact of weather anomalies.

Agroecological zoning was made according to the principle of uniformity of character dynamics of the production potential of agricultural areas. This approach is fundamentally different from that of zoning based on the total yield of crops (Lazarenko, 1995). Classification on the basis of absolute yield value is justified for systems that are close to the steady-state. In the face of global climate change and the transformation of the environmental regimes, this approach is unacceptable. The agroecological zones proposed by us do not differ in the overall level of productivity of grain and leguminous during the study period. Features of these zones are due to the values of principal components and reflect the nature of the relationship between different spatial units. Spatial distribution of principal components indicates a continual pattern, but their overlapping allows us to determine spatially discrete units, which we identified as agroecological zones. Each zone is characterized by a certain character and dynamics of production capacity and has an invariant pattern of response to varying climatic, environmental, and agroeconomic factors.

5 CONCLUSIONS

The global principal components analysis revealed the presence of dynamic processes of cereal and grain legumes yields which have the oscillatory nature with different frequencies. We associate oscillatory processes of the varying frequency with causes of different nature.

The oscillating processes with a period of 10 years or more may be of climatic origin. So, the oscillatory process with the longest period (13 years – longer periods are hypothetical due to relatively limited time series) is characteristic for the principal component 1 (PCA 1), which explains the largest part of cereal and grain legumes yields variability (22.6 %). It is possible to assume that among agro-ecological factors climate changes mostly influence crop productivity. For all other principal components, oscillation processes are more frequent (from 3 to 9 years). High frequency yield components may have the character of noise or may have environmental origins as a consequence of such phenomena as the impact of diseases and pests, or the impact of weather anomalies.

Due to geographically weighted principal components analysis, spatial units with a similar oscillating component of the cereal and grain legumes yields variation were revealed. Since we consider only the environmental component of yield variation, territorial clusters, within which the yields dynamics are the same, can be considered as agroecological zones for crops cultivation.

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Replace of the EPOST glyphosate with pre herbicides and application of different LPOST glyphosate rates for weed control in established vineyard

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Replace of the EPOST glyphosate with pre herbicides and application of different LPOST glyphosate rates for weed control in established vineyard

Abstract: Two-year field trials were conducted at two wine-growing districts (Kavadarci and Skopje in 2016 and 2017) to evaluate wheather oxyfluorfen, pendimethalin and flazasulfuron can replace early post-emergence (EPOST) application of glyphosate in established vineyard until its application in early summer period. The weed vegetation in vineyards in both years was consisted mainly of *Chenopodium album* L., *Setaria viridis* (L). Beauv., *Papaver rhoeas* L., *Xanthium strumarium* L., *Lolium multiflorum* Lam., *Sorghum halepense* (L.) Pers. and *Cynodon dactylon* (L.) Pers. Pendimethalin, oxyfluorfen and flazasulfuron efficiently reduced predominant annual weeds and *S. halepense* seedlings in 2017, but not in 2016. PRE herbicides, regardless year, had no significant effect on *C. dactylon*. Opposite, the efficacy of EPOST applied glyphosate was significantly lower in 2017 compared to 2016. LPOST glyphosate applied at 2.0 l ha^{-1} provided at least 94 % control of dominant annual broadleaf and grass weeds. LPOST application of glyphosate at 2.0 l ha^{-1} and glyphosate at 2.0 and 4.0 l ha^{-1} resulted in unsatisfactory weed control of predominant perennial *S. halepense,* and *C. dactylon*, respectively. LPOST glyphosate applied at 4.0 l ha^{-1} provided control of *S. halepense* by 84 % or more. LPOST glyphosate applied at 8.0 l ha^{-1} reduced the amount of *S. halepense* and *C. dactylon* at least 97 %. Grapevine yield of both varieties was not lower in all herbicide treatments in 2016 compared with 2017. However, yield in the PRE herbicide treatments fb 2.0 and 4.0 l ha^{-1} glyphosate was collectively 15-19 % and 17-19 % lower compare to PRE herbicide treatments fb 8.0 l ha^{-1} glyphosate and standard two applications of glyphosate, respectively for both years and districts. No impacts to grapevine growth were observed from PRE herbicide treatments at either district.

Key words: established vineyard; weeds; herbicides; weed control

Zamenjava EPOST glifosta s s tretiranjem z izbranimi herbicidi pred kalitvijo plevelov in uporaba različnih odmerkov LPOST glifosta za uravnavanje plevelov v vinogradu

Izvleček: Dvoletni poskus je bil izveden v dveh vinogradniških območjih Severne Makedonije (Kavadarci in Skopje, 2016 in 2017) za ovrednotenje zgodnje poletne uporabe herbicidov oksifluorfena, pendimetalina in flazasulfurona kot nadomestilo kasnejše uporabe glifosata (EPOST) v ustaljenem vinogradu. Plevelno vegetacijo v vinogradu so v obeh letih poskusa sestavljale v glavnem naslednje vrste: bela metlika (*Chenopodium album* L.), zeleni muhvič (*Setaria viridis* (L). Beauv.), poljski mak (*Papaver rhoeas* L.), navadni bodič (*Xanthium strumarium* L.), mnogocvetna ljulka (*Lolium multiflorum* Lam.), divji sirek (*Sorghum halepense* (L.) Pers.) in prstasti pesjak (*Cynodon dactylon* (L.) Pers.). Pendimetalin, oksifluorfen in flazasulfuron so učinkovito zavrli rast predvsem enoletnih plevelov in divjega sirka v letu 2017, in ne v letu 2016. PRE herbicidi niso imeli ne glede na leto nobenega značilnega vpliva na prstasti pesjak. Nasprotno je bil učinek EPOST uporabe glifosata v letu 2017 značilno manjši v primerjavi z letom 2016. LPOST uporaba glifosata v odmerku 2,0 l ha^{-1} je najmanj za 94 % zavrla dominatne enoletne širokolistne in travnate plevele. LPOST uporaba glifosata v odmerkih 2,0 in 4,0 l ha^{-1} je bila neučinkovita pri zatiranju trajnih plevelov kot sta divji sirek in prstasti pesjak. LPOST uporaba glifosata v odmerku 4,0 l ha^{-1} je zavrla divji sirek več kot 84 % . Uporaba LPOST glifosata v odmerku 8,0 l ha^{-1} je zmanjšala zapljeveljenost z divjim sirkom in prstastim pesjakom za najmanj 97 %. Pridelek grozdja obeh sort se v letu 2016 ni zmanjšal zaradi uporabe herbicidov v primerjavi z letom 2017. Kljub temu se je pridelek pri obravnavanju s PRE herbicidi v odmerkih 2,0 in 4, l ha^{-1} glifosata skupno zmanjšal za 15-19 % in 17-19 % v primeri z obravnavanjem s PRE herbicidi v odmerku 8,0 l ha^{-1} glifosata in standa rdno uporabo glifosata v obeh letih in na obeh območjih. Na rast vinske trte ni imelo obravnavanje s PRE herbicid nobenega vpliva na obeh območjih.

Ključne besede: ustaljen vinograd; pleveli; herbicidi; uravnavanje plevelov

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1 INTRODUCTION

In Republic of North Macedonia weeds management system in established vineyard based on multiple applications of glyphosate, which is required to maintain effective weed control throughout the season. Another approach that may improve weed control and aid in the stewardship of glyphosate use is to apply a pre-emergence (PRE) herbicides prior to a single late post-emergence (LPOST) application of glyphosate. However, to prevent yield loss due to competition with weeds is required a high efficacy of herbicides against weeds during the critical weed control period of different crops (Knežević et al., 2002). This is an important component of an Integrated Weed management (IWM) system and is a major factor in deciding the optimal timing of herbicide application (Swanton et al., 1999; Boerboom, 2000). In established vineyard, early season weed control is important (Mitchem and Monks, 2005), because vigorous weed growth may allow economically important weed species to reduce grape vine growth and yields by competing for water, nutrients, and sunlight (Kadir and Al-Khatib, 2006). In addition, weed competition can impair berry quality and interfere with the harvest (Zabadal and Dittmer, 1994). Studies have shown that full-season competition due to unmanaged weeds could cause reductions in grapevine yield of up to 37 %, cane mass of up to 68 %, in number of clusters per vine of up to 28 %, and in berry mass of up to 3 % (Byrne and Howell, 1978 cit. by Sanguankeo et al., 2009).

Although cultivation, mowing, and mulching are important weed-management practices in grape vine production (Pool et al., 1990; Kadir et al., 2004), herbicides, particularly use of glyphosate, are the major components of a weed-control program in this crop (Kaps and Odneal, 1991; Kadir and Bauernfeind, 2005). The reliance on glyphosate for weed control in established vineyard in Republic of North Macedonia based on effective weed control throughout the season. But, glyphosate lacks residual soil activity (Baylis, 2000), and multiple applications may be required to provide adequate control of weed species throughout the season (Nurse et al., 2006). The possibility of glyphosate resistance in some weed species (Boerboom and Owen, 2006) means stewardship of glyphosate use will be important to reduce the reliance on this herbicide mode of action for weed control (Lopes Ovejero et al., 2013). An alternative approach that may reduce the amount of glyphosate used and improve season long weed control is the application of a pre-emergence residual herbicide prior to the in crop application of glyphosate (Monsanto Company, 2005).

A few soil applied pre-emergence herbicides are currently registered for use in established vineyard in R. N. Macedonia. Among them, pendimethalin, oxyfluorfen, and flazasulfuron are the most frequent applied. There is limited information whether oxyfluorfen, pendimethalin and flazasulfuron can replace early post-emergence (EPOST) application of glyphosate in established vineyard until its LPOST application in early summer period.

Pendimethalin, a dinitroaniline, and oxyfluorfen a diphenyl ether, are selective pre- and postemergence herbicides that are used to control many annual broadleaf weeds and grasses in many crops including, vineyards (Kaps and Odnea 1991; Mitchem and Monks, 2005; Patil et al., 2008; Alister et al., 2009). Flazasulfuron is a new, recently registered selective, systemic sulfonylurea in viticulture in R. N. Macedonia. It may be used as a pre- or early post-emergence herbicide at very low rates 0.15-0.20 l ha^{-1} and has a wide herbicidal spectrum (Tomlin, 2000; Grove, 2011). Pendimethalin, oxyfluorfen, and flazasulfuron are lipophilic, with a $LogK_{ow}$ of 5.18, 4.47 and 1.30, respectively (Đurović et al., 2008; Anonymous, 2012). This chemical property is associated with a strong organic soil adsorption that results in limited soil mobility (Ying et al., 2000; Barba et al., 2003; Yen et al., 2003; Leak, 2013). Soil residual activity may be maintained for 10-15 weeks (Dev et al., 1992; Raimondi et al., 2010; Grey and McCullough, 2012), but, late germinating weeds may not be controlled satisfactorily. Nonetheless, in an established vineyard pendimethalin, oxyfluorfen, and flazasulfuron may replace early post-emergence (EPOST) application of glyphosate until its late post-emergence LPOST application in full vegetative growth stages (vigorously growing) of perennial weeds, but limited data exists about that.

Therefore, the main objectives were (i) to determine whether acceptable weed control of oxyfluorfen, pendimethalin and flazasulfuron applied PRE may replace EPOST application of glyphosate in established vineyard until the early summer when usually LPOST glyphosate is applied, and (ii) to evaluate efficacy of different LPOST glyphosate rates depending on the weed species in the established vineyard flora.

2 MATERIALS AND METHODS

The field trials were conducted in years 2016 and 2017 on commercial established vineyards at Kavadarci and Skopje wine-growing district in central and northern Macedonia, on vertisol and chromic cambisol, respectively (Filipovski, 2006) (Table 1). The experimen-

Table 1: Soil characteristics of the field trials in the wine growing regions of Kavadarci and Skopje district

Region	Soil	coarse	fine sand	clay + silt %	organic matter	pH-water
Kavadarci	Vertisol	3.5	30.0	60.3	2.4	7.2
Skopje	Chromic cambisol	10.4	41.7	40.5	2.6	6.4

Table 2: Treatments, trade names, rates and time of application of herbicides in the wine growing regions of Kavadarci and Skopje district in 2016 and 2017

Treatments	Trade name	Rate	Time of application
Pendimethalin 455 g l^{-1}	Stomp Aqua	5.0 l ha^{-1}	PRE - em
Oxyfluorfen 240 g l^{-1}	Goal	6.0 l ha^{-1}	PRE - em
Flazasulfuron 250 g l^{-1}	Chikara	0.2 l ha^{-1}	PRE - em
Glyphosate 360 g l^{-1}	Dominator	3.0 l ha^{-1}	EPOST- em
Glyphosate 360 g l^{-1}	Dominator	2.0 l ha^{-1}	LPOST - em
Glyphosate 360 g l^{-1}	Dominator	4.0 l ha^{-1}	LPOST - em
Glyphosate 360 g l^{-1}	Dominator	8.0 l ha^{-1}	LPOST - em
Control	-	-	-

Abbreviations: PRE - pre-emergence; EPOST - early-post-emergence; LPOST - late-post-emergence

tal design was a randomized complete block with four replicates.

The trials were conducted in different sites of the same vineyards in 2016 and 2017. Both sites were spontaneously populated by *Papaver rhoeas* L., *Chenopodium album* L. *Setaria viridis* L., *Xanthium strumarium* L., *Lolium multiflorum* Lam., *Sorghum halepense* (L.) Pers. and *Cynodon dactylon* (L.) Pers. 'Cabernet Sauvignon' and 'Black Magic' grape vines were used at Kavadarci district and Skopje district, respectively. The vineyards were established in 2010 at a spacing of 1.0 m between vines and 2.3 m between rows. The vineyard was drip irrigated, with sprinklers available for frost protection. Drip irrigation and fertilization were applied uniformly across all treatments, based on conventional practices for commercial production.

Herbicides were applied in-row (width 1.0 m) with a CO_2-pressurized backpack sprayer calibrated to deliver 300 l ha^{-1} aqueous solution at 220 kPa.

Prior to the commencement of the trials, diquat (dibromide salt as Di-Quattro®, 200 g a.i. l^{-1}, Agriphar S.A., Belgium) was applied at 2.0 l ha^{-1} to control present established weeds. The PRE herbicide treatments were applied in the early spring, March 3 and February 26 at Kavadarci district and March 8 and March 1 at Skopje district in 2016 and 2017, respectively, when grapevines were still in dormancy. The LPOST application included different rates (2.0, 4.0 and 8.0 l ha^{-1}) of glyphosate (isopropylamine salt). The LPOST glyphosate (isopropylamine salt) treatments were applied 84 days after PRE application (one day before EPOST glyphosate application), i.e. in full vegetative growth stages (vigorously growing) of perennial weeds. For efficacy comparison, two applications of glyphosate (standard application) were made; initially with 3.0 l ha^{-1} when weeds were 10-12 cm tall (EPOST), and repeated with 8.0 l ha^{-1} 45 days after EPOST glyphosate application (one day before LPOST glyphosate application), i.e. in full vegetative growth stages (vigorously growing) of perennial weeds (LPOST). Untreated control was included in the studies, as well.

The control plots were left untreated during the entire experimental period. Grapevine injury was visually evaluated based on a 0=100 % rating scale, where 0 % is no injury to grapevine plants, and 100 % is complete collapse of grapevine (Frans et al., 1986). Grapevine injury was estimated 28 and 56 days after PRE treatments. Weed control efficacy was estimated 84 days after PRE applications, 45 days after EPOST glyphosate application, and 28 days after LPOST application of different glyphosate rates from 1m^2 area within each plot at both district during two-year experimental period. Herbicide efficacy was calculated by equitation (Chinnusamy et al., 2013):

$$W_{CE} = \frac{Wup - Wtp}{Wup} \times 100$$

where:
W_{CE} - weed control efficiency
Wup - number of weeds in the untreated plots
Wtp - number of weeds in the treated plots

Table 3: Total monthly precipitation and average air temperature from February to October in the wine growing regions of Kavadarci and Skopje district in 2016 and 2017 and average year 1990-2010

	Kavadarci district				Skopje district			
	2016		2017		2016		2017	
Months	P (mm)	T (°C)	P (mm)	T (°C)	P (mm)	T (°C)	P (mm)	T (°C)
February	13	7.1	19	5.5	7	7.9	26	6.8
March	8	9.9	33	8.6	17	11.5	39	10.7
April	16	13.5	43	12.7	11	10.9	48	12.9
May	35	19.1	22	17.9	40	14.2	25	14.8
June	68	22.9	56	21.7	61	21.0	85	21.0
July	56	25.1	28	23.2	65	22.7	46	22.9
August	5	25.8	28	24.9	16	24.8	37	22.6
September	21	20.6	104	19.3	57	17.9	168	17.1
	Average 1990-2010							
Months	P (mm)		T (°C)		P (mm)		T (°C)	
February	32		5.5		35		5.0	
March	37		8.2		41		9.9	
April	37		13.3		37		12.2	
May	61		18.4		60		16.8	
June	39		22.3		46		21.0	
July	30		24.6		32		23.2	
August	26		24.5		31		23.0	
September	30		20.1		41		18.4	

Abbreviations: P – precipitations; T - temperature

Number of grape cluster per vine, cluster mass and grape vine yield of 'Cabernet Sauvignon' and 'Black Magic' were measured in four vines that were randomly selected within each experimental unit. The grapes harvested on the wine were counted and weighed on site. Number of grape clusters, their mass and grape vine yield were determined when all the experimental units reached the minimum harvest criterion (i.e., at full maturity), in July for 'Black Magic", and in September for 'Cabernet Sauvignon', respectively.

Total monthly rainfalls are shown in Table 2. The early spring of 2016 was dry in both districts. Precipitations in February, March and April were very low (13, 8 and 16 mm in Kavadarci district, and 7, 17 and 11 mm in Skopje districts). However, May, June (especially), and even July were unusually wet months. Opposite, spring of 2017 was humid. Precipitation occurred during February, March and April were a little bit above the average for both districts. In Kavadarci district, precipitation occurred during the three days in the middle of February, and during the first two and the last four days of March. In April and May, it rained on seven and nine days at intervals throughout each month, respectively. In June, precipitation occurred in the second decade of the month. Similar, in Skopje district same year, precipitation occurred in the third decade of February, and at the end of the first and beginning of the second decade of March. In April and May, it rained on seven and ten days at intervals throughout each month, respectively. Summer months in 2017, particularly June, were very humid, 53 % above the 30 years average for the Skopje district (110 mm).

All treatments in both years were applied at times when herbicide applications typically occur in North Macedonia vineyard production.

The data from both years were combined, tested for homogeneity of variance and normality of distribution (Ramsey and Schafer, 1997) and were log-transformed as needed to obtain roughly equal variances and better symmetry before ANOVA was performed. Data were transformed back to their original scale for presentation. Data were pooled across locations and years and means were separated by using LSD test at 5 % of probability.

3 RESULTS AND DISCUSSION

3.1 WEED CONTROL

Weed density 56 days after PRE herbicide application in nontreated control plot was 98 and 148 plants/m^2 in 2016 and 2017, respectively, at Kavadarci district, and 113 and 178 plants/m^2 in 2016 and 2017, respectively, at Skopje district. Efficacy of PRE herbicides and EPOST applied glyphosate varied among weed species, treatments and years, respectively. Inconsistent weather patterns between the 2 years of the study likely influenced the weed control. Limited precipitation after PRE application may have contributed to the poor performance of PRE herbicides at both districts in 2016 (Table 3). Opposite, the humid spring in 2017 (Table 3), and continuous new weed plants emergence, particularly following EPOST glyphosate application, probably was the most likely reason for lower efficacy of EPOST applied glyphosate in 2017 compared to its application in 2016 at both districts (Table 4 and 5).

3.1.1 Chenopodium album

The interaction between treatment and year in two distinct years showed significant results for control of *C.album* in Kavadarci district with PRE herbicides and EPOST glyphosate application, contrary *C. album* control not showed significant results among years for different LPOST glyphosate application. In Kavadarci district in 2016, pendimethalin, oxyfluorfen and flazasulfuron provided satisfied efficacy of *C. album* between 69 and 82 %. Opposite, all PRE herbicides provided greater efficacy than 84 % of *C. album* 84 days after PRE treatments in 2017 at the same district (Table 4). Similar, pendimethalin alone or with lower rates of flumioxazin, controlled *C. album* between 82 and 87 % (Taylor-Lovell et al., 2002), and oxyfluorfen provided *C. album* control from 88 to 95 % (Jursík et al., 2011). The differences in control of *C. album* between 2016 and 2017 demonstrated that inadequate or delayed precipitation can reduce PRE herbicide effectiveness and decrease weed control (Armel et al., 2003; Lyon and Wilson, 2005; Stewart et al., 2012). LPOST application of glyphosate, regardless rates and years, increased *C. album* control up to 100 %. EPOST applied glyphosate provided 95 and 88 % control of *C. album* in 2016 and 2017, respectively. The higher precipitation in the spring 2017, may have promoted late emergence of *C. album* plants It is widely known that glyphosate is used in combination with PRE herbicide (Lopes Ovejero et al., 2013), because does not have residual control, therefore would not have controlled late emerging plants of this weed. It was concluded that PRE herbicide treatments followed by different efficacy of LPOST glyphosate applications, was the most effective for controlling *C. album* in both 2016 and 2017.

3.1.2 *Setaria viridis* and other weed species

The interaction between treatment and year in two distinct years showed significant results for control of *S. viridis* and other weed species in Kavadarci district with PRE herbicides and EPOST glyphosate application. *S. viridis* and other weed species control did not differ between years for different LPOST glyphosate applications. At Kavadarci district in 2016, *S. viridis* and other weed species control was between 70 and 77 % with pendimethalin and oxyfluorfen. The greatest control was achieved with flazasulfuron (80-83 %) (Table 4). In 2017, pendimethalin and oxyfluorfen controlled *S. viridis* and other weed species 86 to 93 %, and flazasulfuron 94 to 98 %. Obtaining the greatest weed control in the plots treated with flazasulfuron was not surprising, because flazasulfuron has longer residual activity than pendimethalin and oxyfluorfen and good grass weed control (Nieto et al., 1998; Singh et al., 2012). Control of *S. viridis* and other weed species improved after the LPOST glyphosate applications ranging from 99 to 100 % and 98 to 100 % in 2016 and 2017, respectively. From the other side, control of *S. viridis* and other weed species ranged 94 to 87 % for EPOST glyphosate application in both years (Table 4). The lower control ratings of *S. viridis* and other weed species in 2017 compared to 2016 may have been the result of more precipitation, which have promoted late weed emergence after the EPOST glyphosate application.

3.1.3 Papaver rhoeas

The interaction between treatment and year in two distinct years showed significant results for control of *P. rhoeas* with PRE herbicides and EPOST glyphosate application at both districts, but *P. rhoeas* control did not differ between years for different LPOST glyphosate applications, as well. Flazasulfuron controlled *P. rhoeas* more than pendimethalin and oxyfluorfen 84 DAT (Tables 4 and 5). At Kavadarci district flazasulfuron controlled *P. rhoeas* by 83 and 85 % in 2016. Bonasia et al. (2012) reported similar levels of *P. rhoeas* control in lampascione - (*Muscari comosum* (L.) Mill.) with flazasulfuron. Pendimethalin and oxyfluorfen did not control *P. rhoeas* more than 78 and 77 %, respectively the same

year. Because PRE herbicides require precipitation to move into the zone of active weed seeds germination, an increase of precipitation in 2017 in compare with 2016, may explain the variability among PRE treatments. Flazasulfuron, pendimethalin, and oxyfluorfen had 12 to 19 % higher efficacy of *P. rhoeas* compared to the same treatments previous year (Table 5). Similar, at Skopje district efficacy of PRE herbicides significantly lower in 2016 compare to 2017. Pendimethalin, oxyfluorfen and flazasulfuron controlled *P. rhoeas* slightly better than did same herbicides at Kavadarci district in 2016, but provided similar efficacy in 2017 (Table 4). Efficacy of EPOST applied glyphosate was significantly lower in 2017 compared to 2016, because of humid spring, which have promoted late emergence of *P. rhoeas* plants and lacks of glyphosate soil activity. However, application of different rates of LPOST glyphosate provided consistent control of *P. rhoeas* (95-100 %) at both districts and in both years (Tables 4 and 5).

3.1.4 Sorgum halepense

A significant treatment by year interaction resulted in two distinct years for *S. halepense* control in Kavadarci district with PRE herbicides and EPOST glyphosate application. *S. halepense* control did not differ among years for different LPOST glyphosate applications. In 2016 pendimethalin and oxyfluorfen provided no more than 76 % control of *S. halepense* seedlings (Table 4). Flazasulfuron provided significantly higher efficacy (79-88 %) in control of *S. halepense* seedlings. In 2017, all PRE treatments provided greater control of *S. halepense*. Pendimethalin provided at least 80 % control of *S. halepense* seedlings. In investigations of Grey and Webster (2013), pendimethalin provides 90 % control of *S. halepense* seedlings. *S. halepense* seedlings control ranged from 78 to 82 % with oxyfluorfen. Flazasulfuron provided control as good as or better than did pendimethalin and oxyfluorfen (92-93 %). McGovern et al. (2010) found similar results with flazasulfuron applied at 0.025 and 0.050 kg/ha a.i., which produced very good initial *S. halepense* control at 30 DAA (95 %) that was maintained through 90 DAA (93 and 90 % control), respectively. EPOST application of glyphosate at 3.0 l ha^{-1} reduced *S. halepense* by 90 and 84 % in 2016 and 2017, respectively. Opposite, the lowest LPOST glyphosate application (2.0 l ha^{-1}) did not control *S. halepense* more than 52 %, while LPOST glyphosate applied at 4.0 l ha^{-1} provided control of *S. halepense* by 84 % or more, 28 DAT. The highest rate of LPOST glyphosate (8.0 l ha^{-1}) provided 100 % *S.* halepense control, in each year.

3.1.5 Xanthium strumarium

A significant treatment by year interaction resulted in two distinct years for *X. strumarium* control in Skopje district with PRE herbicides and EPOST glyphosate application, but *X. strumarium* control did not differ among years for different LPOST glyphosate applications (Table 4). Pendimethalin, oxyfluorfen and flazasulfuron provided inconsistent control of *X. strumarium* and varied greatly between years and PRE treatments (ranging from 53 to 89 % control). Variability in control between years demonstrated that less rainfall before and, particularly after the PRE applications in 2016 did not dissolve the herbicides in soil water solution so that they could not be taken up by the emerging weeds after germination (Novosel et al., 1998; Chomas and Kells, 2004). Pendimethalin, oxyfluorfen and flazasulfuron provided 53 to 78 % *X. strumarium* control in 2016, but in 2017 the same PRE treatments controlled *X. strumarium* 73 to 89 %. Opposite, efficacy of the EPOST glyphosate was significantly higher in 2016 than in 2017. However, LPOST glyphosate treatments consistently provided the highest levels of *X. strumarium* control, between 94 and 100 % (Table 5).

3.1.6 Lolium multiflorum

A significant treatment by year interaction resulted in two distinct years for *L. multiflorum* control in Skopje district with PRE herbicides. However, *L. multiflorum* control did not differ among years for EPOST and different LPOST glyphosate applications (Table 5). In 2016, PRE herbicides provided control of *L. multiflorum* between 75 and 83 %. Control improved in 2017, because Skopje district received 22 and 37 mm more precipitation in March and April, respectively compared to same months in 2016. It is likely that these humid conditions contributed to the increased efficacy of PRE herbicides. Pendimethalin provided at least 91 % control of *L. multiflorum*. Alshallash (2014) reported for effective control of *L. multiflorum* with pendimethalin. *L. multiflorum* control ranged from 86 to 93 % with oxyfluorfen, while flazasulfuron provided control better than did pendimethalin and oxyfluorfen (96-100 %). Excellent control with flazasulfuron was expected as this herbicide provides excellent control of this species (Nieto et al., 1998). Control of *L. multiflorum* improved after the LPOST glyphosate applications, particularly in 2016, ranging from 95 to 100 % and 97 to 100 % in 2016 and 2017, respectively. EPOST glyphosate provided 97 % control of *L. multiflorum* for both years (Table 5).

3.1.7 Cynodon dactylon

C. dactylon control did not differ among years for PRE herbicides, EPOST and different LPOST glyphosate applications (Table 4). PRE herbicides, regardless year, had very poor or no effect on *C. dactylon*. Control of *C. dactylon* was less than 41 % and 32 % with any PRE treatment in 2016 and 2017, respectively (Table 5). In general, preemergence herbicides do not control *C. dactylon*, because the principle means of its propagation is through the rhizomes and stolons (Holm et al., 1977; Kostov, 2006). However, only the highest rate of LPOST glyphosate (8.0 l ha^{-1}) showed high efficacy for *C. dactylon* by 97 % or more, 28 DAT in both years. LPOST glyphosate applied at 2.0 and 4.0 l ha^{-1}, did not control *C. dactylon* more than 71 and 69 % in 2016 and 2017, respectively. Poor control of *C. dactylon* was obtained by EPOST glyphosate application, as well, which was ranged 45 to 48 % in both years (Table 5).

3.2 IMPACT ON GRAPEVINE YIELD

Number of cluster per vine, cluster mass and grape vine yield of 'Cabernet Sauvignon' and 'Black Magic' at both districts varied among treatments and years, mainly due to poor performance of PRE herbicides in 2016 as well as non sufficient control of perennial weeds, particularly *Cynodon dactylon* with LPOST glyphosate application at 2.0 and 4.0 l ha^{-1} in 2016 and 2017, respectively (Table 6).

Generally, vine yield components were lower in 2016 compared with 2017, but without significant effect years x herbicides interaction. In 2016 there was significant effect for the number of cluster per vine at both districts, but not effect was recorded in 2017. Herbicide treatments had effect on cluster mass in both years and districts. For example, cluster mass in the PRE herbicide treatments fb 2.0 and 4.0 l ha^{-1} glyphosate were significantly lower compare with standard two applications of glyphosate and PRE herbicides fb 8.0 l ha^{-1} glyphosate, respectively. Grape vine yield was insignificantly lower in all herbicide treatments in 2016 compared with 2017. Yield in the PRE herbicide treatments fb 2.0 and 4.0 l ha^{-1} glyphosate was collectively 15-19 % and 17-19 % lower compare to PRE herbicide treatments fb 8.0 l ha^{-1} glyphosate and standard two applications of glyphosate, respectively for both years and districts. Similar results were obtained by Sanguankeo et al. (2009). In a rainy year, the herbicides treatments did not differ in grape yield, but in a dry year, in herbicide treatments the grape yield reductions was around 22 %.

3.3 IMPACT ON GRAPEVINE GROWTH

Grapevine growth was ordinary throughout the both growing seasons, and no impacts were observed on vines growth in plots with PRE herbicide treatments at ither district (data not shown). These results are expected because vineyards were well established (since 2010) and their roots were relatively deep in the soil (Kadir et al., 2004). In addition, herbicides used in the study have poor water solubility and leachability (Yen et al., 2003; Leak, 2013). Furthermore, soils at both sites (vertisol and chromic cambisol) contain relatively large amount of silt + clay (60.3 and 40.5 %, respectively), which would result in less herbicide leaching.

4 CONCLUSIONS

Results of this research demonstrate that the efficacy of PRE herbicides pendimethalin, oxyfluorfen and flazasulfuron in established vineyards are strongly depended by the amount of precipitation and weed population. Limited precipitation after PRE application contributed to the poor performance of these herbicides at both districts in 2016. Therefore, pendimethalin, oxyfluorfen and flazasulfuron effectively reduced predominant *Papaver rhoeas, Chenopodium album, Setaria viridis, Xanthium strumarium, Lolium multiflorum* and *Sorghum halepense* in 2017, but not in 2016. This suggests that the application of PRE herbicides for residual weed control is unnecessary and does not improve weed control in comparison to EPOST glyphosate application under dry conditions only. The precipitation amount should be considered when selecting the most appropriate PRE weed management strategy in established vineyard as a replace for the first glyphosate application.

However, there was benefit from the application of the PRE herbicides applied in early spring prior to LPOST glyphosate application in 2017. The excellent weed control afforded by PRE herbicides in this year resulted in very few weeds being present at the time of LPOST glyphosate applications.

Because of that, stewardship of glyphosate use will be important to reduce the reliance on this herbicide mode of action for weed control in established vineyard. Preceding glyphosate application with PRE herbicides may also replace the application of EPOST glyphosate until its LPOST application in the full vegetative growth stages (vigorously growing) of perennial weeds, i.e. in early summer period.

Table 4: *Chenopodium album, Setaria viridis, Papaver rhoeas* and *Sorgum halepense* efficacy 84 days after PRE, 45 days after EPOST and 28 days after LPOST glyphosate treatments, respectively at Kavadarci district in 2016 and 2017

					Kavadarci district													
					Chenopodium Album				*Setaria viridis*				*Papaver rhoeas*				*Sorgum* halepense	
					2016		2017		2016		2017		2016		2017		2016		2017	
Treatment	Rate (l ha^{-1})		Time of application		% Efficacy (%)															
Glyphosate fb glyphosate	3.0	8.0	EPOST	LPOST	95^{a}	100^{a}	88^{bc}	100^{a}	94^{a}	100^{a}	87^{e}	100^{a}	96^{a}	100^{a}	90^{bd}	100^{a}	90^{a}	100^{a}	84^{b}	100^{a}
Pendimethalin fb glyphosate	5.0	2.0	PRE	LPOST	77^{bc}	97^{b}	86^{bc}	96^{b}	76^{cde}	100^{a}	88^{e}	98^{b}	77de	100^{a}	90^{bd}	98^{b}	70^{efg}	52^{d}	76de	45^{d}
Oxyfluorfen fb glyphosate	6.0	2.0	PRE	LPOST	69^{d}	98^{b}	85^{c}	98^{ab}	68^{f}	99^{a}	89de	98^{b}	72^{e}	98^{b}	85^{d}	98^{b}	64^{g}	40^{e}	72^{e}	48^{d}
Flazasulfuron fb glyphosate	0.2	2.0	PRE	LPOST	75^{bcd}	98^{b}	97^{a}	96^{b}	80^{bcd}	100^{a}	96^{ab}	99^{ab}	83^{bc}	100^{a}	95^{ab}	100^{a}	76de	44^{e}	85^{b}	50^{d}
Pendimethalin fb glyphosate	5.0	4.0	PRE	LPOST	72^{cd}	100^{a}	89^{bc}	100^{a}	77^{bcd}	100^{a}	90^{cde}	100^{a}	78^{cd}	100^{a}	88^{cd}	100^{a}	75^{def}	89^{bc}	85^{b}	90^{b}
Oxyfluorfen fb glyphosate	6.0	4.0	PRE	LPOST	78^{bc}	100^{a}	84^{c}	100^{a}	77^{bcd}	100^{a}	86^{e}	100^{a}	75de	100^{a}	88^{cd}	100^{a}	69^{fg}	85^{c}	82^{bc}	84^{c}
Flazasulfuron fb glyphosate	0.2	4.0	PRE	LPOST	80^{b}	100^{a}	90^{abc}	100^{a}	82^{bc}	100^{a}	94^{ac}	100^{a}	84^{b}	100^{a}	91^{bc}	100^{a}	83^{bc}	92^{b}	92^{a}	84^{c}
Pendimethalin fb glyphosate	5.0	8.0	PRE	LPOST	69^{d}	100^{a}	84^{c}	100^{a}	74^{def}	100^{a}	86^{e}	100^{a}	76de	100^{a}	95^{ab}	100^{a}	79^{cd}	100^{a}	80^{bcd}	100^{a}
Oxyfluorfen fb glyphosate	6.0	8.0	PRE	LPOST	72^{cd}	100^{a}	90^{abc}	100^{a}	70^{ef}	100^{a}	93^{bcd}	100^{a}	77de	100^{a}	94^{ab}	100^{a}	74^{def}	100^{a}	78^{cd}	100^{a}
Flazasulfuron fb glyphosate	0.2	8.0	PRE	LPOST	82^{b}	100^{a}	93^{ab}	100^{a}	83^{b}	100^{a}	98^{a}	100^{a}	85^{b}	100^{a}	97^{a}	100^{a}	88^{ab}	100^{a}	93^{a}	100^{a}
LSD (0.05)					7.29	1.71	7.12	2.50	6.68	1.10	4.55	1.92	5.41	1.20	5.89	1.55	6.22	4.07	5.44	5.29
Random effect interactions																				
PRE herbicides x year							*				*				*				*	
EPOST glyphosate x year							*				*				*				*	
LPOST glyphosate x year							NS				NS				NS				NS	

Abbreviations: PRE-pre-emergence; EPOST-early-post-emergence; LPOST-late-post-emergence; fb- followed by; NS-not significant; * Significant at the 5 % level according to a Fisher's protected LSD test at $p < 0.05$.

EPOST glyphosate treatment was applied to 10-12 cm tall weeds

LPOST glyphosate treatments were applied at full vegetative growth stages of the perennial weeds, regardless growth stages of annual weeds

Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at $p < 0.05$.

Table 5: *Xanthium strumarium, Lolium multiflorum, Papaver rhoeas* and *Cynodon dactylon* efficacy 84 days after PRE, 45 days after EPOST and 28 days after LPOST glyphosate treatments, respectively at Skopje district in 2016 and 2017

					Skopje district															
					Xanthium strumarium				Lolium multuflorum				Papaver rhoeas				Cynodon dactylon			
	Rate		Time of application		2016		2017		2016		2017		2016		2017		2016		2017	
Treatment	l ha^{-1})				% Efficacy (%)															
Glyphosate fb glyphosate	3.0	8.0	EPOST	LPOST	95^{a}	100^{a}	89^{a}	100^{a}	97^{a}	100^{a}	97^{abc}	100^{a}	98^{a}	100^{a}	91^{bc}	100^{a}	45^{a}	100^{a}	48^{a}	100^{a}
Pendimethalin fb glyphosate	5.0	2.0	PRE	LPOST	58^{ef}	95^{c}	80^{bc}	98^{ab}	80^{bc}	97^{b}	97^{abc}	98^{b}	80^{c}	100^{a}	95^{ab}	100^{a}	21^{c}	42de	18de	36^{e}
Oxyfluorfen fb glyphosate	6.0	2.0	PRE	LPOST	69^{d}	96^{bc}	85^{ab}	94^{c}	70^{ef}	95^{c}	91^{e}	97^{b}	75^{cd}	96^{b}	88^{cd}	95^{b}	0^{f}	36^{e}	11^{ef}	44^{d}
Flazasulfuron fb glyphosate	0.2	2.0	PRE	LPOST	71^{cd}	98^{ab}	86^{ab}	96^{bc}	83^{b}	100^{a}	100^{a}	100^{a}	89^{b}	100^{a}	98^{a}	100^{a}	14^{cd}	44^{d}	22^{cd}	40de
Pendimethalin fb glyphosate	5.0	4.0	PRE	LPOST	63^{e}	100^{a}	73^{d}	100^{a}	77^{cd}	100^{a}	91^{e}	100^{a}	75^{cd}	100^{a}	84de	100^{a}	8^{def}	62^{c}	10^{f}	69^{b}
Oxyfluorfen fb glyphosate	6.0	4.0	PRE	LPOST	72^{cd}	100^{a}	88^{a}	100^{a}	67^{f}	100^{a}	86^{f}	100^{a}	68^{f}	100^{a}	80^{e}	100^{a}	17^{cd}	58^{c}	4^{f}	55^{c}
Flazasulfuron fb glyphosate	0.2	4.0	PRE	LPOST	75^{bc}	98^{ab}	90^{a}	100^{a}	79^{bcd}	100^{a}	96^{bcd}	100^{a}	85^{bc}	100^{a}	98^{a}	100^{a}	41^{a}	71^{b}	32^{b}	69^{b}
Pendimethalin fb glyphosate	5.0	8.0	PRE	LPOST	53^{f}	100^{a}	76^{cd}	100^{a}	71^{ef}	100^{a}	93de	100^{a}	71^{df}	100^{a}	84de	100^{a}	5^{ef}	98^{a}	4^{f}	100^{a}
Oxyfluorfen fb glyphosate	6.0	8.0	PRE	LPOST	57^{f}	100^{a}	81^{bc}	100^{a}	75^{cde}	100^{a}	94^{cde}	100^{a}	77^{cd}	100^{a}	85de	100^{a}	9^{def}	100^{a}	10^{f}	98^{a}
Flazasulfuron fb glyphosate	0.2	8.0	PRE	LPOST	78^{b}	100^{a}	89^{a}	100^{a}	74de	100^{a}	99^{ab}	100^{a}	81^{c}	100^{a}	95^{ab}	100^{a}	31^{b}	97^{a}	31^{b}	98^{a}
LSD (0.05)																				
Random effect interactions					5.93	2.55	6.16	3.01	5.37	1.94	3.90	1.71	6.52	1.80	5.31	2.04	9.25	7.22	7.54	7.28
PRE herbicides x year							*				*				*				NS	
EPOST glyphosate x year							*				NS				*				NS	
LPOST glyphosate x year							NS				NS				NS				NS	

Abbreviations: PRE-pre-emergence; EPOST-early-post-emergence; LPOST-late-post-emergence; fb- followed by; NS-not significant; * Significant at the 5 % level according to a Fisher's protected LSD test at $p < 0.05$.
EPOST glyphosate treatment was applied to 10-12 cm tall weeds
LPOST glyphosate treatments were applied at full vegetative growth stages of the perennial weeds, regardless growth stages of annual weeds
Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at $p < 0.05$

Table 6: Number of cluster per vine, cluster mass and grape vine yield of 'Cabernet Sauvignon' and 'Black Magic' at Kavadarci and Skopje district, respectively under different PRE, EPOST and LPOST weed management treatments in 2016 and 2017

Treatment	Rate (l ha^{-1})		Time of application		Number of cluster per vine				Cluster mass (g)				Grape vine yield (t ha^{-1})			
					Kavadarci district		Skopje district		Kavadarci district		Skopje district		Kavadarci district		Skopje district	
					2016	2017	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
Glyphosate fb glyphosate	3.0	8.0	EPOST	LPOST	22^{bc}	22^{a}	16^{ab}	17^{a}	120^{a}	126^{a}	423^{a}	415^{a}	10.3^{a}	11.1^{a}	28.5^{a}	29.3^{a}
Pendimethalin fb glyphosate	5.0	2.0	PRE	LPOST	21^{c}	24^{a}	14^{b}	15^{a}	105^{bc}	88^{c}	337^{ef}	358^{bcd}	8.4^{d}	8.1^{e}	22.3^{c}	23.1^{d}
Oxyfluorfen fb glyphosate	6.0	2.0	PRE	LPOST	23^{abc}	22^{a}	16^{ab}	16^{a}	96^{cd}	102^{b}	328^{f}	334^{d}	8.5^{cd}	8.7^{cde}	22.9^{c}	23.8^{cd}
Flazasulfuron fb glyphosate	0.2	2.0	PRE	LPOST	22^{bc}	23^{a}	15^{ab}	16^{a}	98^{cd}	100^{bc}	369^{bcd}	346^{c}	8.2^{d}	9.6^{de}	23.2^{c}	24.2^{cd}
Pendimethalin fb glyphosate	5.0	4.0	PRE	LPOST	23^{abc}	24^{a}	15^{ab}	16^{a}	103^{cd}	106^{b}	388^{bc}	374^{b}	9.3^{abcd}	9.6^{bcd}	25.7^{b}	26.4^{b}
Oxyfluorfen fb glyphosate	6.0	4.0	PRE	LPOST	24^{ab}	22^{a}	15^{ab}	16^{a}	93^{d}	102^{b}	362^{cde}	382^{b}	8.7^{bcd}	9.9^{cde}	25.1^{b}	25.3^{bc}
Flazasulfuron fb glyphosate	0.2	4.0	PRE	LPOST	24^{ab}	24^{a}	15^{ab}	17^{a}	105^{bc}	106^{b}	348^{def}	368^{bc}	9.7^{abc}	10.1^{abc}	24.8^{b}	25.4^{bc}
Pendimethalin fb glyphosate	5.0	8.0	PRE	LPOST	25^{a}	23^{a}	16^{a}	17^{a}	102^{cd}	104^{b}	393^{b}	381^{b}	9.9^{ab}	10.5^{ab}	27.7^{a}	28.3^{a}
Oxyfluorfen fb glyphosate	6.0	8.0	PRE	LPOST	22^{bc}	21^{a}	17^{a}	16^{a}	116^{ab}	129^{a}	385^{bc}	401^{a}	10.2^{a}	10.7^{ab}	28.1^{a}	28.4^{a}
Flazasulfuron fb glyphosate	0.2	8.0	PRE	LPOST	22^{bc}	22^{a}	17^{a}	17^{a}	117^{a}	122^{a}	379^{bc}	392^{a}	10.4^{a}	10.9^{ab}	28.4^{a}	28.9^{a}
LSD (0.05)					2.58	3.13	2.17	2.61	11.79	12.70	26.95	24.96	1.25	1.45	1.19	1.24
Random effect interactions																
PRE herbicides fb LPOST glyphosate x year							NS				NS				NS	
EPOST glyphosate x year							NS				NS				NS	
LPOST glyphosate x year							NS				NS				NS	

Abbreviations: PRE-preemergence; EPOST-early-posteemergence; LPOST-late-postemergence; fb- followed by; NS-not significant; * Significant at the 5 % level according to a Fisher's protected LSD test at $p < 0.05$.
EPOST glyphosate treatments were applied to 10-12 cm tall weeds
LPOST glyphosate treatments were applied at full vegetative growth stages of the perennial weeds, regardless growth stages of annual weeds
Means followed by the same letter within a column are not significantly different according to Fisher's Protected LSD at $p < 0.05$

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First morphological characterization of autochthonous olive (*Olea europaea* L.) denominations from central and eastern of Algeria

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First morphological characterization of autochthonous olive (*Olea europaea* L.) denominations from central and eastern of Algeria

Abstract: Olive (*Olea europaea* L.) resources in Algeria are very little investigated. In fact, a total of 60 denominations have been the subject of characterization studies and they are cited in the bibliography but only 36 varieties are described and listed in the catalog of Algerian varieties of olive tree. In this work, a study on the diversity by mean of a field survey followed by morphological characterization, an estimate of the Shannon diversity index of a set of denominations collected in central and eastern of Algeria were carried out. The survey allowed us to note the existence of 33 denominations never mentioned in the bibliography. Morphological characterization based on the characteristics of tree, fruit and endocarp allowed us to a morphological description of 23 denominations. The Principal Component Analysis, Multiple Correspondence Analysis and Ascending Hierarchical Classification analysis allowed us to classify them into a few groups. The value of the relative diversity of all denominations is slightly below the average (0.42). The results obtained in this work provide very useful information on certain morphological characteristics of the studied denominations and indicate the critical conditions in which several denominations are found, which constitutes a great risk of genetic erosion.

Key words: denominations; diversity; genetic erosion; morphological characterization; olive tree; survey

Prvo morfološko ovrednotenje samoniklih akcesij oljk (*Olea europaea* L.) iz osrednje in vzhodne Alžirije

Izvleček: Populacije oljke (*Olea europaea* L.) so v Alžiriji slabo preučene. Dejansko je bilo celokupno preučenih le 60 akcesij, ki so navedene v literaturi, a od teh je bilo opisanih le 36 sort, ki so navedene v katalogu alžirskih oljčnih sort. V tej raziskavi je pregledu diverzitete na terenu sledila raziskava morfoloških lastnosti in določitev Shannonevega indeksa raznolikosti akcesij nabranih v osrednji in vzhodni Alžiriji. Na osnovi raziskave smo lahko zabeležili 33 novih tipov, ki še niso bili omenjeni v literaturi. Morfološko ovrednotenje je temeljilo na znakih kot so lastnosti drevesa, ploda in endokarpa, kar nam je omogočilo morfološki opis 23 tipov. Z analizo glavnih component, korespondenčno analizo in analizo hierarhične klasifikacije smo te akcesije lahko razvrstili v pet skupin. Vrednost relativne raznolikosti je za vse akcesije nekoliko pod poprečjem (0,42). Rezultati, pridobljeni v tej raziskavi, dajejo koristne informacije o morfoloških lastnostih preučevanih akcesij in nakazujejo kritične razmere, v katerih so bile številne akcesije najdene, kar predstavlja veliko tveganje za genetsko erozijo.

Ključne besede: akcesije; raznolikost; genetska erozija; morfološka oznaka; oljka; pregled

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1 INTRODUCTION

Algeria, like all Mediterranean countries, has a long history with olive (*Olea europaea* L.) growing. With its high diversity of bioclimatic stages, it constitutes a large reservoir of olive resources. In fact the western region is dominated by the Sigoise variety and some introduced varieties, but the central and eastern regions have a very high varietal richness.

In general, confusions about cultivar names exist in all olive-growing countries (Trujillo et al., 2013). Synonymies (different names for the same cultivar) and homonyms (the same name for different cultivars) are extremely common among and in olive-growing countries (Barranco et al., 2000a).

Several morphological characterization studies on Algerian olive resources are carried out but very few of them are published (Mendil and Sebai, 2006; Sidhoum and Gaouar, 2013; Sidhoum and Gaouar, 2017; Boucheffa et al., 2018; Sidhoum et al., 2018; Abdessemed et al., 2018; Boukhari and Gaouar, 2018). Hauville (1953) reported the presence in Algeria of 150 olive cultivars more or less abundant. A project entitled "Conservation, Characterization, Collection and Use of Genetic Resources of the Olive Tree", launched by the International Olive Council, led to the characterization and conservation of 36 local cultivars listed in the catalog of Algerian olive varieties (Mendil and Sebai, 2006). During the last ten years, several studies of genetic characterization by molecular markers have been carried out on Algerian olive resources. These studies allowed to characterize a set of 60 cultivars (Dominguez-Garcia et al., 2011; Haouane et al., 2011; Trujillo et al., 2013; Abdessemed et al., 2015; Boucheffa et al., 2016; Boucheffa et al., 2018; Di Rienzo et al., 2018). However, these numbers seems to be very far from the actual number of cultivars existing in Algeria: first, comparisons between the results that had been obtained in these different works are essential in order to detect probable cases of synonymies and/or homonyms. Secondly, several other older native denominations exist in different regions and remain uncharacterized, unexploited and threatened with extinction. Thus, on one side, among several traditional olive-growing regions in central and eastern Algeria (such as Tizi-ouzou, Bejaia, Setif, Skikda, Guelma, Batna and El Taref), there are a considerable number of cultivars with unique characteristics which never cited in the bibliography. On another side, historical events experienced by Algeria through the ages (conquests and migrations of populations) have allowed the olive tree to settle in regions distant from the Mediterranean basin in semi-arid to arid climates. Nowadays, we find in some remote corners of the high plateaus stripped of all perennial vegetation, many vestiges of olive presses dating from the Roman era while the olive oil was the subject of an intense trade between Algeria and Rome (Alloum, 1974). The availability of subterranean water resources in these areas is also a key factor in the development of this crop. This is the case of the regions of Khirane and Zaouia (Wilaya of Khenchela) and Ain Zaatout (village of Ath Ferrah, Wilaya of Biskra) (located between latitudes 35°09'N and 34°54'N) where this crop is known since at least the Roman era, which is attested by the presence of old oil mills and trees several centuries and millennia (Camps-Fabrer, 1954). These cultivars are very important for their adaptation to the semi-arid and arid climate of the region and their agronomic characteristics as well as their socio-economic and ecological interest for the region.

The great lack of information on the diversity of this crop and the accelerated climate change experienced by Algeria, coupled with mismanagement problems at the olive grower scale (inappropriate conduct, lack of production tools and modern knowledge) and also at the level of the central and local administration (encourage the cultivation of certain Algerian cultivars like Chemlal or introduced to the detriment of native cultivars, absence of efficient national program of preservation of the plant genetic resources etc ...), threaten the varietal richness by the risk of the disappearance of old cultivars with restricted diffusion which may have very interesting characteristics of production, quality, resistance, and adaptation. The problem resides in the permanent loss of some of the olive resources that are still poorly known and whose consequences are not sufficiently evaluated at present.

Native cultivars are important not only for the preservation of biodiversity but also for their specific adaptation to local growing conditions (Poljuha et al., 2008). In this context this study works on highlighting the following objectives: prospecting as well as morphological characterization of olive resources in central and eastern of Algeria in order to provide more information on the actual state of olive diversity in Algeria. This is an essential element for the establishment of possible preservation and genetic improvement programs.

2 MATERIALS AND METHODS

2.1 STUDY AREAS, FIELD SURVEYS AND SAMPLING

The field trips were made with the help of the Technical Institute of Fruit Tree and Grapevine (ITAFV) in different regions of the center and east of the country (El Taref, Bouira, Setif, Tizi- ouzou, Bejaia, Khenchela,

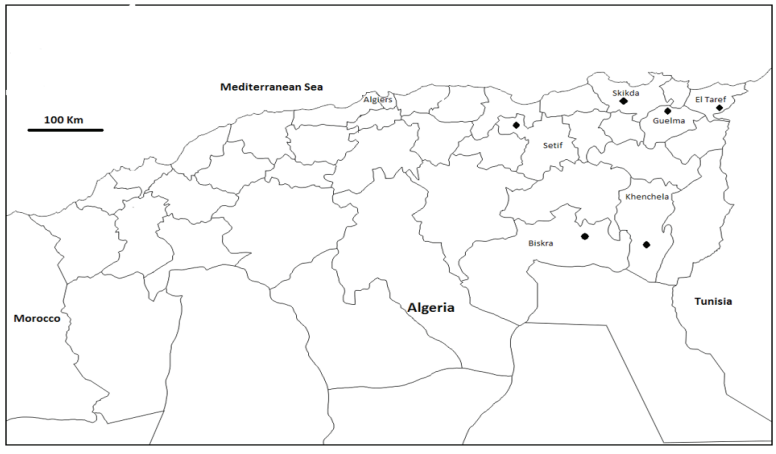

Figure 1: Sampling areas

Table 1: GPS coordinates and irrigation conduct of the study areas

Areas	GPS location	Irrigation
Ain Zaatout (Biskra)	35°08'40,4"N 5°49'58,8"E	Insufficient gravity irrigation
Khirane (Khenchela)	35°00'16,4"N 6°45'18,6"E	Insufficient gravity irrigation
Zaouia (Khenchela)	34°57'40,0"N 7°02'10,6"E	Insufficient gravity irrigation
Beni Meloul (Setif)	36°23'10,8"N 5°01'10,9"E	Without irrigation
Fedj Ziadi (El Taref)	36°25'09,7"N 8°18'40,9"E	Without irrigation
Gastu (Skikda)	36°42'18,6"N 7°17'56,7"E	Without irrigation
Josef (Skikda)	36°37'19,1"N 6°48'57,8"E	Without irrigation
Fejouj (Guelma)	36°36'26,2"N 7°20'05,4"E	Without irrigation

Biskra, Skikda and Guelma) during the harvest period and during two successive olive growing seasons (2014-2016). The geographic positions of the study areas are shown in Figure 1.

The GPS coordinates as well as the irrigation conduct of the study areas, are summarized in Table 1. This step allowed us to record and collect samples of all unknown denominations and to have data on the cradle of each denomination, its distribution and its agronomic potential, as well as the local know-how that will be useful for better preservation and use of the resource.

2.2 MORPHOLOGICAL CHARACTERIZATION

The morphological characterization concerned the quantitative and qualitative descriptors of the tree, the fruit and the endocarp described in the methodology established by the International Olive Council in its methodology for the primary characterization of olive varieties. Observations and measurements were made on 40 fruits and 40 endocarps of each tree at the rate of 5 trees per denomination. Twenty five (25) characters from the tree, fruit and endocarp were used in this study, of which 10 are quantitative and 15 are qualitative (Table 3). Analysis of variance (ANOVA), homogeneous groups (alpha = 0.05), principal component analysis (PCA) and ascending hierarchical classification (AHC) were calculated using Statistica V10 software while the multiple correspondence analysis (MCA) was performed by Xlstat (2014).

Moreover, In order to estimate the phenotypic diversity of quantitative traits, the Shannon-Weaver index (Shannon and Weaver, 1964) is calculated on the basis of the frequencies of the classes corresponding to each trait.

The Shannon-Weaver index is given by the following formula:

$$H = -\sum_{i=1}^{n} \text{Pi Ln Pi}$$

Pi: frequency of class i
n: number of individuals of a class in the sample
A value of the relative diversity index (*J*) is obtained

Table 2: List of the 23 denominations studied

	Denomination	Abbreviation
1	Ahia ousbaa	AHIA
2	Akenane	AK
3	Alslith	ALS
4	Azeboudj de Ain Zaatout	AZB-AZ
5	Azeboudj Boudhoudhane	AZB-B
6	Azeboudj de Khirane	AZB-K
7	Azeboudj de Elouandoura	AZB-E
8	Azizawth	AZIZ
9	Balbal	BAL
10	Barouni	BAR
11	Abeskri de Ain Zaatout	ABS
12	Blanquette de Gastu	B-GASTU
13	Bouchouka	BOUCH
14	Bouguenfou	BOUG
15	Chetoui	CHET
16	Derdi	DER
17	Guerboua	GUER
18	Melissi	MEL
19	Rougette de Fejouj	R-FEJOUJ
20	Serti	SERT
21	Taliani	TAL
22	Serradj	SERR
23	Azerradj de Ain Zaatout	AZR

Table 3: List of studied characters

Organs		Characters
Endocarp characters	PN	Endocarp mass
	LN	Endocarp length
	DN	Endocarp width
	LN/DN	Length to diameter ratio of the endocarp
	SyAN	Symmetry in the A position of the endocarp
	SyBN	Symmetry in the B position of the endocarp
	PDmaxN	Position of the maximum diameter of the endocarp
	FsomN	Form of the summit of the endocarp
	FbaseN	Form of the base of the endocarp
	SurfN	Surface of the endocarp
	NS	Number of furrows
	ESN	End of the summit of the endocarp
Fruit characters	PO	Fruit mass
	LO	Fruit length
	DO	Width of the fruit
	LO/DO	Length to diameter ratio of the fruit
	SyAO	Symmetry in the A position of the fruit
	PDmaxO	Symmetry in the B position of the fruit
	FsomO	Form of the summit of the fruit
	FbaseO	Form of the base of the fruit
	Mam	Presence of the nipple
	RPN	Pulp / endocarp ratio
Tree characters	Vig	Vigor of the tree
	DensF	Density of foliage
	Port	The port of the tree

by dividing the value of (*H*) on its maximum value *Hmax* (*Hmax* = Ln (n)):

$$J = \frac{H}{Hmax}$$

3 RESULTS AND DISCUSSION

3.1 FIELD PROSPECTING

The field prospecting that we carried out led us to record the existence of 59 denominations other than the 36 varieties already described in the catalog of Algerian olive varieties. 33 of them have not been the subject of any study and are never cited in the bibliography ((b) in Table 4) and 23 of them were considered in our study ((d) in Table 4).

This study has allowed us to highlight that: (1) the presence in some olive-growing regions (El Tarf, Bouira, Setif, Tizi-ouzou, Bejaia, Skikda and Guelma) of several denominations not known until now. (2) There are several small isolated historical areas of olive growing in the region of southern Aurés (semi-arid to arid climate regions) as Khirane (wilaya of Khenchela) and Ain Zaatout (wilaya of Biskra) rich in olive resources not yet recorded.

The olive tree has practically disappeared today from the Aurasian landscape (the Aurés region) although 150 years ago it was still very much alive below 1000 m, the presence of numerous ruins of presses throughout the massif up to the altitude of 1500 m seems to indicate that the culture of this tree was very developed there in Antiquity (Morizot, 1993).

It is to highlight that new plantings have been put in place, in several regions of the country, during the last two decades in the framework of several programs for the establishment of 1.000.000 hectares of olive tree: PPDRI (Proximity Rural Development Project) FNRDA (National Regulatory Fund and agricultural development) PSD (Sector Development Program) (Frah et al.,

Table 4: List of Algerian olive resources

N°	Denomination	Area	N°	Denomination	Area
01	Abani[a.c]	Khenchela	49	Bouichret[a.c]	Bejaia
02	Aberkane[a.c]	Bejaia	50	Boukaïla[a.c]	Constantine
03	Abeskri de Ain Zaatout[b.d]	Biskra	51	Bouricha[a.c]	Skikda
04	Abeskri de Khirane[c]	Khenchela	52	Braouki[c]	Skikda
05	Aedli[b]	Tizi-ouzou	53	Chemlal[a.c]	Kabylie
06	Aelah[a.c]	Khenchela	54	Chetoui[c.d]	Skikda
07	Aghchren de Titest[a.c]	Setif	55	Derdi[b.d]	Skikda
08	Aghchren de Elousseur[a.c]	Setif	56	Derdouri[c]	Skikda
09	Aghenfas[a.c]	Setif	57	El-Kharfi[c]	Skikda
10	Aghenfous[c]	Setif	58	Ferkani[a.c]	Tebessa
11	Agrarez[a.c]	Bejaia	59	Gelb al faroudje[c]	Batna
12	Aguenaou[a.c]	Setif	60	Grosse de Hamma[a.c]	Constantine
13	Aharoun[a.c]	Bouira	61	Guerboua [b.d]	El Taref
14	Ahia-Ousbaa[c.d]	Khenchela	62	Hamra[a.c]	Jijel
15	Ahorri[b]	Tizi-ouzou	63	Hamraya[b]	Khenchela
16	Aîmel[a.c]	Bejaia	64	Hebraya[b]	Khenchela
17	Akenane[c.d]	Biskra	65	Ifiri[c]	Béjaia
18	Akerma[a.c]	Bejaia	66	Issoual[b]	Béjaia
19	Akounyane[b]	Tizi-ouzou	67	Kahlaya[b]	Khenchela
20	Alslith (Lasli)[b.d]	Khenchela	68	Kerdoussi[c]	Skikda
21	Altifane[b]	Setif	69	Laaninbi[c]	Skikda
22	Amezzir[b]	Tizi-ouzou	70	Lahmar[c]	Skikda
23	Arihani[b]	Tizi-ouzou	71	Limli[a.c]	Bejaia
24	Arjouni[b]	Bouira	72	Lokchiri[c]	Skikda
25	Attounsi[c]	Setif	73	Longue de Meliana[a.c]	Ain defla
26	Azeboudj de Ain Zaatout[b.d]	Biskra	74	Mekki[a.c]	Khenchela
27	Azeboudj Boudoudane[c.d]	Khenchela	75	Melissi[b.d]	Setif
28	Azeboudj de Khirane[b.d]	Khenchala	76	Neb jmel[a.c]	Khenchela
29	Azeboudj de Louandoura[b.d]	Khenchala	77	Oukhelfa[b]	Khenchela
30	Azeradj[a.c]	Bejaia	78	Reyab[b]	Khenchela
31	Azeradj de Ain Zaatout[b.d]	Biskra	79	Ronde de Meliana[a.c]	Ain defla
32	Azeradj Tamorka[c]	Setif	80	Rougette de Metidja[a.c]	Blida
33	Azevli[b]	Tizi-ouzou	81	Rouihni[c]	Skikda
34	Azizawth (Khadraïa)[c.d]	Khenchela	82	Roujette de Fejouj[b.d]	Guelma
35	Balbal[d]	Skikda	83	Serradj[b.d]	Skikda
36	Balbal2[c]	Skikda	84	Serti[b.d]	Skikda
37	Barouni[b.d]	El Taref	85	Sigoise[a.c]	Mascar
38	Beskri[c]	Batna	86	Souidi[a.c]	Khenchela
39	Blanquette de Castu[c.d]	Guelma	87	Tabelout[a.c]	Bejaia
40	Blanquette de Guelma[a.c]	Guelma	88	Takesrit[a.c]	Bejaia
41	Blilti[c]	Skikda	89	Taliani[b.d]	El Taref

Continued

42	Bouchouk[c]	Setif	90	Tefah[a,c]	Bejaia
43	Bouchouk Guergour[a]	Setif	91	Telthi[c]	Batna
44	Bouchouk Lafayette[a,c]	Setif	92	Thawraghth[b]	Setif
45	Bouchouk Soummam[a,c]	Bejaia	93	Thazougaghth[b]	Setif
46	Bouchouka[b,d]	Skikda	94	Zeletni[a,c]	Khenchela
47	Boughefous[a,c]	Setif	95	Zitoune[b]	Skikda
48	Bouguenfou[b,d]	Khenchela			

[a] : Cultivars described in the catalog of Algerian olive varieties (36 cultivars) (Mendil et Sebai, 2006).
[b]: Denominations never mentioned in the bibliography (33 denominations) (Original, 2020).
[c]: Denominations already characterized by molecular markers (60 denominations) ((Dominguez-Garcia et al., 2011; Haouane et al., 2011; Trujillo et al., 2013; Abdessemed et al., 2015; Boucheffa et al., 2016; Boucheffa et al., 2018; Di Rienzo et al., 2018).
[d]: Denominations sampled for morphological characterization in this work (23 denominations).

2015). However, almost all these new olive groves consist of either introduced cultivars or dominant Algerian cultivars (Chemlal, Sigoise ... etc). This action, although it has many positive points on the development of Algerian olive growing, it could worsen the situation of rare indigenous cultivars with very limited distribution.

3.2 MORPHOLOGICAL CHARACTERISTICS

Table 5a and Table 5b summarize the results of the measurements carried out on 23 denominations studied. ANOVA test reveals very highly significant differences between the denominations for all traits studied. The coefficient of variation varies from a minimum of 9.84 % for the character PO/OD to a maximum of 55.36 % for the character PO. The PN, NS, RPN and PO characters show high variations with values of 25.80 %, 37.68 %, 42.08 % and 55.36 % respectively.

The BAL denomination has the highest average for the characters PO, LO, DO, LN and DN (8.66 g, 32.33 mm and 22.75 mm, 21.93 mm and 8.98 mm respectively), while the GUER denomination has the highest average for the RPN ratio and DN (9.84 and 9.73 mm respectively), and the lowest average for LN and LN/DN (12.44 mm and 1.29 respectively). The denominations GUER and BAL, with a large caliber, are widely distinguished from the other denominations, they are characterized by very superior quantitative values and they are very appreciated as table olive by the olive growers and local consumers. It should be noted that these two denominations are not very existent even in their growing regions.

The BOUG denomination is characterized by the lowest averages for PO, LO, DO, and LN (1.50 g, 16.99 mm, 12.01 mm, 12.59 mm respectively). In contrast, the SERR denomination has the lowest averages for PN and DN (0.28 g and 6.12 mm, respectively). The SERT denomination has the highest average for LN/DN and LO/DO ratios (2.60 and 1.62, respectively). The TAL denomination has the highest average for NS (10.51) while the AZIZ denomination has the lowest average for this trait (5.25). Finally, the SERR and AZB-E denominations are characterized by the lowest average for PN (0.28 g).

The denominations AHIA, AK, ALS, BAL, B-GASTU, CHET, GUER, R-FEJOUJ, TAL and SERR show the highest RPN character values. This character informs us about the richness of the fruit in pulp which is the main and most important part. Based on this trait, the denominations given could give important oil yield results or can be used as valuable table olive.

By using the morphological description of the denominations studied, based on 20 traits of the tree, the fruit and the endocarp, a morphological description is given in Table 6a and Table 6b for the 23 denominations studied. This allowed us to give an identity for each of them and to differentiate them from those of the catalog of Algerian olive varieties which goes in favor of the hypothesis of unique cultivar of each denomination. This study allowed us to enrich the list of olive varieties that exist in Algeria with 33 candidate denominations that can constitute varieties in their own right. In this case, a study by molecular markers is necessary to detect cases of taxonomic confusion (synonymy or homonymy).

3.2.1 Principal Component Analysis (PCA)

Figure 2 shows the projection of the characters on the plane generated by the first two principal components accumulating 74.37 % of total inertia. The variables PO, PN, LO, LN, DO, DN and LO/DO explain most of the variance on the first axis (negatively correlated). While the second axis is explained by the variables LN/DN, NS and RPN (correlated negatively). BAL and GUER de-

Table 5a: Results relating to quantitative trait measurements

Denomination	PN (g) ***	LN (mm) ***	DN (mm) ***	LN/DN ***	NS ***
AHIA	0.30±0.05 bc	14.10±2.21 d	6.62±0.32 f	2.13±0.34 g	6.10±1.21 b
AK	0.37±0.09 f	14.57±1.98 e	7.15±0.54 h	2.04±0.22 e	7.37±1.14 c
ALS	0.34±0.05 e	14.06±0.92 cd	6.92±0.38 g	2.04±0.19 e	8.61±1.36 g
AZB-AZ	0.29 ±0.05 ab	13.81±0.81 c	6.23±0.38 bcd	2.22±0.15 h	7.43±1.00 c
AZB-B	0.55±0.13 k	17.74±1.52 k	7.60±0.48 m	2.34±0.17 mp	8.04±1.56 f
AZB-K	0.38±0.05 f	15.91±2.12 g	6.96±0.30 g	2.29±0.33 km	7.59±1.11 cde
AZB-E	0.28±0.05 a	13.13±1.06 b	6.2±0.43 abc	2.12±0.13 f	8.73±1.23 gh
AZIZ	0.34±0.06 e	12.72±1.16 a	6.94±0.41 g	1.83±0.13 c	5.25±1.17 a
BAL	0.91±0.14 s	21.93±1.63 p	8.98±0.85 s	2.46±0.27 q	12.66±1.85 s
BAR	0.47±0.07 h	15.90±1.33 g	7.35±0.38 k	2.16±0.17 g	9.91±1.21 p
ABS	0.48±0.10 h	15.87±2.23 g	7.94±0.59 p	2.00±0.26 de	7.53±1.55 cd
B-GASTU	0.32±0.06 de	14.98±1.30 f	6.35±0.39 e	2.36±0.21 p	8.65±1.44 g
BOUCH	0.71±0.09 q	19.06±1.37 m	8.31±0.45 q	2.30±0.17 km	7.49±1.78 c
BOUG	0.31±0.31 ab	12.59±1.53 a	6.16±0.50 ab	2.04±0.16 e	9.14±1.98 k
CHET	0.31±0.06 c	14.92±1.54 f	6.26±0.53 cde	2.47±1.34 p	9.01±1.57 hk
DER	0.43±0.08 g	14.22±1.13 d	7.13±0.45 h	2.00±0.14 d	7.87±1.17 df
GUER	0.69±0.13 p	12.44±1.43 a	9.73±0.95 t	1.29±0.21 a	8.68±1.70 g
MEL	0.57±0.09 m	16.67±1.05 h	7.64±0.45 m	2.19±0.15 gh	7.82±1.72 def
R-FEJOUJ	0.29±0.05 abc	14.24±1.36 d	6.24±0.34 bcd	2.29±0.22 k	9.59±1.58 m
SERT	0.56±0.07 km	18.92±1.46 m	7.30±0.34 k	2.60±0.20 s	10.41±1.51 q
TAL	0.31±0.03 cd	15.63±0.94 g	6.32±0.25 de	2.48±0.17 q	10.51±1.35 q
SERR	0.28±0.05 a	14.90±1.21 f	6.12±0.37 a	2.44±0.18 q	10.43±1.54 q
AZR	0.33±0.05 e	12.77±0.94 a	7.34±0.31 k	1.74±0.14 b	7.48±0.99 c
CV %	42.08	17.42	13.74	15.02	25.80
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

***: very highly significant ANOVA result (alpha = 0.05);
a, b, c, d, e, f…: homogeneous groups

nominations are not included in this analysis because they have extreme values.

The PCA also shows the grouping of the characters studied into two groups. The first includes the characters DN, PN, LO / DO LN, LO, PO and DO. The second group contains the characters LN / DN, NS and RPN.

Projection of individuals (Figure 3) shows that the denominations AZB-B, ABS, BOUCH and SERT are characterized by the highest values for the characters PO, PN, LO, LN, DO, DN and LO/DO. In contrast, the denominations AZB-AZ, AZB-E and BOUG are characterized by the lowest values for these same traits. The B-GASTU, CHET, R-FEJOUJ, TAL and SERR denominations show high NS, LN/DN and RPN, while the AZIZ, DER, MEL and AZR denominations show reduced values for the same characters.

The results of PCA show that it is possible to distinguish between the denominations using the characters of the fruit, the endocarp, in particular: PN, LN, DN, LN / DN, LO, PO and RPN. On another side, the grouping of characters by the PCA into two groups can most probably be a reflection of a pleiotropic effect and therefore exploitable for possible genetic improvement tests.

For the denominations AZB-B, ABS, BOUCH and SERT, despite the high values of mass, length and width of the fruits, their interest is affected by the high values of the endocarp for these same characters, which also results in lower RPNs. On the other hand the denominations AZB-AZ, AZB-E and BOUG record reduced values for these same characters of the fruit, which can be explained by the fact that they are feral forms, not cultivated and not exploited because either of their small fruits or their relatively large endocarp.

Table 5b: Results relating to quantitative trait measurements

Denomination	PO (g) ***	LO (mm) ***	DO (mm) ***	LO/DO ***	RPN ***
AHIA	2.63±0.57 k	20.40±2.19 f	15.17±1.02 m	1.35±0.13 fg	7.88±1.91 p
AK	2.99±0.56 m	21.17±2.09 hk	16.34±1.14 s	1.30±0.10 b	7.40±1.81 km
ALS	2.68±0.39 k	20.45±1.42 f	15.44±0.80 p	1.33±0.08 de	6.97±1.40 h
AZB-AZ	1.72±0.23 b	18.23±1.01 b	13.05±0.75 c	1.40±0.07 mp	5.14±1.40 e
AZB-B	3.51±0.52 q	24.75±1.53 s	15.82±0.76 q	1.57±0.08 t	5.68±1.44 fg
AZB-K	2.10±0.36 de	20.87±2.19 gh	13.37±0.85 d	1.56±0.16 t	4.62±1.14 d
AZB-E	1.64±0.24 b	17.87±1.06 b	12.56±0.70 b	1.42±0.06 q	5.00±1.23 e
AZIZ	2.18±0.43 e	18.62±1.46 d	14.37±0.97 g	1.30±0.09 bc	5.61±1.38 f
BAL	8.66±1.27 t	32.33±1.81 u	22.75±1.27 u	1.42±0.08 q	8.71±2.04 q
BAR	2.45±0.32 gh	20.50±1.81 fg	14.82±0.69 h	1.38±0.10 km	4.29±1.06 c
ABS	3.27±0.62 p	22.58±2.07 q	16.55±1.06 t	1.37±0.12 ghk	5.99±1.34 g
B-GASTU	3.09±0.50 m	21.74±1.65 p	16.19±0.86 s	1.34±0.09 ef	8.67±1.75 q
BOUCH	3.34±0.66 p	24.64±1.76 s	16.19±1.23 s	1.52±0.08 s	3.81±1.12 b
BOUG	1.50±0.40 a	16.99±1.88 a	12.01±1.08 a	1.41±0.08 pq	4.51±1.35 cd
CHET	2.46±0.66 gh	20.30±2.44 f	14.87±1.34 hk	1.36±0.08 gh	7.01±1.83 h
DER	1.93±0.40 c	18.81±1.67 d	13.71±1.08 e	1.37±0.09 hk	3.66±1.22 ab
GUER	7.21±1.03 s	25.53±1.47 t	22.96±1.12 u	1.11±0.06 a	9.84±2.31 s
MEL	2.49±0.34 h	21.62±1.29 mp	14.13±0.85 f	1.53±0.09 s	3.44±0.78 a
R-FEJOUJ	2.35±0.43 fg	19.66±1.80 e	14.97±0.93 hk	1.31±0.11 cd	7.13±1.65 hk
SERT	3.38±0.52 p	25.43±1.56 t	15.67±0.86 q	1.62±0.08 u	5.15±1.15 e
TAL	2.62±0.37 k	21.32±1.20 km	15.07±0.82 km	1.42±0.07 pq	7.49±1.41 m
SERR	2.30±0.58 f	20.23±2.05 f	14.30±1.36 fg	1.42±0.08 pq	7.30±2.08 hkm
AZR	1.98±0.18 cd	18.58±0.91 cd	14.31±0.51 fg	1.30±0.07 bc	5.11±1.00 e
CV %	55.36	16.80	16.65	9.84	37.68
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

***: very highly significant ANOVA result (alpha = 0.05);
a, b, c…: homogeneous groups

3.2.2 Multiple Correspondence Analysis (MCA)

Based on qualitative traits, the MCA (Figure 4) ranks the 23 denominations in five (05) groups:

Group 1: composed by GUER denomination characterized by slightly asymmetric endocarp in position B and symmetrical fruits in position A.

Group 2: composed by AHIA and AZB-K denominations which are characterized by fruits with nipples.

Group 3: composed by denominations AK, AZIZ, AZB-E, ABS, BOUG and AZR which are characterized by symmetrical endocarp in position B, and slightly asymmetric fruits in position A with a central position of the maximum diameter and a rounded form of the summit without nipple.

Group 4: composed by ALS, AZB-AZ, BAR, CHET, DER, MEL, R-FEJOUJ and SERT denominations which are characterized by slightly asymmetric endocarp in position A and symmetrical in position B with a rough surface.

Group 5: composed by denominations AZB-B, BAL, BOUCH, B-GASTU, TAL and SERR which are characterized by endocarps slightly asymmetric in position A, symmetrical in position B with a central position of maximum transverse diameter and a pointed form of the summit, and fruits with a central position of the maximum transverse diameter and trees with strong vigor.

3.2.3 Ascending Hierarchical Classification (AHC)

The ascending hierarchical classification with morphological data (Figure 5) produces a dendrogram representing the 23 denominations classified into six (06) different groups:

Table 6a: Morphological description of the 23 denominations studied

	Characters	AHIA	AK	ALS	AZB-AZ	AZB-B	AZB-K	AZB-E	AZIZ	BAL	BAR	ABS	B-GASTU
Endocarp	PN	M à E	M	M	R à M	E	M	R à M	M	E	E	E	M
	LN/DN	El à Al	El	El	El à Al	Al	El à Al	El	O à El	Al	El à Al	El	Al
	SyAN	L.As	L.As	L.As	L.As	L.As	L.As	Sy	L.As	L.As	L.As	L.As	L.As
	SyBN	Sy	Sy	Sy	Sy	Sy	Sy	Sy	Sy	Sy	Sy	Sy	Sy
	PDmaxN	V.So	C	C	C	C	C	C	C	C	C	V.So	C
	FSoN	P	P	Ar	P	P	P	Ar	Ar	P	Ar	Ar	P
	FbaseN	P	P	Ar	P	P	P	Ar	P	P	P	P	P
	SurfN	Ru	Ru	Ru	Ru	Rab	Ru	Ru	Ru	Rab	Ru	Rab	Ru
	NS	M à E	M	M	M	M	M	M	R	E	M	M	M
	ESN	Av.Mu	Ss.Mu	Av.Mu	Ss.Mu	Av Mu	Av.Mu	Ss.Mu	Ss.Mu	Av.Mu	Av.Mu	Av.Mu	Av.Mu
Fruit	PO	M	M	M	R	M	R à M	R	R à M	très E	M	M	M
	LO/DO	O	O	O	O	Al	Al	O	O	O	O	O	O
	SyAO	L.As	L.As	L.As	L.As	As	L.As	L.As	L.As	L.As	L.As	L.As	As
	PDmaxO	C	C	C	C	C	C	C	C	C	C	C	C
	FSoO	Ar	Ar	Ar	Ar	Ar	P	Ar	Ar	P	Ar	Ar	P
	FbaseO	Ar	Ar	Ar	Ar	T	P	Ar	T	Ar	T	T	T
	Mam	Abs	Abs	Abs	Abs	Abs	Prés	Abs	Abs	Abs	Abs	Abs	Eb
Tree	Vig	M	Ft	Ft	Ft	Ft	M	Ft	Ft	Ft	M	M	Ft
	DensF	La	M	La	La	M	La	La	La	M	Co	M	M
	Port	Dr	Et	Dr	Dr	Et	Re	Re	Et	Dr	Dr	Re	Et
	Use	H	H	DF	-	-	-	-	H	H	H	DF	DF

M : medium; E : high ; très E : very high ; R : reduced ; El : elliptical; Al : lengthened; O : oval ; S : spherical ; Sy : symmetrical ; As : asymmetrical ; L.As : slightly asymmetrical ; C : central ; V.So : towards the summit; V.Ba : towards the base ; P : sharp; Ar : rounded ; T : tranquered ; Ru : rough ; Rab : dresser ; Li : smooth ; Av.Mu : with mucron ; Ss.Mu : without mucron ; Abs : absent ; prés : present ; Eb : draft ; Ft : strong ; Fb : weak ; La : cowardly ; Co : compact ; Dr : trained ; Et : spread out ; Re : falling back ; H : oil ; DF : dual purposes.

Table 6b: Morphological description of the 23 denominations studied

	Characters	BOUCH	BOUG	CHET	DER	GUER	MEL	R-FEJOUJ	SERT	TAL	SERR	AZR
Endocarp	PN	R à M	R à M	M à.E	E	E	R à M	E	M	R à M	M	E
	LN/DN	El	Al	El	S	El à Al	El à Al	Al	Al	Al	O à El	Al
	SyAN	Sy	L.As	L.As	L.As	L.As	L.As	L.As	L.As	L.As	L.As	L.As
	SyBN	Sy	Sy	Sy	L.As	Sy	Sy	Sy	Sy	Sy	Sy	Sy
	PDmaxN	C	C	V.So	C	C	C	C	C	C	V.So	C
	FSoN	Ar	P	Ar	Ar	Ar	P	P	P	P	Ar	P
	FbaseN	Ar	P	P	Ar	P	Ar	Ar	Ar	Ar	Ar	P
	SurfN	Ru	Ru	Ru	Rab	Ru	Ru	Ru	Rab	Ru	Li	Rab
	NS	M à E	M à E	M	M	M	M à E	E	E	E	M	M
	ESN	Ss.Mu	Av.Mu	Av.Mu	Ss.Mu	Av.Mu	Av.Mu	Av.Mu	Av.Mu	Av.Mu	Ss.Mu	Ss.Mu
Fruit	PO	R	M	R à M	très E	M	M	M	M	M	R à M	M
	LO/DO	O	O	O	S	Al	O	Al	O	O à Al	O	Al
	SyAO	L.As	L.As	L.As	Sy	As	L.As	As	L.As à As	As	L.As	As
	PDmaxO	C	C	V.So	C	V.So	C	V.So	C	C	C	C
	FSoO	Ar	P	Ar	Ar	Ar	Ar	P	P	P	Ar	P
	FbaseO	Ar	Ar	Ar	Ar	Ar	T	T	T	Ar	T	P
	Mam	Abs	Abs	Abs	Abs	Eb	Abs	Abs	Eb	Abs	Abs	Abs
Tree	Vig	Ft	Ft	Ft	M	Fb	Ft	Fb	Ft	Ft	Ft	Ft
	DensF	M	Co	Co	M	Co	Co	La	Co	Co	M	Co
	Port	Re	Et	Re	Et	Et	Dr	Dr	Et	Et	Et	Et
Use	DF	-	DF	H	H	H	H	H	H	DF	H	

M : medium; E : high ; très E : very high ; R : reduced ; El : elliptical; Al : lengthened; O : oval ; S : spherical ; Sy : symmetrical ; As : asymmetrical ; L.As : slightly asymmetrical ; C : central ; V.So : towards the summit; V.Ba : towards the base ; P : sharp; Ar : rounded ; T : tranquered ; Ru : rough ; Rab : dresser ; Li : smooth ; Av.Mu : with mucron ; Ss.Mu : without mucron ; Abs : absent ; prés : present ; Eb : draft ; Ft : strong ; Fb : weak ; La : cowardly ; Co : compact ; Dr : trained ; Et : spread out ; Re : falling back ; H : oil ; DF : dual purposes.

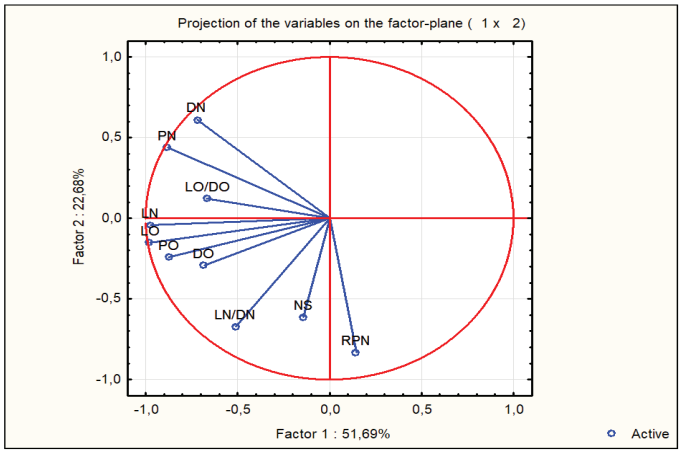

Figure 2: Projection of characters on the plane generated by the first two main components (PCA)

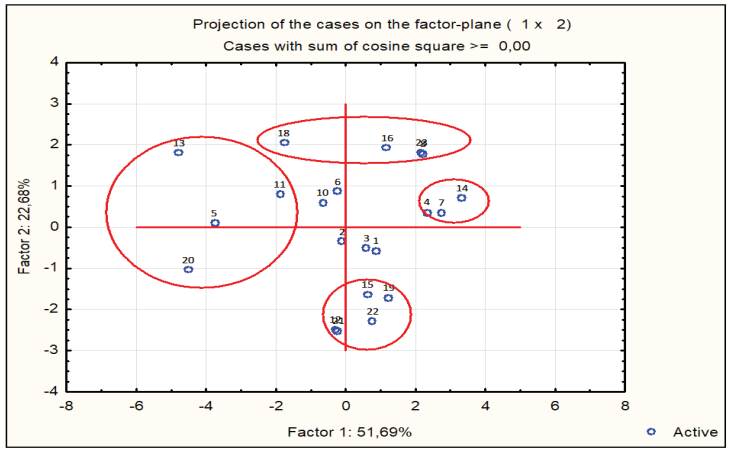

Figure 3: Projection of individuals on the plane generated by the first two main components (PCA).
(1) : Ahia ousbaa ; (2) : Akenane ; (3) : Alslith ; (4) : Azeboudj de Ain Zaatout; (5) : Azeboudj Boudhoudhane; (6) : Azeboudj de Khirane; (7) : Azeboudj de Elouandoura; (8) : Azizawth; (9) : Balbal ; (10) : Barouni ; (11) : Abeskri de Ain Zaatout ; (12) : Blanquette de Gastu; (13) : Bouchouka ; (14) : Bouguenfou ; (15) : Chetoui ; (16) : Derdi ; (17) : Guerboua ; (18) : Melissi ; (19) : Rougette de Fejouj ; (20) : Serti ; (21) : Taliani ; (22) : Serradj ; (23) : Azerradj de Ain Zaatout.

Group 1: composed only by the BAL denomination that is characterized by the highest values for the majority of the characters except for LO/DO.

Group 2: composed only by the GUER denomination that is characterized by high values for PO, LO, DO, PN, LN, DN. This denomination is also characterized by reduced values for LO/DO and LN/DN.

Group 3: composed by the three denominations: BAR, MEL and AZB-K that are characterized by high LO/DO, LN/DN and reduced RPN ratio.

Group 4: composed by six denominations: AZIZ, AZR, DER, BOUG, AZB-E and AZB-AZ that are characterized by reduced values for PO, PN, LO, LN, DO and DN.

Group 5: composed by three denominations: AZB-B, BOUCH and SERT which are characterized by a high PO, LO, DO and LO/DO. On the other hand, they have reduced RPN ratio.

Group 6: composed by the nine remaining denominations (09) namely: AHIA, AK, ALS, ABS, B-GASTU, CHET, R-FEJOUJ, TAL and SERR. These denominations are characterized by intermediate values for PO, LO, DO LO/DO and LN.

The results of the Ascending Hierarchical Classification are consistent with those obtained by principal components analysis. These two tests (based on quantitative traits), with Multiple Correspondence Analysis (based on qualitative characteristics) classified the 23 denominations into five to six groups with relatively similar morphological characteristics.

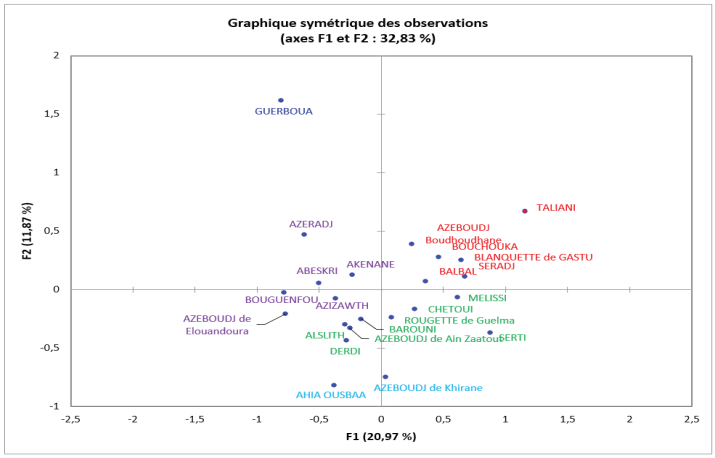

Figure 4: Multiple correspondence analysis (MCA) of the 23 denominations.
(1) : Ahia ousbaa ; (2) : Akenane ; (3) : Alslith ; (4) : Azeboudj de Ain Zaatout; (5) : Azeboudj Boudhoudhane; (6) : Azeboudj de Khirane; (7) : Azeboudj de Elouandoura; (8) : Azizawth; (9) : Balbal ; (10) : Barouni ; (11) : Abeskri de Ain Zaatout ; (12) : Blanquette de Gastu; (13) : Bouchouka ; (14) : Bouguenfou ; (15) : Chetoui ; (16) : Derdi ; (17) : Guerboua ; (18) : Melissi ; (19) : Rougette de Fejouj ; (20) : Serti ; (21) : Taliani ; (22) : Serradj ; (23) : Azerradj de Ain Zaatout.

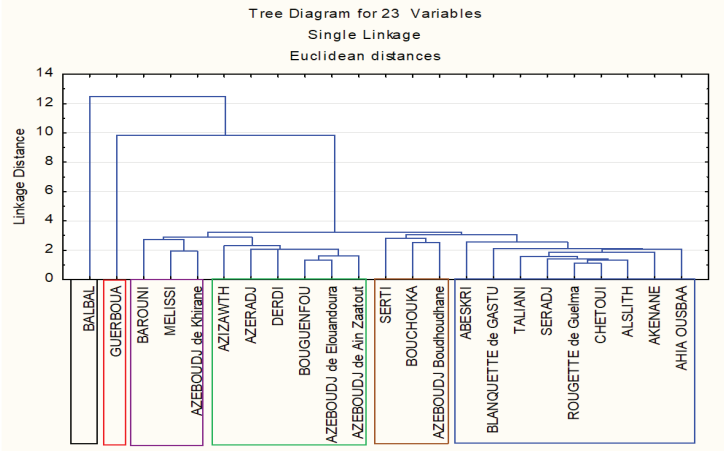

Figure 5: Ascending hierarchical classification (AHC)
(1) : Ahia ousbaa ; (2) : Akenane ; (3) : Alslith ; (4) : Azeboudj de Ain Zaatout; (5) : Azeboudj Boudhoudhane; (6) : Azeboudj de Khirane; (7) : Azeboudj de Elouandoura; (8) : Azizawth; (9) : Balbal ; (10) : Barouni ; (11) : Abeskri de Ain Zaatout ; (12) : Blanquette de Gastu; (13) : Bouchouka ; (14) : Bouguenfou ; (15) : Chetoui ; (16) : Derdi ; (17) : Guerboua ; (18) : Melissi ; (19) : Rougette de Fejouj ; (20) : Serti ; (21) : Taliani ; (22) : Serradj ; (23) : Azerradj de Ain Zaatout.

3.2.4 Diversity Index (Shannon-Weaver)

The Shannon-Weaver relative diversity indices (J) of all traits and denominations as well as the means are presented in Table 7.

The average relative diversity of all denominations and characters is 0.42.

The characters NS, SyAO, and SyAN have the highest average values of relative diversity (0.63, 0.66, and 0.73 respectively), followed by PN, LN/DN, LO/OD, FbaseO, PDmaxN, and FbaseN which have lower average values (between 0.42 for PDmaxN and 0.54 for LO/OD). The other characters have low average values (between 0.24 for SyBN and 0.35 for FsomO and FsomN).

The denominations AHIA, AK, AZB-K and BOUG have the highest average values of relative diversity (between 0.55 and 0.58). The other denominations are characterized by lower mean values (between 0.31 for GUER and 0.47 for ALS).

The average relative diversity of all denominations obtained (0.42) is lower than that recorded by (Sidhoum et al., 2018) who conducted studies on samples composed of local and introduced denominations in western Algeria, and by (Laaribi et al., 2014) on hybrids of Chemlali in

Table 7: Shanon's relative variability index (J)

	Characters																	
	PN	LN/DN	PO	LO/DO	NS	ESN	SyAO	PDmaxO	FsomO	FbaseO	Mam	SyAN	SyBN	PDmaxN	FsomN	FbaseN	SurfN	Average
AHIA	0.65	0.60	0.36	0.74	0.62	0.23	0.71	0.32	0.58	0.83	0.88	0.84	0.31	0.58	0.63	0.04	0.41	**0.55**
AK	0.93	0.65	0.23	0.71	0.62	0.96	0.61	0.27	0.60	0.63	0.16	0.87	0.35	0.50	0.60	0.46	0.57	**0.57**
ALS	0.45	0.66	0.16	0.51	0.74	1.00	0.63	0.35	0.06	0.53	0.35	0.84	0.00	0.61	0.34	0.59	0.15	**0.47**
AZB-AZ	0.63	0.50	0.23	0.61	0.59	0.17	0.18	0.53	0.30	0.63	0.00	0.63	0.00	0.18	0.62	0.00	0.11	**0.35**
AZB-B	0.55	0.39	0.28	0.24	0.82	0.00	0.54	0.31	0.62	0.95	0.00	0.98	0.34	0.80	0.63	0.03	0.16	**0.45**
AZB-K	0.41	0.58	0.47	0.57	0.62	1.00	0.76	0.03	0.63	0.89	0.63	0.85	0.26	0.63	0.63	0.57	0.41	**0.58**
AZB-E	0.62	0.38	0.14	0.56	0.65	0.81	0.34	0.00	0.30	0.68	0.00	0.52	0.11	0.63	0.30	0.34	0.36	**0.40**
AZIZ	0.62	0.48	0.46	0.59	0.34	0.80	0.52	0.17	0.04	0.77	0.00	0.71	0.23	0.52	0.29	0.60	0.22	**0.43**
BAL	0.00	0.33	0.00	0.56	0.18	0.29	0.86	0.12	0.63	0.00	0.00	0.86	0.43	0.17	0.63	0.47	0.00	**0.33**
BAR	0.58	0.56	0.21	0.59	0.58	0.00	0.20	0.11	0.48	0.00	0.00	0.59	0.44	0.54	0.48	0.33	0.52	**0.36**
ABS	0.59	0.73	0.10	0.89	0.47	0.04	0.38	0.27	0.61	0.28	0.02	0.91	0.13	0.64	0.61	0.44	0.32	**0.44**
B-GASTU	0.69	0.40	0.10	0.65	0.74	0.00	0.89	0.13	0.52	0.53	0.64	0.54	0.15	0.09	0.52	0.55	0.00	**0.42**
BOUCH	0.00	0.41	0.33	0.45	0.89	0.11	0.94	0.18	0.03	0.34	0.64	0.59	0.44	0.12	0.03	0.19	0.00	**0.34**
BOUG	0.77	0.49	0.30	0.63	0.92	0.96	0.83	0.30	0.61	0.52	0.00	0.47	0.03	0.60	0.61	0.57	0.68	**0.55**
CHET	0.68	0.36	0.44	0.61	0.75	0.00	0.89	0.34	0.25	0.85	0.58	0.47	0.34	0.11	0.25	0.46	0.51	**0.46**
DER	0.71	0.37	0.48	0.48	0.51	0.00	0.54	0.65	0.05	0.26	0.66	0.49	0.15	0.63	0.05	0.60	0.00	**0.39**
GUER	0.11	0.44	0.23	0.00	0.82	0.00	0.47	0.00	0.00	0.00	0.00	0.95	0.84	0.45	0.00	0.97	0.00	**0.31**
MEL	0.31	0.48	0.21	0.35	0.89	0.00	0.78	0.67	0.46	0.63	0.00	0.64	0.09	0.64	0.62	0.51	0.30	**0.45**
R-FEJOUJ	0.66	0.48	0.33	0.86	0.63	0.00	0.88	0.17	0.37	0.00	0.00	0.82	0.00	0.39	0.00	0.48	0.68	**0.40**
SERT	0.27	0.03	0.35	0.03	0.56	0.00	0.27	0.73	0.27	0.00	0.51	0.73	0.18	0.30	0.00	0.59	0.68	**0.32**
TAL	0.53	0.10	0.16	0.63	0.45	0.00	0.81	0.37	0.31	0.60	0.63	0.73	0.62	0.08	0.00	0.49	0.31	**0.40**
SERR	0.59	0.23	0.44	0.63	0.54	0.00	0.75	0.06	0.16	0.63	0.36	0.75	0.24	0.15	0.00	0.46	0.00	**0.35**
AZR	0.50	0.48	0.50	0.59	0.47	0.34	0.83	0.66	0.21	0.38	0.52	0.91	0.00	0.41	0.42	0.13	0.32	**0.45**
Average	0.52	0.44	0.28	0.54	0.63	0.29	0.64	0.29	0.35	0.48	0.29	0.72	0.25	0.42	0.36	0.43	0.29	0.42

Tunisia (self-pollinations and cross-pollinations with local and introduced denominations). The diversity index can inform us about the degree of selection to which the different characters studied are submitted. In fact a high diversity index obtained for the characters: NS, SyAO, and SyAN will probably be due to a weak selection and/or genes that code for not very important characters, while a low diversity index obtained for the rest of the characters could be explained by either a strong selection and/or by genes which code for important characters. The highest average values of relative diversity obtained for the denominations AHIA, AK, AZB-K and BOUG can be explained by the fact that denominations AHIA and AK are less subject to selection and/or spread over more diverse environments than other denominations, while for AZB-K and BOUG this is explained by the fact that they are feral forms and therefore not subject to selection.

4 CONCLUSIONS

Native olive resources are of considerable interest in the development of the olive sector in Algeria. This work aims to update and provide as much information as possible about the diversity of olive denominations using morphological characters.

Field surveys in several regions of central and eastern Algeria led to the registration of 33 denominations never mentioned in the bibliography. This number does not obviously reflect the actual number of existing denominations because several other regions are not yet prospected and cases of synonymy and homonymy can exist between different denominations. Morphological characterization allowed a morphological description based on the characters of the tree, fruit, and endocarp of the 23 denominations studied, and principal component analysis and ascending hierarchical classification allowed to classify them into six groups. The value of the relative diversity of all denominations is slightly below the average (0.42).

The results obtained in this work provide very useful information on the richness of Algerian olive resources and on certain morphological characteristics of the studied denominations, but only a genetic characterization by the molecular markers could confirm or invalidate the unique genetic identity of each denomination (a project of characterization by the SSRs markers is in progress).

Finally, in view of the critical conditions and the situation in which many of these denominations are found, it is urgent and imperative to carry out programs of introduction and preservation in-situ and ex-situ of these olive-growing resources, in particular those of Khirane and Ain Zaatout (wilaya of Khenchela and wilaya of Biskra respectively) which are in very extreme environmental conditions.

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Assessment genetic diversity of einkorn genotypes (*Triticum monococcum* L.) by gliadin electrophoresis

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Assessment genetic diversity of einkorn genotypes (*Triticum monococcum* L.) by gliadin electrophoresis

Abstract: The genetic diversity of gliadins in twenty two einkorn accessions preserved under long-term seed storage condition in the National gene bank of Bulgaria were evaluated, using acid polyacrylamide-gel electrophoresis (Acid-PAGE). In total, 64 polymorphic bands and 22 gliadin patterns were identified. Thirty four different mobility bands and 21 gliadin patterns were identified in the ω-gliadin zone, 12 bands and 16 patterns were noted in the γ-gliadins, 17 patterns and 12 mobility bands were found for β-gliadins and six bands with five different α -gliadin patterns were determined. The genetic diversity index (H) was the highest for ω-gliadins (0.950), followed by β-gliadins (0.924) and γ- (0.914), respectively and the lowest value was detected in α-gliadin patterns (0.120). Cluster analysis based on the UPGMA method and Nei and Li similarity coefficients classified all the genotypes into 3 main groups. No relationships between genetic diversity, geographic origin and the genotypes were observed. The results of cluster analysis justify the high level of genetic variation among investigated einkorn accessions.

Key words: A-PAGE electrophoresis; einkorn; genetic diversity; gliadins

Ocenjevanje genetske raznolikosti genotipov enozrne pšenice (*Triticum monococcum* L.) z elektroforezo gliadinov

Izvleček: Genetska raznolikost gliadinov 22 akcesij enozrne pšenice, ki je bila shranjena v razmerah dolgotrajnega shranjevanja v nacionalni genski banki Bolgarije je bila ovrednotena s poliakrilamidno elektroforezo v kislem pH območju (Acid-PAGE). Celokupno je bilo ugotovljenih 64 polimorfnih prog in 22 vzorcev gliadinov. Določeno je bilo 32 različnih spremenljivih prog in 21 vzorcev gliadinov je bilo določenih znotraj ω-gliadinov, 12 prog in 16 vzorcev je bilo znotraj γ-gliadinov, 17 vzorcev in 12 spremenljivih prog je bilo v območju β-gliadinov in 6 prog s petimi različnimi vzorci v območju α -gliadinov. Indeks genetske raznolikosti (H) je bil največji za ω-gliadine (0,950), ki su mu sledile vrednosti za β- (0,924) in γ-gliadine (0,914). Najmanjše vrednosti indeksa so bile ugotovljene za α-gliadine (0,120). Klasterska analiza, ki je temeljila na UPGMA metodi in koeficientu podobnosti po Nei in Li je razvrstila vse genotipe v tri glavne skupine. Ugotovljeno ni bilo nobene povezave med genetsko raznolikostjo, geografskim poreklom in genotipi. Rezultati klasterske analize so potrdili veliko genetsko variabilnost med analiziranimi akcesijami enozrne pšenice.

Ključne besede: elektroforeza A-PAGE; enozrna pšenica; genetska raznolikost; gliadini

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1 INTRODUCTION

Genetic diversity has played a vital role in the success of crop improvement. Knowledge of genetic diversity has been successfully used for efficient germplasm management and utilization, genetic fingerprinting and genotype selection (Engles et al., 2002; Aliyeva et al., 2012).

The electrophoresis of seed storage proteins used for variety fingerprinting is reliable, simple, repeatable and economic procedure. It can be utilized by wheat breeders to detect variability among wheat genotypes to identify new sources of variation that could be used in crop improvement programs (Aliyeva et al., 2012).

Gliadins are alcohol-soluble storage proteins that accounts for about 40 %, by mass, of all proteins of wheat flour. They are one of the most polymorphic proteins in nature and one of the major protein components of the human diet (Dziuba et al., 2014; Metakovsky et al., 2018). They are monomeric proteins with a molecular mass in the range of 35,000 to 70,000 daltons. (Shewry et al., 2002; Kuktaite, 2004; Meintjes, 2004; D'Ovidio & Masci, 2004; Shuaib et al., 2007). When fractionated in acidic medium, they are divided into 4 major groups- α, β, γ and ω (Konarev, 1983; Bushuk, 1991). The genes controlling α- and β-gliadins were found to be located in the short arms of the chromosomes of the sixth (Gli-2) homologous group - 6A, 6B and 6D, and of the γ- and ω-gliadins of the first (Gli-1) homeology group - 1A, 1B and 1D (Payne et al., 1987; Dachkevitch et al., 1993; Nieto-Taladriz et al., 1996; Branlard, 2004). A small group of gliadin fractions are encoded by genes located at Gli-3, Gli-4, Gli-5 and Gli-6 loci (Pogna et al., 1993; Rodriguez & Carrillo, 1996; Metakovsky et al., 1997 a, 1997 b). An original methodology for their electrophoretic separation (A-PAGE) and a nomenclature for their designation (Bushuk & Zilman, 1978; Bushuk & Sapirstein, 1991) have been developed to detect the complex polymorphism of gliadins and to identify them. Intensive work with gliadins has been carried out in Ukraine and Russia (Metakovsky et al., 1984, 1986; Metakovsky & Sozinov, 1987; Metakovsky & Novoselskaya, 1991; Novoselskaya-Dragovich et al., 2003). Genetic polymorphism has been used to evaluate genetic diversity in wheat samples in Austria, Yugoslavia, Canada, Italy, France, Spain (Metakovsky et al., 1991, 1993, 1994, 1997 a, 1997 b, 2000; Metakovsky & Branlard, 1998; Ruiz et al. , 2002 a, 2002 b; Branlard et al., 2001), Japan (Tanaka et al., 2003), China (Wu et al., 2007), India (Sewa et al., 2005), Pakistan (Anjum et al., 2000), Bulgaria (Stoyanova, 2002), Africa (Mohd et al., 2007). Based on the gliadin spectra, catalogs have been drawn up in some countries to identify varieties (Sapirstein & Bushuk, 1986; Lookhart et al., 1983; Metakovsky et al., 1991, 1994, 2018; Velkov, 1991; Stoyanova & Kolev, 1996; Ruiz et al., 2002 a, 2002 b).

The allelic compositions at Gli-A1 and Gli-A2 loci in *Triticum monococcum* L. ssp. *monococcum*, *Triticum monococcum* ssp. *boeoticum* (Boiss.) C. Yen and *Triticum urartu* Thumanjan ex Gandilyan had investigated (Metakovsky & Baboev, 1992, Saponaro et al., 1995; Zhao-cai et al., 2006). The high-quality gliadin alleles as Gli-A2b were found in einkorn wheat (Zhao-cai et al., 2006). In 40 accessions of *Triticum boeoticum* Boiss. and *Triticum urartu* Thumanjan ex Gandilyan collected from different regions of Iran, Ahmadi and Pour-Aboughadareh (2015) found that 92 % of the accessions carried Gli-A2 allele detected by Long et al. (2005) and Kawaura et al. (2005). The variation in gliadin seed storage proteins in Spanish einkorn was high, with seven allelic variants for the Gli-Am1 locus and fourteen for the Gli-Am2 locus found among the evaluated accessions (Alvarez et al., 2006; Alvarez & Guzmán, 2013).

The aim of this study was to compare the genetic diversity between einkorn genotypes with different geographic origin by electrophoretic patterns of seeds proteins.

2 MATERIAL AND METHODS

Twenty two einkorn accessions with different geographic origin preserved under long-term seed storage condition in the National gene bank of Bulgaria more than 20 years were evaluated by gliadin electrophoresis (Table 1).

Acid-PAGE (acid polyacrilamyde gel electrophoresis) was carried out according to the standard reference method of ISTA (Draper 1987; Anonymous 2003). Proteins were extracted from a bulk sample of 50 mg finely ground powdered seeds with 300 µl extracting solution (0.05 g Pyronin G; 25 ml 2-chloroethanol), stained overnight at room temperature, and centrifuged for 30 min at 17 000 g and 14 °C. Then, 10 µl of the extracts were loaded into wells. Gliadin electrophoresis was performed on a vertical polyacrylamide gel with a thickness of 1.5 mm and an electrode buffer with a pH of 3.2 using a Consort E835 vertical unit (with gel cassette 200 × 200 mm). Electrophoresis was carried out at 20 mA for 5 hours and 15 minutes. Staining of gels was performed in a solution of Coomasie Brilliant Blue G-250: Coomasie Brilliant Blue R-250 (1:3), dissolved in trichloroacetic acid/methanol for 48 hours.

Specialized software BIO-1D++, version 11.07 was used to create databases for the gliadin spectra of the studied genotypes. Based on the results of electrophoretic band spectra, genetic similarity coefficient of Nei &

Table 1: List of accessions included in the study

Number of the accessions	Genus	Species	Spaut.	Subtaxa	Origin
BGR 19069	*Triticum*	*monococcum*	L.	var. *hornemannii*	Germany
BGR 19065	*Triticum*	*monococcum*	L.	var. *hornemannii*	Georgia
BGR 30035	*Triticum*	*monococcum*	L.	var. *hornemannii*	Russia
BGR 19078	*Triticum*	*monococcum*	L.	var. *laetissimum*	Germany
BGR 30022	*Triticum*	*monococcum*	L.	var. *laetissimum*	Germany
BGR 19061	*Triticum*	*monococcum*	L.	var. *laetissimum*	Spain
BGR 19079	*Triticum*	*monococcum*	L.	var. *nigricultum*	Germany
BGR 11001	*Triticum*	*monococcum*	L.	var. *atriaristatum*	Switzerland
BGR 12386	*Triticum*	*monococcum*	L.	var. *laetissimum*	Germany
BGR 19063	*Triticum*	*monococcum*	L.	var. *flavescens*	Spain
BGR 30030	*Triticum*	*monococcum*	L.	var. *eincorn*	Russia
BGR 30031	*Triticum*	*monococcum*	L.	var. *vulgare*	Russia
BGR 30028	*Triticum*	*monococcum*	L.	var. *hornemannii*	Switzerland
BGR 30026	*Triticum*	*monococcum*	L.	var. *hornemannii*	Switzerland
BGR 28720	*Triticum*	*monococcum*	L.	var. *macedonicum*	Germany
BGR 30036	*Triticum*	*monococcum*	L.	var. *macedonicum*	Russia
BGR 19055	*Triticum*	*monococcum*	L.	var. *flavescens*	Germany
B2E0417	*Triticum*	*monococcum*	L.	*var. vulgare*	Bulgaria
B3E0025	*Triticum*	*monococcum*	L.	*var. vulgare*	Bulgaria
BGR 28717	*Triticum*	*monococcum*	L.	var.*eincorn*	Russia
BGR 30027	*Triticum*	*monococcum*	L.	var.*albohornemannii*	Germany
BGR 26774	*Triticum*	*monococcum*	L.	var.*eredvianum*	Germany

Li (1979) was calculated for all possible pair of electrophoregrams. The similarity matrix was used to construct the dendrogram by the unweighted pair group average method (UPGMA).

The genetic diversity for each gliadin pattern was calculated as per Nei (1973), as $H = 1 - \Sigma pi^2$, where H is the genetic variation index and Pi is the proportion of a particular pattern in each group of α, β, γ and ω gliadins separately. The mean value of H was calculated for all the four groups of gliadins.

3 RESULTS AND DISCUSSION

In the Table 2 were presented number of gliadin bands, patterns, and the genetic variation index in gliadins for 22 investigated accessions of *Triticum monococcum* L. Among the 22 einkorn accessions analysed, 64 different bands were detected assuming that the bands with the same relative mobility represent the same protein. Each zone (α, β, γ and ω) was considered as a single locus and the different patterns as allelic variants. The patterns within each gliadin group of α, β, γ and ω were identified by comparing banding patterns of each einkorn accession with all the other einkorn accessions (Ojaghi & Akhundova, 2010; Aliyeva et al., 2012).

A total of 34 different mobility bands and 21 gliadin patterns were identified in the ω-gliadin zone. Bands varied between two and seven in each ω-gliadin pattern, as patterns with five bands being the most frequent (40.9 %). Twenty one accessions presented its unique ω-gliadin pattern, while two genotypes had the same patterns, respectively 14-BGR 19055 and 15-B2E0417 with pattern 14 (Figure 1).

In the γ-gliadin zone, 12 bands and 16 different patterns were noted. The γ -gliadin pattern 5, were observed in accessions 5-BGR 30022 and 19-BGR 30027. The pattern 6 was detected in 6-BGR 19061 and 7-BGR 19079. The γ -gliadin patterns 10 and 12 were marked respectively in two groups of accessions – 11-BGR 19063, 14-BGR 19055 and 15-B2E0417; 13-BGR 30026, 18-BGR 26774 and 20-BGR 28717 (Figure 1).

Seventeen β- gliadin patterns and totally 12 different mobility bands were found. The bands in the gliadin

Table 2: Number of gliadin bands, patterns, and the genetic diversity in gliadins for 22 accessions of *Triticum monococcum* L.

		Number of gliadin bands				
№	Accession number	α	β	γ	ω	Total bands
1	BGR 19069	3	4	2	5	14
2	BGR 19065	3	6	1	5	15
3	BGR 30035	4	4	4	4	16
4	BGR 19078	3	4	3	2	12
5	BGR 30022	4	3	4	5	16
6	BGR 19061	3	7	4	2	16
7	BGR 19079	3	4	4	3	14
8	BGR 11001	3	4	5	4	16
9	BGR 12386	4	3	4	6	17
10	BGR 19063	3	4	4	4	15
11	BGR 30031	3	5	3	3	14
12	BGR 30028	3	6	4	5	18
13	BGR 30026	4	5	4	4	17
14	BGR 19055	3	5	3	6	17
15	B2E0417	3	4	3	6	16
16	B3E0025	3	5	2	5	15
17	BGR 28720	3	5	4	3	15
18	BGR 26774	3	5	4	7	19
19	BGR 30027	3	5	4	5	17
20	BGR 28717	3	5	4	5	17
21	BGR 30036	3	5	4	5	17
22	BGR 30030	3	6	1	5	15
Range of gliadin bands		3-4	3-7	1-5	2-7	12-19
Number of gliadin patterns		5	17	16	21	22
Genetic variation index (H, %)		0.120	0.924	0.914	0.950	0.727

patterns varied from 3 to 7 bands, as patterns with five bands being the most frequent (45 %). Thirteen accessions had specific patterns in the β- gliadin zone, while 1-BGR 19069 and 4-BGR 19078 with pattern 1, 2-BGR 19065, 20-BGR 28717 and 22-BGR 30030 with pattern 2, 3-BGR 30035 and 10-BGR 19063 with pattern 3, 5-BGR 30022 and 9-BGR 12386 with pattern 4 had the similar patterns (Table 2, Fig. 1).

Six bands were recorded in α -gliadin region and only five different α -gliadin patterns were determined. Two numbers of accessions (3-BGR 30035 and 13-BGR 30026) had specific patterns, respectively patterns with numbers 2 and 5. α -gliadin pattern with number 1 included ten accessions, pattern number three -2 accessions (5-BGR 30022 and 9-BGR 12386) and pattern number four - 8 accessions (6-BGR 19061, 7-BGR 19079, 8-BGR 11001, 10-BGR 19063, 11-BGR 30031, 12-BGR 30028, 14-BGR 19055, 18-BGR 26774). The accessions with three bands in the α-gliadin zone predominate (81.82 %).

Considering the four zones together, 22 gliadin patterns were identified, as in BGR 26774- 19 bands and in BGR 30028- 18 bands were detected. The lowest numbers of bands were found in BGR 19078 genotype (Table 2).

According to the work of Metakovsky and Baboev (1992), two independent gliadin blocks were determined for each einkorn accession. The upper block was composed by ω - and γ -gliadins, and in some cases also by one slow moving β-gliadin, whereas the lower block was formed by β - and α -gliadins. These two blocks were encoded by the *Gli-Alm* and the *Gli-A2m* loci on the short arm of chromosomes 1 and 6, respectively. Ciaffi et al. (1997), in study of 74 accessions of *T. monococcum* from Italy, Greece, Turkey and Russia found more allelic variation at *Gli-A1m* than at *Gli-A2m*. In contras Alvarez et

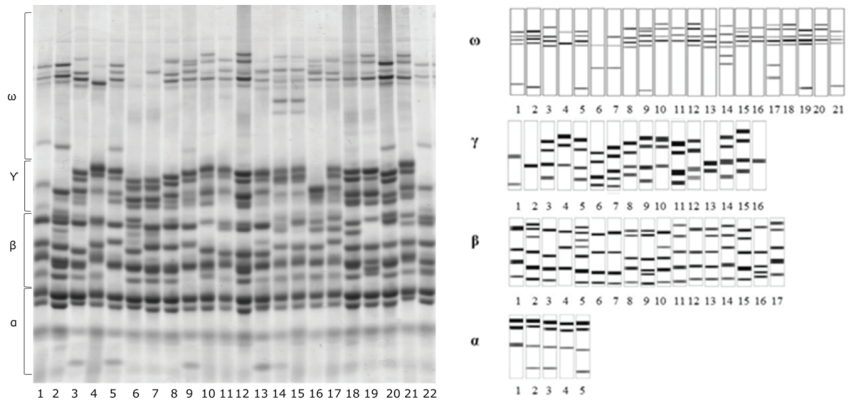

Figure 1: A) Gliadin patterns of accessions of *Triticum monococcum* L. after acid polyacrylamide gel electrophoresis (A-PAGE) (1- BGR 19069, 2- BGR 19065, 3- BGR 30035, 4- BGR 19078, 5-BGR 30022, 6- BGR 19061, 7- BGR 19079, 8- BGR 11001, 9- BGR 12386, 10- BGR 19063, 11- BGR 30031, 12- BGR 30028, 13- BGR 30026, 14- BGR 19055, 15- B2E0417, 16- B3E0025, 17-BGR 28720, 18- BGR 26774, 19- BGR 30027, 20- BGR 28717, 21- BGR 30036, 22- BGR 30030); B). Ideograms of different gliadins patterns in the α, β, γ and ω regions

al. (2006) found 7 and 14 alleles for the *Gli-A1*m and *Gli-A2m* between the 48 evaluated einkorn accessions. Zhao-cai et al. (2006) identified 14 and 19 allele variants, respectively at *Gli-A1m* and *Gli-A2m* of 41 *T. monococcum* accessions. Ruiz et al. (2007) noted that *Gli-A2m* was the most polymorphic and the most useful to distinguish between 17 einkorn varieties.

In this study, it was observed that ω, γ, and β areas had high allelic variants but the least allelic variants were established in area α. The highest allelic variants had ω gliadins (Fig.1).

In investigated 22 einkorn genotypes, no identical gliadin patterns were found in the gliadin spectra. The genetic diversity based on the patterns was calculated for each of the four zones. The genetic variation indexes varied between 0.120 and 0.950. Omega (ω) zone was found to have the biggest diversity (H = 0.950), followed by β (H = 0.924) and γ (H = 0.914) and the least diversity being that of α (H = 0.120). The mean value of H calculated for all the four groups of gliadins was also high (H = 0.727) (Table 2). It indicated that high genetic variation existed in the einkorn wheat (Zhao-Cai et al., 2006).

The genetic similarities (GS) among 22 einkorn accessions estimated by Nei and Li coefficient (1979) are presented in Table 3. It ranged from 0.36 to 0.97. The highest GS (0.97) was found between accessions: BGR 30022 (from Germany) and BGR 12386 (from Germany), BGR 19055 (from Germany) and B2E0417 (from Bulgaria), respectively, which indicates that the genetic diversity within these pairs of accessions was very low. The lowest value of GS was 0.36, which was observed between accession BGR 30028 (from Switzerland) and BGR 30030 (from Russia).

Cluster analysis of gliadin bands using the UPGMA method, as well as Nei and Li (also called Dice) similarity coefficients is presented in Fig. 2. The correlation between the cophenetic value matrix and the original similarity coefficient matrix was high (r = 0.739) indicating a good fit of the cluster analysis performed (Ma et al., 2012). The dendrogram allowed distinguishing three main clusters. Cluster 1 included 6 genotypes. It was divided into three subgroups, The first subgroup included only genotype1-BGR 19069 (var. *hornemannii* from Germany). Subcluster 2 combined 3 genotypes: 2-BGR 19065 (var. *hornemannii* from Georgia), 22-BGR 28717 (var. *eincorn* from Russia) and 20-BGR 30030 (var. *einkorn* from Russia), which had pattern 2 in the β zone and pattern 1 in α zone. Subgroup 3 included 4-BGR 19078 (var. *laetissimum* from Germany) and 21-BGR 30036 (var. *macedonicum* from Russia), which had pattern 1 in α zone. In cluster 2 were included 15 genotypes. The cluster was divided to two subgroups. The first subgroup grouped 3-BGR 30035 (var. *hornemannii* from Russia), 13-BGR 30026 (var. *hornemannii* from Switzerland), 5-BGR 30022 (var. *laetissimum* from Germany), 9-BGR 12386 (var. *laetissimum*, Germany), 10-BGR 19063 (var. *flavescens* from Spain), 12- BGR 30028 (var. *hornemannii* from Switzerland), 18-BGR 26774 (var. *eredvianum* from Germany), 19-BGR 30027 (var. *albohornemannii* from Germany), 6-BGR 19061 (var. *laetissimum* from Spain) and 7-BGR 19079 (var. *nigricultum* from Germany). The second subgroup included 5 genotypes, grouped also into three subgroups. In the first subgroup was separated

Table 3: Average genetic similarity matrix of Nei and Li (1979) coefficient based on A-PAGE gliadin patterns for the 22 einkorn accessions

	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22
L1	1																					
L2	0.55	1																				
L3	0.67	0.58	1																			
L4	0.69	0.67	0.64	1																		
L5	0.67	0.65	0.81	0.64	1																	
L6	0.53	0.58	0.69	0.57	0.63	1																
L7	0.5	0.62	0.8	0.46	0.73	0.87	1															
L8	0.53	0.65	0.56	0.5	0.69	0.69	0.73	1														
L9	0.52	0.63	0.85	0.55	0.97	0.61	0.71	0.67	1													
L10	0.55	0.6	0.77	0.74	0.71	0.65	0.76	0.71	0.75	1												
L11	0.43	0.69	0.73	0.69	0.6	0.67	0.64	0.6	0.58	0.75	1											
L12	0.56	0.48	0.76	0.47	0.65	0.71	0.75	0.76	0.63	0.85	0.69	1										
L13	0.52	0.63	0.85	0.62	0.85	0.73	0.77	0.73	0.88	0.81	0.84	0.74	1									
L14	0.45	0.69	0.67	0.69	0.67	0.67	0.65	0.67	0.65	0.63	0.77	0.69	0.76	1								
L15	0.47	0.58	0.69	0.71	0.69	0.63	0.67	0.69	0.55	0.65	0.73	0.53	0.73	0.97	1							
L16	0.69	0.73	0.77	0.67	0.77	0.71	0.76	0.65	0.75	0.73	0.76	0.73	0.75	0.69	0.65	1						
L17	0.76	0.67	0.71	0.74	0.77	0.65	0.62	0.58	0.75	0.8	0.83	0.67	0.75	0.69	0.65	0.87	1					
L18	0.67	0.53	0.8	0.58	0.74	0.69	0.67	0.69	0.78	0.82	0.73	0.81	0.67	0.67	0.74	0.71	0.76	1				
L19	0.77	0.56	0.85	0.69	0.79	0.67	0.65	0.55	0.76	0.88	0.71	0.8	0.71	0.59	0.61	0.69	0.75	0.89	1			
L20	0.71	0.88	0.67	0.69	0.67	0.55	0.45	0.55	0.76	0.69	0.58	0.51	0.71	0.65	0.61	0.5	0.63	0.61	0.76	1		
L21	0.71	0.56	0.61	0.76	0.55	0.48	0.52	0.48	0.65	0.63	0.52	0.57	0.53	0.53	0.48	0.56	0.81	0.72	0.76	0.71	1	
L22	0.83	0.93	0.58	0.74	0.58	0.52	0.55	0.45	0.5	0.6	0.48	0.36	0.56	0.56	0.58	0.6	0.67	0.41	0.56	0.75	0.75	1

The L1 to L22 correspond to the following number of accessions: L1- BGR 19069, L2- BGR 19065, L3- BGR 30035, L4- BGR 19078, L5-BGR 30022, L6- BGR 19061, L7- BGR 19079, L8- BGR 11001, L9- BGR 12386, L10- BGR 19063, L11- BGR 30031, L12- BGR 30028, L13- BGR 30026, L14- BGR 19055, L15- B2E0417, L16- B3E0025, L17-BGR 28720, L18- BGR 26774, L19- BGR 30027, L20- BGR 28717, L21- BGR 30036, L22- BGR 30030

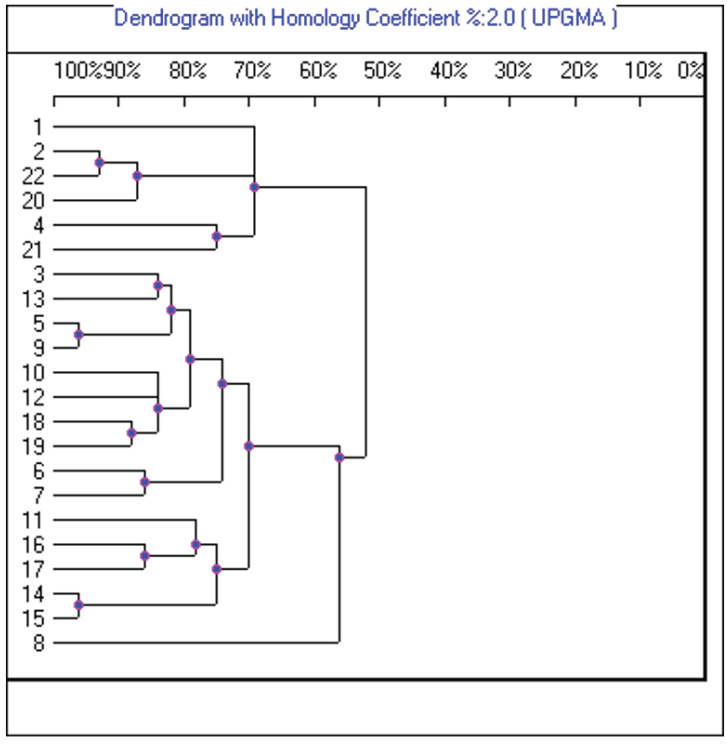

Figure 2: UPGMA dendrogram base on A-PAGE and Nei and Li similarity index (1- BGR 19069, 2- BGR 19065, 3- BGR 30035, 4- BGR 19078, 5-BGR 30022, 6- BGR 19061, 7- BGR 19079, 8- BGR 11001, 9- BGR 12386, 10- BGR 19063, 11- BGR 30031, 12- BGR 30028, 13- BGR 30026, 14- BGR 19055, 15- B2E0417, 16- B3E0025, 17-BGR 28720, 18- BGR 26774, 19- BGR 30027, 20- BGR 28717, 21- BGR 30036, 22- BGR 30030)

11-BGR 30031 (var. *vulgare* from Russia), into second respectively 16-B3E0025 (var. *vulgare* from Bulgaria) and 17-BGR 28720 (var. *macedonicum* from Germany), while in the third were 14-BGR 19055 (var. *flavescens* from Germany) and 15-B2E0417 (var. *vulgare* from Bulgaria), which had pattern 14 in the ω-gliadin zone and pattern 10 in the γ-gliadin. In the last cluster 3 was separated genotype 8-BGR 11001 (var. *atriaristatum* from Switzerland). The results of cluster analysis justify the high level of genetic variation among einkorn genotypes. No relationships between genetic diversity, geographic origin and the genotypes were observed. Zhao-Cai et al. (2007) also confirmed that the genetic relationships in diploid wheats based on the gliadins were associated with the species or subspecies rather than the geographical origin. Khabiri et al. (2013) and Medouri et al. (2015) evaluated the relationships among 17 populations of *Aegilops cylindrica* Host and *Aegilops geniculata* Roth by gliadin polymorphism and found that genetic diversity did not follow the geographical distribution. Zaefizadeh et al. (2010) also found no correlation between genetic diversity and the geographical distribution of durum wheat landraces studied. A considerable differentiation of common wheat germplasms from different countries and breeding centres was discovered earlier using gliadin alleles as wheat genotype markers (Metakovsky et al., 1991, 1994; Chernakov and Metakovsky, 1994; Metakovsky and Branlard, 1998).

Additional investigation must to do to analyze population structure of einkorn wheat and relationships between agro-morphological data and gliadins.

4 CONCLUSION

The investigated 22 einkorn genotypes were characterized with high genetic diversity on the basis of their alcohol soluble proteins-gliadins by the acid-PAGE method. The ω-zone was the most polymorphic, followed by β, γ and α, respectively. The highest genetic similarities (GS) was found between accessions: BGR 30022 and BGR 12386, BGR 19055 and B2E0417, respectively. The lowest GS was observed between accession BGR 30028 and BGR 30030. The results of cluster analysis justify the high level of genetic variation among einkorn accessions. No relationship between genetic diversity, geographic origin and the genotypes was observed.

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Impact of different fermentation characteristics on the production of mycelial biomass, extra-cellular polysaccharides, intra-cellular polysaccharides, and on the antioxidant activities of *Cordyceps militaris* (L.) Fr. (strains AG-1, PSJ-1)

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Impact of different fermentation characteristics on the production of mycelial biomass, extra-cellular polysaccharides, intra-cellular polysaccharides, and on the antioxidant activities of *Cordyceps militaris* (L.) Fr. (strains AG-1, PSJ-1)

Abstract: The mycelial biomass, antioxidant activity and production of extra- and intra-cellular polysaccharides production [EPS, IPS] of *Cordyceps militaris* strains AG-1, PSJ-1 were evaluated under different submerged liquid culture (SLC) conditions**.** At 24 ^{0}C mycelial biomass and polysaccharide production of AG-1, PSJ-1 was optimal using PVC media and static culture conditions; (AG-1: 21.85 ± 1.00; PSJ-1: 18.20 ± 1.84 g l^{-1}), and oven drying at 40 ^{o}C (AG-1: 25.95 ± 0.84, PSJ-1: 23.55 ± 0.69 mg g^{-1}) compared with hot water extraction (AG-1: 7.07 ± 0.15, PSJ-1: 7.39 ± 0.61 mg g^{-1}). Maximum biomass, EPS and IPS production were observed when the initial pH was 6.7: AG-1: 12.92 ± 0.33, 209.70 ± 1.56, 32.62 ± 0.87; PSJ-1: 9.03 ± 0.24 g l^{-1} , 198.16 ± 0.85 mg g^{-1}, 30.63 ± 1.96 mg g^{-1}, respectively. The use 3.5 % coconut oil improved biomass, EPS, IPS production, which were 8.27 ± 0.09, 8.01 ± 0.01 g l^{-1} ; 1208.00 ± 8.60, 1110.40 ± 7.20 mg g^{-1}; 32.43 ± 0.49, 29.74 ± 0.44, for AG-1 and PSJ-1, respectively. Both culture condition and oven drying methods had significant effects on H_2O_2 and radical scavenging activity, $ABTS^+$ radical activity, lipid peroxidation, and also had effects on total flavonoid and, total phenolic contents. The use of crude submerged liquid culture and oven drying on strains AG-1, PSJ-1 led to extracts with potent antioxidant activity, suggesting the therapeutic use of polysaccharides from strains AG-1, PSJ-1.

Key words: antioxidant activities; Cordyceps militaris; extra-cellular polysaccharides and intra-cellular polysaccharides; oven drying; pH

Vpliv različnih postopkov fermentacije na produkcijo biomase micelija, ekstra in itracelularnih polisaharidov in na antioksidacijsko aktivnost kokonovega glavatca (*Cordyceps militaris* (L.) Fr., seva AG-1, PSJ-1)

Izvleček: Biomasa micelija, antioksidacijska aktivnost in produkcija zunaj- in znotraj celičnih polisaharidov [EPS, IPS] so bile ovrednotene pri kokonovem glavatcu **(***Cordyceps militaris* (L.) Fr., seva AG-1, PSJ-1) v razmerah različnih submerznih tekočih kultur (SLC) **.** Pri 24 ^{0}C sta bili biomasa micelija in produkcija polisaharidov pri obeh sevih optimalni v gojišču PVC in stabilnih ramerah; (AG-1: 21,85 ± 1,00; PSJ-1: 18,20 ± 1,84 g l^{-1}), po sušenju v pečici na 40 ^{o}C (AG-1: 25,95 ± 0,84, PSJ-1: 23,55 ± 0,69 mg g^{-1}) v primerjavi z ekstrakcijo z vročo vodo (AG-1: 7,07 ± 0,15, PSJ-1: 7,39 ± 0,61 mg g^{-1}). Največja biomasa in produkcija obeh tipov polisaharidov (EPS in IPS) sta bili doseženi pri začetnem pH 6,7: AG-1: 12,92 ± 0,33; 209,70 ± 1,56; 32,62 ± 0,87; PSJ-1: 9,03 ± 0,24 g l^{-1}; 198,16 ± 0,85 mg g^{-1}; 30,63 ± 1,96 mg g^{-1}. Uporaba kokosovega olja (3,5 %) je povečala biomaso in produkcijo polisaharidov v obeh sevih in sicer: 8,27 ± 0,09; 8,01 ± 0,01 g l^{-1}; 1208,00 ± 8,60: 1110,40 ± 7,20 mg g^{-1}; 32,43 ± 0,49; 29,74 ± 0,44. Oba načina gojenja in metode sušenja v pečici so imeli značilen vpliv na aktivnost nevtralizacije H_2O_2 in prostih radikalov, aktivnost $ABTS^+$ radikala in peroksidacijo lipidov. Imeli so tudi vpliv na vsebnost celokupnih flavonoidov in fenolov. Uporaba surove submerzne tekoče culture in sušenje obeh sevov v pečici sta dali izvlečke s potencialno antioksidacijsko aktivnostjo, kar nakazuje terapevtsko uporabo polisaharidov iz obeh sevov kokonovega glavatca.

Ključne besede: antioksidacijska aktivnost; Cordyceps militaris; zunaj- in znotraj celični polisaharidi; sušenje v pečici; pH

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1 INTRODUCTION

Cordyceps militaris (L.) Fr. (Ascomycota: Hypocreales) is an entomopathogenic fungus that is used in traditional Asia medicine; it is a common parasite of lepidopteran larvae (Shih et al., 2007). The medicinal properties of *Cordyceps militaris* result from its ability to produce bioactive compounds including cordycepin. At present, liquid fermentation is used for biomass production. However, optimization is required to maximize production of bioactive compounds. The efficacy of extracts production depends mainly on the strain used, nutrient sources in the culture medium, and cultivation parameters (Dong et al., 2012). During the fermentation, *C. militaris* grows in liquid suspension, which is thought to be the best way to produce complex organic compounds including cordycepin (Mao et al., 2005). Vegetable oils can enhance growth rates of mycelium in liquid culture (Schisler & Volkoff, 1977) and reports suggest that oils and fatty acids promote production of fungal metabolites (Kojima et al., 1972).

In recent years, considerable research attention had focused on natural compounds with hypoglycemic activity. Polysaccharides extracted from various medicinal fungi species have shown hypoglycemic activity (Kiho et al., 1997). Although many researchers have tried to optimize submerged liquids culture conditions to promote extra–cellular polysaccharides (EPS) production by fungi, the nutritional requirements and medium conditions for submerged cultures are poorly understood, yet the method remains popular (Park et al., 2001). For *C. militaris* in particular, submerged liquid culture has rarely been studied (Nielsen et al., 1995).

Currently, antioxidants extracted from field-collected *C. militaris* fruiting bodies, are investigated extensively for their ability to protect organisms and cells from oxidative damage due to aging and cell degeneration (Cazzi et al., 1997). Traditionally, field-collected *C. militaris* is used widely in both food and pharmaceutical preparations (Isildak et al., 2004). Field-collected and cultivated *C. militaris* is becoming increasingly popular as a functional food (due to antioxidant activity) as well as for its medicinal properties (Elmastas et al., 2007).

In this study, we determined the optimal cultivation conditions (pH and type of vegetable oils used) for production of *C. militaris* mycelial biomass. Moreover, we quantified the effect of cultivation conditions on biomass production of biologically active ingredients, extra and intra-cellular polysaccharide production**,** antioxidant activities, total phenol content, flavonoid content. We also characterized the biological activity of methanol extracts of mycelial and filtrates obtained using the static cultures method.

2 MATERIALS AND METHODS

2.1 FUNGAL STRAINS

Two field-collected strains of *Codiceps militaris* AG-1, and strain PSJ-1 were obtained from the Plant Physiology and Value Added Microorganisms Laboratory, Department of Plant Industry, National Pingtung University of Science and Technology (NPUST), Taiwan. Mycelia from each of the two strains were cultivated and maintained in the collection on mannitol yolk polymyxin selective agar (MYPS) (Dang et al., 2018) at 24 °C.

2.2 MEDIA

Five submerged liquid culture media were used to evaluate the growth and production of secondary metabolites: (1) MYPS media: 4 g l^{-1} malt extract powder; 4 g l^{-1} yeast extract; 6 g l^{-1} peptone; 10 g l^{-1} sucrose; 0.3 g l^{-1} Vitamin B1, and 1000 ml distilled water; (2) PVC media: 30 g l^{-1} glucose; 10 g l^{-1} corn powder; 1 g l^{-1} KH_2PO_4; 0.6 g l^{-1} K_2HPO_4; 0.7 g l^{-1} $MgSO_4 \cdot 7H_2O$; 0.25 g l^{-1} $FeSO_4 \cdot 7H_2O$; 0.5 g l^{-1} vitamin B1, 6 g l^{-1} peptone, and 1000 ml distilled water. (3) PD culture medium: 200 g l^{-1} potatoes; 20 g l^{-1} dextrose; 30 g l^{-1} sucrose; 0.5 g l^{-1} Vitamin B1, and 1000 ml distilled water, (4) Malt-extract medium (ME): 5g l^{-1} malt extract powder; 5g l^{-1} peptone; 20g l^{-1} glucose; 0.3 g l^{-1} Vitamin B1, and 1000 ml distilled water (Atlas, 1993); (5) Czapek-Dox medium (CD): 30 g l^{-1} Sucrose; 2 g l^{-1} $NaNO_3$, 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4.7H_2O$, 0.5 g l^{-1} KCl; 0.3 g l^{-1} vitamin B1 and 1000 ml distilled water (Stevens, 1981). Each cultivation attempt was conducted in five (5) repetitions per medium.

2.3 DETERMINING THE EFFECT OF DIFFERENT MEDIA ON BIOMASS OF *C. militaris* AND QUANTITY OF HOT WATER EXTRACTED

Discs (6 mm diameter cut from culture grown on MYPS plates) of mycelia from the two strains of *C. militaris* were inoculated into the liquid culture medium. Five discs were used for 250 ml of liquid media in each 500 ml Erlenmeyer flask. Flasks were incubated at 24 °C in a temperature controlled rotary shaker (Orbital Shaker Model SK-302AB, Sun Kuan Instruments Co., Kaoshung, Taiwan) for 18 days. The shaking function was set 'static' for the first 5 days and then to 'shake' (93 rpm) for the following 13 days) (Park et al., 2002). After the incubation period mycelia were harvested from each flask by filtration through a pre-weighed filter paper (Whatman No. 1, Whatman Ltd.Toyo Roshi Kaisha, Ltd. Japan), weighed

(wet weight) and then vacuum-dried (dry weight per unit volume of media). The filtrate was concentrated under reduced pressure (rotary evaporator) at 4 °C. Dried filtrates and mycelia were ground into fine powders (20 mesh) in a mill grinder before a hot water extraction (Jo et al., 2010).

2.4 EFFECT OF DIFFERENT PH ON BIOMASS AND ON YIELD OF EXTRAC-CELLULAR AND INTRA-CELLULAR POLYSACCHARIDES AFTER SUBMERGED CULTURE OF *C. militaris*

The effect of pH was done in PVC cultivation medium with pH adjusted to 4.5, 5.5, 6.7, 7.5, or 8.0. 1N HCl or 1N NaOH were used for adjusting the pH. Strains AG-1, PSJ-1 of *C. militaris* in were then grown at each pH in submerged culture; under static conditions for 18 days at 24 °C. After 18 days the biomass (dry weight) was determined and the extra and intra-cellular polysaccharides extracted and weighed. Each treatment was conducted 5 repetitions.

2.4.1 Extra-cellular polysaccharides (epd) and intracellular polysaccharide (ips)

Extra-cellular polysaccharides (EPS) were extracted from the culture filtrate from each flask using standard methods with minor modifications (Fang & Zhong, 2002). Specifically, mycelial biomass in the medium was centrifuged at 10,000 g for 20 min. The supernatant obtained was mixed with three volumes of pure ethanol and incubated at 4 °C for 24 hours. The resulting precipitate (EPS) was then separated by centrifugation at 8000 g for 10 min, washed with ultrapure water and lyophilized prior to weighing. For intra-cellular polysaccharides (IPS), mycelial biomass was subjected to extraction with boiling water for one hour and the mixture was filtered through Whatman No. *1* filter paper. The filtrate was allowed to precipitate with four volumes of 95 % (v/v) ethanol following incubation overnight at 4 °C. The resulting precipitate (IPS) was separated by centrifugation at 8000 g for 10 min, washed with ultrapure water and lyophilized before determining dry mass (Sharma et al., 2015).

2.4.2 Extract of oils on biomass and ips yield after submerged culture of *C. militaris*

The effects of adding different types of oils to the culture medium on biomass and production of extra and intra-cellular polysaccharides during submerged culture was done in PVC medium only. The following oils were used: sunflower oil (HALA Taisun Col Ltd., Taiwan), olive oil (Olitalia sunflower oil, Italia), coconut oil (coconut oil virgin, Viet Delta Industry Co., Ltd, Vietnam), suet volum % (Pingtung City Supermarket, Taiwan), and peanut oil (HACCP, Taiwan) at concentrations of 1.5 %, 2.5 %, and 3.5 %. One type of oil was used per setup. Fermentation was done at 24 °C, at an initial pH of 6.7, in static cultivations conditions for 18 days. Biomass (dry weight) and extra-cellular and intra-cellular polysaccharide production were measured as described previously. Each oil type and % treatment was conducted in five repetitions.

2.5 ANTIOXIDANT ACTIVITIES OF MYCELIAL EXTRACTS OF *C. militaris* GROWN UNDER DIFFERENT SUBMERGED CULTURE CONDITION

The antioxidant activity of mycelial extracts of *C. militaris* was in a submerged culture of three different media (MYPS, PVC, PD) under of three protocoles: (1) shake (shaking regimes for 18 days at 24 °C); (2) static (for 18 days at 24 °C); (3) static + shake (static for the first 5 days and then 'shake' at 93 rpm for the following 13 days). After cultivation mycelia were oven dried, methanol extracts were then evaluated for antioxidant activity *In vitro*. We specifically quantified the total phenolic content and total flavonoid content. For each test, each treatment was conducted in five repetitions.

2.5.1 Mycelial drying method (oven drying)

Fresh mycelia were dried using the oven drying (OD) method (40 °C for 72 h at RH = 65 %). Oven drying was done in hot air oven (Rotek Instruments, B & C Industries, Cochin, India). (Mediani et al., 2013). The samples were ground to powder with a grinder (Yuqi, DM-6, Taiwan), and stored at -20 °C prior to methanol extraction.

2.5.2 Hot water extract

Approximately 2 g from each dryed and grinded sample was extracted with 200 ml of boiling distilled water for 2 h. The extract was filtered through filter paper (Advantec No. 1, Japan) while the residue was re-extracted twice under same conditions. The filtrates obtained from the three separate extractions

were combined, concentrated and lyophilized. The lyophilized extracts were weighed and stored at 4 °C prior to use.

2.5.3 Methanol crude extraction from oven-dried mycelia.

Approximately 2 g from each dryed and grinded (DM-6, Taiwan) sample was extracted with 200 ml of 95 % methanol at 75 ^{0}C after 2 h using a bath/circulator, and filtered through muslin cloth. The extracts were filtered through Whatman No. 2, Whatman Ltd. Toyo Roshi Kaisha, Ltd. Japan) filter paper. The residues were then extracted with an additional 100 ml of methanol. This was then filtered again through 100 mm of filter paper. Extractions from each sample were done twice and the combined filtrates were concentrated, under conditions of reduced pressure, in a rotary evapotator at 40 ^{0}C until dry. The dried extracts obtained were re-dissolved in methanol to a concentration of 100 mg ml^{-1} and were stored at 4 °C prior to the analyses of antioxidant attributes. For downstream experiments the dried methanol, filtered, evaporated under reduced pressure and vacuum-dried at 40 ^{0}C to get the viscous residue needed for estimation of antioxidant activities.

2.5.4 Scavenging of hydroxyl radicals

The hydroxyl radical scavenging activity of *C. militaris* methanol extract was measured according to the method of (Halliwell et al., 1992). Stock solutions of EDTA (1 mM) were prepared in DMSO and $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) in distilled deionized water. For each extract the scavenging activity was determined at different concentrations: 0.5, 1, 2.5, 5, 7.5, and 10 mg ml^{-1} in methanol dissolved in distilled water, 330 µl of phosphate buffer (50 mM, pH 7.5) and 100 µl of ascorbic acid. For each extract/ concentration 1000 µl were added to a solution made up of 100 µl of EDTA, 10 µl of $FeCl_3$, 100 µl of H_2O_2, 360 µl of deoxyribose and incubated at 37 °C for 1 h. After this time 1 ml of the incubated mixture was mixed with 1 ml of 10 % TCA and 1 ml of 0.5 % TBA (in 0.025 M NaOH containing 0.025 % butylated hydroxyl anisole) and the development of pink chromogen was measured spectrophotometrically at 532 nm. The hydroxyl radical scavenging activity of each extract was reported as percentage inhibition of deoxyribose degradation and was calculated according to the formula (1):

$$Scavenged\ (\%) = \frac{(A0 - A1) \times 100}{A0}$$

Where A0 was the control absorbance and A1 was the absorbance of the solution containing either the extract or absorbance of the solution containing either the extract or the standard absorbance.

2.5.5 Scavenging of ABTS$^{\cdot+}$, a radical cation.

The scavenging activity of the extracts was estimated using the ABTS$^{\cdot+}$ decolorization method (Arumagam et al., 2006; Re et al., 1999). A stock solution for evaluation of antioxidant activity was produced my mixing 5 ml of 7 mM ABTS and 88 µl of 140 mM $K_2S_2O_8$; this solution was allowed to complete radical generation for 12–16 h in darkness at room temperature. The stock solution was diluted with ethanol and PBS (pH 7.4) to give an absorbance of 0.75 at 734 nm. The scavenging activity of each strains of *C. militaris* methanol extract was determined at concentrations: 0.5, 1, 2.5, 5, 7.5, and 10 mg ml^{-1}. For each extract/ concentration combination 1 ml of extract was added to 1 ml of diluted stock solution and, 5 min after the initial mixing, the absorbance was measured at 734 nm, using ethanol as the blank solution. All measurements were performed in triplicate. The total antioxidant activity (TAA) percentage was calculated using the formula (2):

$$TAA\ \% = (Ac - \frac{As}{Ac}) \times 100$$

Where A = absorbance of stock solution and As = absorbance of the extract.

2.5.6 Antioxidant activity in relation to lipid peroxidantion

The antioxidant activity of *C. militaris* two strains methanol extracts was determined using a 1,3-diethyl-2-thiobarbituric acid (DETBA) method (Furuta et al., 1997). Extracts were evaluated at different concentrations (0.5, 1, 2.5, 5, 7.5, and 10 mg ml^{-1} in methanol); for each sample was added to 50 ml of linoleic acid emulsion (2 mg ml^{-1} in 95 % ethanol) and a positive control butylated hydroxytoluene (BHT) (0.1 mg ml^{-1}) were used in this study. The mixture was incubated at 80 °C for 60 min; it was then cooled in an ice bath and mixed with 200 ml of 8 % sodium dodecyl sulfate (SDS), 400 ml of deionized water, and 3.2 ml of 12.5 mM DETBA (Aldrich Chemical Co., WI, USA) in sodium phosphate buffer (pH 3.0).

After thoroughly mixing it was incubated at 95 °C

for 15 min and then cooled in an ice bath. Ethyl acetate (4.0 ml) was then added to the mixture, the mixture was centrifuged at 1000 g at 20 °C for 15 min. The fluorescence of the ethyl acetate layer was then measured in a spectrofluorometer with excitation set at 515 nm and emission set at 555 nm. Each value was expressed as the mean of triplicate measurements +/- standard deviation. The percentage of lipid peroxidation was determined against a blank with no sample added (100 %). The antioxidant activity was expressed as the inhibition of lipid peroxidation using the formula (3):

$$\textit{Antioxidant activity}\ (\%) = (1\text{- Percentage of lipid peroxidation}) \times 100$$

2.5.7 IC50 VALUES IN AN ANTIOXIDANT ACTIVITY.

The results of antioxidant activity, hydroxyl radicals, scavenging activity of ABTS+ radical cation, lipid peroxidation, respectively were normalized and expressed as IC50 (mg extract/ml). A lower IC_{50} value (mg extract/ml) corresponds to the higher antioxidant activity of *C. militaris* mycelial (obtained by submerged cultivation) extract.

2.6 QUANTIFYING ANTIOXIDANTS

2.6.1 Total phenolic content (tpc) of the extracts.

Total phenolic contents of each methanol extract was determined using gallic acid as a standard (Chan et al., 2009). A sample of 0.2 ml from each extract (10 mg ml^{-1} in methanol) was mixed with 2 ml of Folin-Ciocalteu's phenol reagent, 2N (Sigma). The mixture was incubated at room temperature for 5 minutes. Then 1.8 ml of 20 % sodium carbonate (Na_2CO_3, Nihon Shiyaku) was added, and the mixture centrifuged at 3000 g for 10 min. The mixture was then incubated for 90 min at room temperature. The absorbance of each reaction mixture was measured at 735 nm using a spectrophotometer (Hitachi U-2800, Japan). Gallic acid was used as a standard to create a calibration curve. The TPC was obtained by interpolation from linear regression analysis in mg gallic acid equivalents (mg GAE)/g of dry extract. All tests are performed in triplicate. Two strains mycelial concentration of the sample solution was determined based on a standard curve regression equation (4):

$A = 0.8533C + 0.0211, r^2 = 0.997$

(Where A is the absorbance and C is the concentration). Then, the extraction rate of TPC in the *C. militaris* sample was calculated.

2.6.2 Total flavonoid content (tfc) of the extracts.

The TFC was determined according to the method of Jia et al. (1999). A sample of 0.2 ml from each extract (100 mg ml^{-1} in methanol) was mixed with 1.5 ml of distilled water. To this 0.1 ml of 10 % aluminium nitrate [Al(NO3)3] and 0.1 ml of 1 M potassium acetate (CH3COOK) was added. After 40 min at room temperature, absorbance was measured at 415 nm. Quercetin was used as a standard for a calibration curve. Flavonoid contents was determined by interpolation from linear regression analysis in mg quercetin equivalents (QE)/g dry extract. All tests were done in triplicate. Two strains mycelial concentration in the sample solution was determined based on a standard curve regression equation (5):

$A = 3.2173A + 0.0618, r^2 = 0.997$

(Where A is the absorbance and C is the concentration). From, this extraction rate of TFC in *C. militaris* samples was calculated.

2.7 EXPERIMENTAL DESIGN AND DATA ANALYSIS

All experiments were done at the Department of Plant Industry, NPUST, Taiwan. One-way analysis of variance (ANOVA) was done followed Duncan's multiple range tests for means comparisons ($p \leq 0.05$). All analysis was done in SAS Version 9.4 (SAS Institute Inc., Cary, NC, USA). For each test, each value is expressed as mean ± SE and with 5 replicates (n = 5).

3 RESULTS AND DISCUSSION

3.1 EFFECT OF CULTIVATION MEDIA ON *C. militaris* BIOMASS PRODUCTION

Mycelial wet mass, dry mass and extra-cellular polysaccharide content of both strains were all the greatest in cultures grown in PVC media compared with the other media evaluated (Table 1 and Fig. 1). Overall, significantly more mycelial biomass was produced by strain AG-1 than by strain PSJ-1 in submerged culture. Overall, the cultivation methods used for both AG-1, PSJ-1 in this study had low or similar quantities of extracts as obtained in other set-ups. For example, studies on *C. militaris* strain BCC2816 reported 36 mg of extract from mycelium grown in 5 l of potato dextrose broth medium at 25 °C (Rukachaisirikul et al., 2004.), while 19.1 g l^{-1} were collected from mycelia produced in 57 ml medium at temperature 28 °C, and pH of 6.2 (Chunyan et

Table 1: Effect of different liquids media and mycelial biomass on static culture fresh mass, oven dries mass, crude hot water extract of strains AG-1 and, PSJ-1 after 18 days of cultivation. Valuea are expressed as mean± standard deviation (n = 5). Means within the same column followed by the same letters are not significantly different from each other at $p \leq 0.05$ according to Duncan's multiple range tests.

	Mycelial Biomass					
	Static culture fresh mass (g l $^{-1}$)		Oven dried mass (40 ^{0}C) (mg l $^{-1}$)		Boiling distilled water crude extract (mg l $^{-1}$)	
Media	AG-1	PSJ-1	AG-1	PSJ-1	AG-1	PSJ-1
MYPS	17.88±0.87^{ab}	15.03±0.71^{ab}	23.73±1.02^{ab}	22.74±0.75^{ac}	7.53±0.32^{a}	6.87±0.69^{ab}
PD	10.71±1.13^{cc}	8.92±1.28^{ab}	22.90±0.06^{ac}	24.02±0.04^{aa}	6.10±0.09^{bc}	5.91±0.44^{ac}
PVC	21.85±1.00^{aa}	18.20±1.84^{aa}	25.95±0.84^{aa}	23.55±0.69^{ab}	7.07±0.15^{ab}	7.39±0.61^{aa}
ME	14.45±1.37^{bb}	13.59±0.78^{ac}	18.76±0.85^{bc}	16.96±0.56^{b}	5.26±0.50^{be}	5.05±0.43^{bc}
CD	10.17±0.85^{cd}	8.63±0.77^{bb}	21.49±0.68^{ab}	18.31±0.75^{c}	5.99±0.04^{bd}	2.80±0.51^{c}

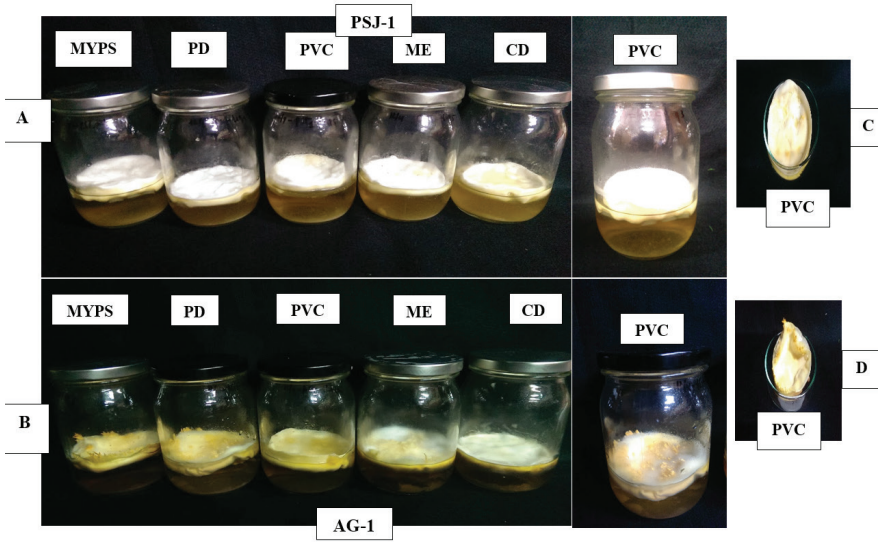

Figure 1: Effect of difficult submerged culture, fresh mycelial weight extract cordycepin production, MBDW: Mycelial Biomass dry weight (g/L), EPS: exopolysaccharide, IPS: Intracellular polysaccharides (mg/g DW). PVC media. of *C. militaris* cultured at 18 days. A: PSJ-1 strain and B: AG-1 strain. C: fresh mycelial of PSG-1 on PVC liquid media; D: fresh mycelial and fruiting body of AG-1 on PVC liquid media.

Table 2: Effect of initial pH and between strains AG-1, PSJ-1 strains on the mycelial biomass, extra-cellular and intra-cellular polysaccharide production after 18 days in static culture in PVC media at 24 0C. Data show the mean±standard deviation (n = 5). Means within the same column followed by the same letters are not significantly different to each other according to Duncan›s multiple range tests ($p \leq 0.05$)

	Mycelial biomass (dry mass; g l $^{-1}$)		Extra- cellular polysaccharides (mg l $^{-1}$)		Intra-cellular polysaccharides (mg l $^{-1}$)	
pH	AG-1	PSJ-1	AG-1	PSJ-1	AG-1	PSJ-1
5	4.63±0.03^{c}	3.95±0.06^{b}	38.9±0.36^{d}	32.06±0.55^{d}	13.31±1.10^{ad}	12.20±0.84^{ad}
5.5	5.71±0.05^{ac}	4.88±0.09^{b}	45.85±0.87^{cd}	35.06±1.18^{d}	15.84±0.79^{cd}	14.69±0.95^{cd}
6	8.61±0.71^{bc}	7.88±0.34^{ab}	168.56±0.94^{ab}	168.16±0.67^{ab}	23.68±0.19^{bc}	22.62±0.12^{bc}
6.7	12.92±0.3^{b}	9.03±0.24^{a}	209.70±1.56^{a}	198.16±0.85^{a}	32.62±0.87^{b}	30.63±1.96^{b}
7.5	6.70±0.69^{ac}	7.93±0.52^{a}	69.50±4.05^{bc}	57.39±2.18^{bc}	9.94±0.86^{ade}	8.66±0.73^{ad}
8	5.28±0.43^{c}	5.07±0.43^{ba}	39.73±2.87^{d}	35.16±1.73^{cd}	8.44±0.74^{ae}	7.83±0.62^{d}

Table 3: Effect of different type of oils and between strains AG-1, PSJ-1 strains on mycelial biomass, extra-cellualar and intra-cellular polysaccharide production of C. militaris after 18 days of cultivation in static culture in PVC media (initial pH 6.5) at 24 °C. Data show the means of five independent experiments±standard deviation (SD). Each value is expressed as mean±standard deviation (n = 5). Within columns the mean values with different capital letters within a row are significantly different ($p \leq 0.05$) to each other according to Duncan's multiple range tests)

		Mycelial dry biomass ($g\ l^{-1}$)		Extra-cellular polysaccharide ($mg\ l^{-1}$))		Intracellular polysaccharide ($mg\ g\ DM^{-1}$)	
Type of oils		AG-1	PSJ-1	AG-1	PSJ-1	AG-1	PSJ-1
Sunflower oil	1.5 %	3.49 ± 0.04^{cdm}	3.22 ± 0.01^{ik}	254.80 ± 12.69^{ag}	223.00 ± 5.84^{gh}	6.62 ± 0.20^{ci}	6.48 ± 0.05^{bh}
	2.5 %	4.17 ± 0.08^{ik}	3.96 ± 0.03^{fg}	291.00 ± 3.74^{af}	245.20 ± 2.49^{g}	6.75 ± 0.07^{ci}	6.36 ± 0.03^{bh}
	3.5 %	4.67 ± 0.03^{hi}	4.21 ± 0.06^{ef}	348.80 ± 2.40^{de}	316.20 ± 3.15^{de}	14.56 ± 0.28^{ade}	12.85 ± 0.21^{ef}
Oliver oil	1.5 %	3.32 ± 0.07^{d}	3.12 ± 0.08^{m}	113.38 ± 2.94^{hi}	107.10 ± 1.00^{ik}	6.86 ± 0.05^{ci}	6.26 ± 0.03^{bh}
	2.5 %	3.68 ± 0.03^{cm}	3.47 ± 0.07^{hi}	139.80 ± 1.71^{hi}	125.00 ± 1.52^{I}	8.80 ± 0.08^{bch}	7.45 ± 0.04^{bgh}
	3.5 %	3.90 ± 0.02^{ck}	3.72 ± 0.03^{gh}	154.20 ± 2.22^{gh}	127.60 ± 1.36^{hI}	5.59 ± 0.10^{ci}	5.21 ± 0.19^{b}
Coconut oil	1.5 %	5.87 ± 0.02^{bg}	5.55 ± 0.07^{cd}	434.00 ± 1.73^{cd}	406.60 ± 2.87^{bc}	17.98 ± 0.14^{ad}	16.54 ± 0.61^{e}
	2.5 %	6.67 ± 0.07^{af}	6.14 ± 0.02^{c}	701.20 ± 7.75^{bc}	683.60 ± 4.95^{ab}	18.17 ± 1.17^{de}	17.01 ± 0.97^{de}
	3.5 %	8.27 ± 0.09^{E}	8.01 ± 0.0^{a}	1208.00 ± 2.30^{b}	1110.40 ± 3.16^{a}	23.61 ± 1.31^{cd}	20.39 ± 1.55^{cd}
Suet	1.5 %	5.33 ± 0.10^{h}	5.14 ± 0.05^{de}	276.40 ± 3.17^{afg}	246.00 ± 1.64^{fg}	7.97 ± 0.90^{chi}	7.00 ± 0.92^{bh}
	2.5 %	6.50 ± 0.13^{afg}	6.26 ± 0.04^{bc}	309.00 ± 2.40^{ef}	294.20 ± 2.03^{ef}	10.45 ± 1.18^{defg}	9.58 ± 0.64^{agh}
	3.5 %	6.18 ± 0.05^{abg}	6.05 ± 0.02^{c}	411.60 ± 3.75^{de}	389.00 ± 3.03^{cd}	12.18 ± 1.04^{abf}	11.10 ± 0.66^{afg}
Peanut oil	1.5 %	5.19 ± 0.04^{hi}	5.06 ± 0.02^{df}	55.48 ± 0.57^{m}	53.53 ± 0.35^{m}	9.95 ± 0.12^{bhi}	9.37 ± 0.07^{agh}
	2.5 %	6.92 ± 0.02^{f}	6.78 ± 0.01^{ab}	60.40 ± 0.16^{ik}	59.53 ± 0.20^{m}	6.90 ± 0.05^{ci}	6.72 ± 0.05^{bgh}
	3.5 %	8.46 ± 0.14^{ef}	8.22 ± 0.05^{a}	103.60 ± 2.23^{i}	100.48 ± 1.70^{km}	46.17 ± 0.81^{d}	43.51 ± 0.21^{c}

al., 2009). Our results are in agreement with (Ing-Lung Shih et al., 2007), whose studies showed that metabolites and cell growth of 15.5 $g\ l^{-1}$ (shaking) after 36 days, and 14.0 $g\ l^{-1}$ (static) after 30 days.

3.2 EFFECT OF PH ON THE MYCELIAL *C. militaris* BIOMASS AND EXTRA- AND INTRA-CELLULAR POLYSACCHARIDES PRODUCTION

Initial pH affected the growth of mycelial, as well as the production of extra and intra-cellular polysaccharides, for both AG-1, PSJ-1 strains when cultivated using the submerged culture method (Table 2). Submerged liquids culture dry mass, EPS, and IPS were the highest when the initial pH ranged from 6.0 to 6.7, and were the lowest at the highest and the lowest pHs evaluated (5 and 8). Previous reports have suggested that low pH may increase quantities of extracellular polysaccharides in continuous production of many types of Basidiomycetes and Ascomycetes, but this may have been due to oil supplementation (Hsieh et al., 2005; Park et al., 2020; Kim et al., 2001). The optimal pH for cordycepin production by different strains of *C. militaris* has been reported to be in the range of 4-7 (Kang et al., 2014; Zhong et al., 2011). Other studies reported that *C. militaris* strain BCC2816 achieved the highest dry mycelial mass (19.1 $g\ l^{-1}$), and optimal cordycepin yield (1.8 $mg\ l^{-1}$) at 28 °C, pH 6.2 (Chunyan et al., 2009). In general, the effects of pH in static culture of strains AG-1, PSJ-1 in the present study were lower compared with other studies. For instance, *C. militaris* strain 3936 produced cordycepin in the pH range of 4.5 to 7.0, with the highest levels at pH 5.5 (213 $mg\ l^{-1}$), which was also consistent with earlier reports (Leung et al., 2007).

3.3 EFFECT OF DIFFERENT OILS ON BIOMASS AND ON EPS AND IPS YIELD AFTER SUBMERGED CULTURE OF TWO *C. militaris* STRAINS

Addition of oil sources led to significant increased levels of mycelial biomass, EPS and IPS (Table. 3). The greatest mycelial dry mass was achieved in coconut oil (Table. 3). Mycelial biomass, EPS and IPS production were higher when media were supplemented with sunflower oil, olive oil, coconut oil, suet, and peanut oil; for all oils growth increased as toe % oil increased. The results are in agreement with (Hsieh et al., 2006), who showed that mycelial growth was increased when media were supplemented with > 1 % olive oil; this was also associated with greater IPS production. Previous reports have also shown that production of extra-cellular polysac-

Table 4: Hydroxyl radical scavenging ability at different concentrations of *C. militaris* two strains grown using nine submerged culture methods that incorporated different media and shaking regimes. The concentration causing 50 % inhibition (IC50) is recorded. Mean values with different capital letters within a row are significantly different to each other ($p < 0.05$). Means within the same column of *C. militaris* two strains followed by the same letters are not significantly different at $p \leq 0.05$ according to Duncan's multiple range tests. Extracts were oven dried (n = 5).

		Sample concentration (mg ml^{-1})							
Submerged culture method	Strains	0.50	1.00	2.50	5.00	7.50	10.00	IC50 (µg ml^{-1})	R^2
MYPS (shake)	AG-1	19.16 ± 0.39^{b}	28.69 ± 1.03^{ac}	41.46 ± 1.04^{a}	56.56 ± 1.01^{c}	66.32 ± 0.61^{ad}	80.26 ± 0.62^{ad}	2.30	$R^2 = 0.9966$
	PSJ-1	8.94 ± 0.86^{ad}	8.71 ± 0.39^{a}	45.58 ± 0.34^{b}	61.21 ± 0.60^{b}	69.66 ± 0.83^{df}	77.00 ± 0.41^{acd}	2.01	$R^2 = 0.9191$
PVC (shake)	AG-1	4.99 ± 0.63^{e}	40.77 ± 1.20^{b}	15.68 ± 0.42^{c}	69.57 ± 1.51^{B}	76.77 ± 0.61^{b}	81.42 ± 1.01^{a}	2.49	$R^2 = 0.7894$
	PSJ-1	10.86 ± 1.01^{ad}	25.44 ± 1.73^{c}	34.96 ± 0.64^{ac}	57.03 ± 0.60^{bc}	76.54 ± 0.62^{c}	78.16 ± 0.62^{ac}	2.33	$R^2 = 0.9697$
PD (shake)	AG-1	10.10 ± 0.39^{d}	21.49 ± 0.83^{d}	35.89 ± 0.83^{b}	47.74 ± 1.07^{d}	76.07 ± 0.61^{b}	78.63 ± 0.22^{bd}	2.16	$R^2 = 0.9694$
	PSJ-1	8.25 ± 1.39^{abcd}	38.91 ± 0.63^{b}	12.19 ± 1.23^{e}	47.97 ± 0.59^{ae}	68.41 ± 1.01^{bdf}	76.54 ± 0.24^{ad}	1.84	$R^2 = 0.7819$
MYPS (static)	AG-1	19.16 ± 1.19^{b}	29.15 ± 0.82^{ac}	40.53 ± 0.23^{a}	51.92 ± 0.70^{acd}	76.54 ± 0.85^{b}	82.58 ± 0.41^{ac}	2.34	$R^2 = 0.9752$
	PSJ-1	26.36 ± 1.26^{c}	10.80 ± 0.79^{ae}	34.50 ± 1.19^{ac}	49.13 ± 0.41^{ad}	73.52 ± 0.41^{ac}	79.79 ± 0.41^{ac}	2.19	$R^2 = 0.8607$
PVC (static)	AG-1	10.34 ± 0.63^{cd}	31.01 ± 1.04^{bc}	43.32 ± 0.62^{a}	56.79 ± 1.06^{c}	71.89 ± 0.61^{c}	84.67 ± 0.40^{c}	3.57	$R^2 = 0.9941$
	PSJ-1	8.48 ± 0.26^{abcd}	17.77 ± 0.42^{d}	31.48 ± 0.61^{ad}	45.64 ± 0.83^{e}	69.80 ± 0.83^{adf}	82.58 ± 0.41^{b}	3.01	$R^2 = 0.9825$
PD (static)	AG-1	13.82 ± 0.59^{bc}	26.60 ± 1.27^{ace}	36.59 ± 0.39^{b}	55.40 ± 1.20^{c}	68.18 ± 1.01^{cd}	78.40 ± 0.39^{bd}	3.04	$R^2 = 0.0772$
	PSJ-1	5.46 ± 0.59^{bd}	28.92 ± 0.82^{bc}	33.10 ± 0.39^{a}	50.52 ± 0.41^{ad}	62.83 ± 0.62^{e}	75.84 ± 0.62^{d}	2.79	$R^2 = 0.9803$
MYPS (static + shake)	AG-1	6.39 ± 0.27^{ae}	23.11 ± 0.64^{de}	34.50 ± 1.44^{b}	54.47 ± 0.84^{ac}	72.59 ± 0.62^{bc}	77.70 ± 0.41^{be}	2.46	$R^2 = 0.9842$
	PSJ-1	4.76 ± 0.60^{b}	13.36 ± 0.23^{de}	29.15 ± 0.62^{d}	56.56 ± 0.61^{c}	71.43 ± 0.70^{abc}	78.40 ± 0.41^{ac}	2.40	$R^2 = 0.9714$
PVC (static + shake)	AG-1	9.87 ± 0.24^{d}	25.20 ± 0.23^{ade}	32.87 ± 0.60^{b}	52.15 ± 0.60^{ac}	64.46 ± 0.41^{a}	80.72 ± 0.23^{ad}	2.91	$R^2 = 0.9922$
	PSJ-1	11.50 ± 0.42^{cd}	26.13 ± 1.19^{c}	37.51 ± 0.63^{bc}	56.33 ± 1.02^{c}	65.85 ± 0.80^{bef}	73.29 ± 0.47^{de}	2.65	$R^2 = 0.9847$
PD (static + shake)	AG-1	9.64 ± 1.00^{ad}	28.46 ± 1.00^{ac}	36.35 ± 0.61^{b}	50.29 ± 0.61^{ad}	59.58 ± 0.80^{e}	77.24 ± 0.84^{b}	2.55	$R^2 = 0.9881$
	PSJ-1	7.32 ± 0.38^{abd}	19.40 ± 0.47^{d}	33.33 ± 0.23^{b}	52.85 ± 0.46^{cd}	67.02 ± 0.82^{bdF}	78.63 ± 0.22^{ac}	2.40	$R^2 = 0.9948$
Control	BHT	88.65 ± 0.30^{a}	88.87 ± 0.04^{a}	89.01 ± 0.06^{a}	89.56 ± 0.25^{a}	89.72 ± 0.14^{a}	90.16 ± 0.06^{a}		

Table 5: Scavenging activity of ABTS.+ radical cation and inhibition concentration at 50 % (IC50) values of mycelial extracts of *C. militaris* two strains grown using nine submerged culture methods that incorporated different media and shaking regimes. Mean values with different upper-case letters within a row are significantly different to each other ($p < 0.05$). Means within the same column of *C. militaris* two strains followed by the same lower-case letters are not significantly different to each other at $p \leq 0.05$ according to Duncan's multiple range tests. Extracts were oven dried n = 5).

		Sample concentration (mg ml^{-1})							
Submerged culture method	Strains	0.50	1.00	2.50	5.00	7.50	10.00	IC50 (µg ml^{-1})	R^2
MYPS (shake)	AG-1	5.92 ± 1.15^{ee}	16.26 ± 1.08^{be}	40.01 ± 2.14^{ac}	13.75 ± 1.60^{d}	65.98 ± 1.47^{b}	66.26 ± 0.81^{a}	3.46	R^2 = 0.7283
	PSJ-1	16.26 ± 1.84^{acd}	41.68 ± 1.14^{b}	45.03 ± 0.68^{ac}	35.54 ± 1.41^{cd}	62.63 ± 0.68^{ab}	63.46 ± 1.83^{c}	3.06	R^2 = 0.7949
PVC (shake)	AG-1	8.16 ± 1.57^{ab}	23.80 ± 2.09^{ab}	34.14 ± 1.46^{a}	45.31 ± 1.20^{b}	57.04 ± 1.67^{a}	73.53 ± 1.58^{cb}	4.56	R^2 = 0.9944
	PSJ-1	8.72 ± 1.39^{bd}	22.97 ± 2.14^{c}	39.45 ± 1.67^{ad}	48.10 ± 1.81^{ad}	61.51 ± 0.93^{ab}	66.26 ± 0.64^{ad}	3.10	R^2 = 0.9927
PD (shake)	AG-1	7.32 ± 1.46^{b}	22.96 ± 1.12^{ab}	34.42 ± 1.19^{a}	58.72 ± 1.57^{ab}	63.75 ± 1.09^{ab}	66.54 ± 0.93^{a}	2.58	R^2 = 0.95
	PSJ-1	4.53 ± 1.14^{b}	22.97 ± 2.37^{c}	41.96 ± 1.20^{acd}	50.90 ± 1.01^{abd}	57.04 ± 1.30^{ab}	65.98 ± 2.40^{ac}	2.87	R^2 = 0.0935
MYPS (static)	AG-1	10.95 ± 1.30^{ab}	22.40 ± 1.86^{b}	52.29 ± 0.93^{b}	37.77 ± 1.03^{bc}	62.85 ± 1.99^{ab}	76.32 ± 1.20^{b}	4.09	R^2 = 0.8763
	PSJ-1	16.82 ± 1.19^{ac}	31.62 ± 1.93a	44.47 ± 1.20^{ac}	58.44 ± 0.77^{b}	65.98 ± 1.60^{b}	71.29 ± 0.90^{ab}	3.22	R^2 = 0.9724
PVC (static)	AG-1	17.38 ± 1.11^{a}	36.37 ± 1.19^{a}	43.08 ± 0.93^{bc}	51.18 ± 1.74^{ab}	65.42 ± 1.67^{b}	76.58 ± 0.60^{b}	4.57	R^2 = 0.9821
	PSJ-1	9.28 ± 1.40^{abcd}	24.64 ± 1.74b^{bc}	35.81 ± 1.65^{d}	45.87 ± 1.47^{d}	65.98 ± 1.47^{b}	73.52 ± 1.82^{b}	4.03	R^2 = 0.9896
PD (static)	AG-1	8.16 ± 0.84^{ab}	18.21 ± 3.74^{bd}	34.14 ± 1.46^{a}	46.71 ± 1.65^{a}	61.51 ± 1.41^{ab}	72.13 ± 1.09^{ab}	3.45	R^2 = 0.9969
	PSJ-1	12.63 ± 0.83^{acd}	25.20 ± 1.19^{c}	48.94 ± 1.37^{bc}	34.98 ± 2.42^{c}	64.31 ± 1.56^{b}	71.29 ± 0.81^{ab}	2.79	R^2 = 0.8668
MYPS (static + shake)	AG-1	6.21 ± 0.80^{bd}	40.84 ± 1.65^{a}	14.86 ± 1.35^{d}	20.17 ± 0.73^{cd}	63.19 ± 0.84^{ab}	72.96 ± 1.11^{cb}	3.87	R^2 = 0.6346
	PSJ-1	15.43 ± 2.88^{acd}	26.88 ± 1.19^{ac}	41.12 ± 0.87^{ac}	53.97 ± 1.29^{abd}	66.26 ± 1.34^{b}	69.61 ± 0.81^{abc}	3.22	R^2 = 0.9706
Liquid (static + shake)	AG-1	6.21 ± 0.32^{bd}	20.73 ± 1.01^{cb}	38.33 ± 1.13^{ac}	59.56 ± 0.73^{a}	67.94 ± 1.67^{b}	74.64 ± 2.23^{ab}	4.49	R^2 = 0.9706
	PSJ-1	9.56 ± 1.13^{abcd}	34.98 ± 1.46^{abc}	60.12 ± 2.61^{b}	48.94 ± 1.59^{ad}	66.26 ± 1.43^{b}	70.11 ± 1.02^{abc}	3.65	R^2 = 0.811
PD (static + shake)	AG-1	10.39 ± 0.56^{b}	37.21 ± 1.07^{a}	42.80 ± 0.73^{c}	49.78 ± 2.12^{b}	63.19 ± 0.93^{ab}	72.96 ± 0.17^{cb}	3.50	R^2 = 0.9449
	PSJ-1	17.10 ± 1.29^{c}	31.62 ± 2.04^{ac}	45.87 ± 1.47^{ac}	57.32 ± 2.20^{b}	66.26 ± 1.57^{b}	71.85 ± 1.25^{ab}	3.42	R^2 = 0.976
Control	BHT	76.00 ± 1.73^{a}	76.00 ± 1.15^{a}	77.33 ± 0.88^{a}	78.33 ± 1.20^{a}	78.67 ± 0.88^{a}	79.33 ± 0.67^{A}		

charides and mycelial growth of the *C. militaris* significantly increased (nearly tripling) following the addition af vegetable oils to the medium (Park et al., 2002).

3.4 SCAVENGING OF HYDROXYL RADICALS

In our study, we found that all mycelial extracts were able to scavenge hydroxyl radicals but that the scavenging activity was affected by concentration (Table 4). At 10 mg ml^{-1}, both AG-1, PSJ-1 strains had the greatest hydroxyl radical scavenging activities compared with other concentrations; values ranged from: 77.70 ± 0.41 to 84.67 ± 0.40 % AG-1 and 73.29 ± 0.47 to 79.79 ± 0.41 % for PSJ-1. In terms of grown of the strain in different shaking regimes, IC_{50} values ranged from AG-1: 2.16-3.57 mg ml^{-1}, PSJ-1: 1.84-3.01 mg ml^{-1}. The results are similar to previous studies, where 76 % of the highest scavenging capacity of hydroxyl radicals was achieved at a concentration of 3.67 g l^{-1} (sample/water). (Shen & Shen, 2001). Also in previous studies, boiling water extracts of cultured and natural *C. sinensis* (Berk.) Sacc. mycelia achieved lower radical scavenging activities (IC_{50}: 0.96 ± 0.06 mg l^{-1} and 1.03 ± 0.03 mg ml^{-1} at concentration of 0.25–2.0 mg ml^{-1}, respectively) (Dong & Yaoa, 2007). In contrast, other studies achieved greater hydroxyl radical scavending ability using hot water extractions of mycelium (37.1 ± 3.8 % to 74.2 ± 4.7 %). (Yuxiang et al., 2006).

3.5 SCAVENGING ACTIVITY OF THE CATION RADICAL $ABTS^{\cdot+}$.

$ABTS^{\cdot+}$ scavenging activity for mycelial extracts from strains AG-1, PSJ-1 increased as concentrations increased (Table 5), and was greatest at the 10 mg ml^{-1} concentration, ranging from 66.54 ± 0.93 to 77.15 ± 0.81 for AG-1 and from 65.98 ± 2.40 to 76.58 ± 0.60 for PSJ-1. The $ABTS^{\cdot+}$ radical scavenging activity was achieved by a mycelial extract from strain AG-1, grown on PVC without shaking (static); overall IC_{50} values for AG-1 ranged from 2.58-4.57 mg ml^{-1}. For PSJ-1 IC_{50} values obtained in the following treatments: MYPS shake (3.06 mg ml^{-1}), PVC shake (3.10 mg ml^{-1}), MYPS static (3.22 mg ml^{-1}), PD static (2.79 mg ml^{-1}), MYPS static+shake (3.22 mg ml^{-1}), PVC static+shake (3.65 mg l^{-1}); and PD static+shake (3.42 mg ml^{-1}), as shown in Table 5. In the studies of others, using different extraction and fermentation methods of *Cordyceps militaris* SN-18 also exhibited excellent $ABTS^{\cdot+}$ radical scavenging activity with an EC_{50} of: 5.25 ± 0.03 mg ml^{-1} at 4 mg ml^{-1} (Yu Xiao et al., 2014). According to Sapan et al. (2015), EPS and IPS extracts from mycelia of *C. cicadae* exhibited strong $ABTS^{\cdot+}$ radical scavenging activity with EC_{50}: 6.38 ± 0.12 mg ml^{-1} (EPS) and 5.23 ± 0.25 mg ml^{-1} (IPS), at a concentration of 8.0 mg ml^{-1}.

3.6 INHIBITION OF LIPID PEROXIDATION

Lipid peroxidation by extracts from strains AG-1, PSJ-1 increased with increasing concentration. A concentration of 10 mg ml^{-1} of strains AG-1, PSJ-1 resulted in mycelial extracts that exhibited the highest antioxidant activity compared with the other concentration, and the results were comparable to butylated hydroxyl toluene used as a positive control (BHT) (Table 6). At 10 mg ml^{-1} there was no significant difference in the inhibition ambition of strains AG-1, PSJ-1 ($p < 0.05$). Lipid peroxidation inhibition activity of the mycelia extract from strain AG-1 grown on the PD medium under 'shake' conditions was significantly stronger than the other extracts with the lowest IC_{50} values (1.88 mg ml^{-1}) (Table 6). Based on estimated IC_{50} from the inhibition assays of various *Cordyceps* strain, concentrations ranged from 0.08 to 5 mg ml^{-1}. These values are in line with the doses of *Cordyceps* prescribed medicinally, which ranges from 1-10 g per dose, (Zhu et al. 1998). For example, using a concentration of 15.0 mg ml^{-1} of *C. militaris* extract, inhibition of lipid peroxidation reached nearly 50 %, at 4.0 mg ml^{-1} (Chun-Lun Wang et al., 2015).

3.7 TOTAL PHENOLIC CONTENT (TPC).

In this study the TPC of mycelial extract from strain AG-1 ranged from 1.75 ± 0.07 to 3.74 ± 0.18 mg g^{-1} (Table 7) and were highest when grown in PVC medium under static conditions (3.74 ± 0.18 mg g^{-1} extract). For AG-1, the TPC was significantly higher in treatments PVC static, MYPS static, PD static, PVC static+shake, MYPS static+shake, PD static+shake, PVC shake, MYPS shake, and PD shake than PD static treatment. For strain PSJ-1 the TPC of mycelial extracts were lower than those from strain AG-1, with values that range from 1.94 ± 0.21 to 3.23 ± 0.10 mg g^{-1}. The results of AG-1, PSJ-1 were similar to some previous reports: 3.91 ± 0.16 mg GAE/g extract (Chun Lun Wang et al., 2015); 3.9 ± 0.2 mg GAE/g extract (Lee et al., 2013). This confirms that antioxidant potential is positively correlated with TPC (Kaur & Kapoor, 2002). The TPC in our study was much higher, and thus incomparable to the results of previous researchers. In one study TPC obtained from methanolic extracts from natural and cultured *C. sinensis* were: 17.07 ± 0.38 mg GAE/g extract while in another they varied from 12.02-12.14 mg GAE/g (Junqiao Wang et al., 2015). Thus, the TPC of extracts from AG-1, PSJ-1

Table 6: Inhibition of lipid peroxidation and inhibition concentration at 50 % (IC50) values of mycelial extracts of *C. militaris* two strains grown using nine submerged culture method that incorporated different media and shaking regimes. Mean values with different upper-case letters within a row are significantly different to each other ($p < 0.05$). Means within the same column of *C. militaris* two strains followed by the same lower-case letters are not significantly different to each other at $p \leq 0.05$ according to Duncan's multiple range tests. Extracts were oven dried (n = 5).

		Sample concentration (mg ml^{-1})							
Submerged culture method	Strains	0.50	1.0	2.50	5.00	7.50	10.00	IC50 (µg ml^{-1})	R^2
MYPS (shake)	AG-1	8.33 ± 1.45^{abc}	14.67 ± 1.76^{cd}	28.00 ± 1.15^{b}	37.67 ± 1.86^{cd}	58.00 ± 1.53^{ab}	66.33 ± 1.20^{ad}	1.79	$R^2 = 0.9827$
	PSJ-1	11.67 ± 1.20^{acd}	22.00 ± 1.15^{a}	35.00 ± 1.00^{ac}	47.33 ± 1.20^{b}	57.33 ± 1.33^{ad}	65.33 ± 1.20^{abc}	1.69	$R^2 = 0.9509$
PVC (shake)	AG-1	13.33 ± 0.88^{ac}	30.67 ± 1.20^{abc}	40.00 ± 1.15^{a}	50.33 ± 0.88^{ac}	61.00 ± 1.53^{b}	70.67 ± 0.67^{ac}	2.02	$R^2 = 0.944$
	PSJ-1	18.00 ± 1.15^{b}	35.33 ± 0.88^{c}	21.67 ± 1.20^{e}	47.67 ± 1.45^{b}	61.00 ± 1.15^{ab}	68.00 ± 0.58^{ab}	1.74	$R^2 = 0.9468$
PD (shake)	AG-1	10.33 ± 0.88^{abc}	37.67 ± 1.45^{b}	37.00 ± 1.00^{a}	57.00 ± 0.58^{b}	60.00 ± 0.58^{b}	62.00 ± 0.58^{d}	1.88	$R^2 = 0.8298$
	PSJ-1	8.33 ± 0.88^{d}	14.00 ± 0.58^{b}	25.33 ± 1.20^{bde}	36.33 ± 2.33^{a}	46.00 ± 1.53^{cd}	58.67 ± 0.67^{c}	1.51	$R^2 = 0.9898$
MYPS (static)	AG-1	12.00 ± 1.15^{ac}	5.33 ± 0.33^{e}	28.00 ± 1.00^{b}	48.00 ± 1.00^{c}	57.33 ± 0.88^{ab}	68.00 ± 1.15^{ac}	2.06	$R^2 = 0.9787$
	PSJ-1	12.33 ± 0.67^{acd}	21.33 ± 0.67^{ae}	39.00 ± 1.00^{ac}	41.33 ± 0.33^{ab}	58.00 ± 1.00^{abd}	66.00 ± 1.53^{abc}	1.67	$R^2 = 0.9319$
PVC (static)	AG-1	14.67 ± 0.88^{c}	28.33 ± 1.67^{ac}	37.00 ± 1.00^{Aa}	49.67 ± 0.33^{ac}	60.00 ± 1.15^{b}	77.67 ± 1.45^{b}	2.14	$R^2 = 0.983$
	PSJ-1	12.33 ± 0.33^{acd}	27.33 ± 1.45^{ad}	40.33 ± 1.45^{c}	47.00 ± 1.00^{b}	58.33 ± 1.20^{abd}	70.33 ± 0.33^{b}	1.83	$R^2 = 0.94$
PD (static)	AG-1	5.80 ± 0.53^{b}	25.40 ± 0.80^{c}	39.67 ± 0.33^{a}	54.33 ± 0.67^{ab}	61.33 ± 0.67^{b}	69.00 ±1.00^{ac}	1.95	$R^2 = 0.8806$
	PSJ-1	9.33 ± 0.67^{cd}	19.00 ± 0.58^{abe}	29.33 ± 0.67^{abd}	42.00 ± 1.15^{ab}	63.33 ± 0.88^{b}	68.67 ± 0.67^{ab}	1.85	$R^2 = 0.9742$
MYPS (static + shake)	AG-1	7.67 ± 1.76^{ab}	33.67 ± 0.33^{ab}	16.33 ± 0.88^{bc}	23.00 ± 1.00^{e}	58.00 ± 0.58^{ab}	68.67 ± 0.88^{ac}	1.84	$R^2 = 0.9232$
	PSJ-1	13.00 ± 1.53^{abc}	21.67 ± 0.88^{ae}	36.00 ± 0.58^{ac}	46.67 ± 1.33^{b}	53.33 ± 0.88^{d}	66.00 ± 2.08^{abc}	1.73	$R^2 = 0.945$
Liquid (static + shake)	AG-1	11.00 ± 0.58^{abc}	27.67 ± 1.33^{c}	41.00 ± 0.58^{a}	53.93 ± 1.03^{ab}	60.67 ± 0.88^{b}	71.33 ± 1.76^{bc}	2.02	$R^2 = 0.8872$
	PSJ-1	12.33 ± 0.33^{acd}	22.00 ± 1.00^{ae}	34.33 ± 0.33^{acd}	44.33 ± 1.45^{ab}	56.33 ± 0.88^{ad}	69.00 ± 3.21^{ab}	1.93	$R^2 = 0.9633$
PD (static + shake)	AG-1	13.00 ± 1.00^{ac}	6.00 ± 0.58^{de}	31.00 ± 0.58^{ab}	50.00 ± 1.15^{ac}	54.00 ± 0.58^{a}	65.33 ± 0.67^{ad}	1.92	$R^2 = 0.9519$
	PSJ-1	14.67 ± 0.33^{ab}	24.33 ± 1.45^{ad}	35.67 ± 1.76^{ac}	42.33 ± 0.33^{ab}	53.33 ± 1.33^{ad}	62.33 ± 0.88^{ac}	1.67	$R^2 = 0.961$
Control	BHT	78.27 ± 0.58^{a}	78.67 ± 0.88^{a}	78.67 ± 0.33^{a}	79.67 ± 0.33^{a}	82 ± 0.58^{a}	83.00 ± 0.58^{a}		

Table 7: The total phenolic contents and total flavonoids contents of mycelial extracts of *C. militaris* grown using nine submerged culture method that incorporated different media and shaking regimes. Mean values with different upper-case letters within a row are significantly different to each other ($p < 0.05$). Means within the same column of *C. militaris* strains AG-1, PSJ-1 followed by the same lower-case letters are not significantly different to each other at $p \leq 0.05$ according to Duncan's multiple range tests. Extracts were all oven dried (40 °C) (n = 5).

	Total phenolic contents (mg ml^{-1})		Total flavonoids content (mg ml^{-1})	
Submerged culture method	AG-1	PSJ-1	AG-1	PSJ-1
MYPS (shake)	1.94 ± 0.10^{bd}	2.02 ± 0.17^{b}	4.91 ± 0.08^{b}	4.86 ± 0.13^{b}
PVC (shake)	3.04 ± 0.41^{ab}	2.37 ± 0.08^{b}	5.55 ± 0.14^{ba}	5.48 ± 0.31^{b}
PD (shake)	1.75 ± 0.07^{d}	1.94 ± 0.21^{b}	4.93 ± 0.28^{ab}	4.80 ± 0.11^{b}
MYPS (static)	2.76 ± 0.10^{abc}	2.37 ± 0.21^{ab}	5.43 ± 0.15^{ba}	5.28 ± 0.11^{b}
PVC (static)	3.74 ± 0.18^{a}	3.23 ± 0.10^{a}	6.01 ± 0.22^{a}	5.70 ± 0.21^{b}
PD (static)	2.72 ± 0.16^{bc}	2.25 ± 0.10^{b}	5.33 ± 0.17^{ab}	5.22 ± 0.18^{b}
MYPS (static + shake)	2.10 ± 0.18^{cd}	1.98 ± 0.20^{b}	4.88 ± 0.22^{b}	4.90 ± 0.13^{b}
PVC (static + shake)	2.33 ± 0.07^{bcd}	2.25 ± 0.14^{b}	5.48 ± 0.28^{ab}	5.05 ± 0.10^{b}
PD (static + shake)	2.02 ± 0.27^{bd}	1.94 ± 0.17^{b}	4.86 ± 0.07^{b}	4.70 ± 0.07^{b}

in this study depended not only on the use of different submerged culture method but also on the mycelial drying methods used.

3.8 TOTAL FLAVONOID CONTENT (TFC).

The TFC of mycelial extracts from AG-1 ranged from 4.86 ± 0.07 to 6.01 ± 0.22 mg g^{-1} (Table 7). Extracts from mycelial grown in PVC medium and static conditions also gave the highest TFC for AG-1 (6.01 ± 0.22 mg g^{-1} extract). For AG-1 TFC was significantly greater in mycelial growm in the PVC static, MYPS static, PD static, PVC static+shake, MYPS static+shake, PD static+shake, PVC shake, MYPS shake, and PD shake than in mycelial grown in the PD static treatment. TFC of PSJ-1 mycelial were lower than those of strain AG-1, and rangerd from 4.70 ± 0.07 to 5.70 ± 0.21 mg g^{-1} (Table 7). In our study TFC was significantly dependent on the strain used and on the nine different submerged culture strategies. Previous work on strains AG-1, PSJ-1 have already shown that TFC is less dependent on drying method than the indicated TFC of *C. militaris* waster medium was 4.26 ± 0.05 mg of QE g^{-1} (distilled water) (Chun Lun Wang et al., 2015), and that while the TPC from the growing medium of *C. militaris* was 3.91 ± 0.16 mg GAE g^{-1} the TFC was higher at 4.26 ± 0.05 mg of QE g^{-1} (Chun Lun Wang et al., 2015). However, other studies have shown that the TFC of mycelial of *C. militaris* grown on fermented of unfermented wheat were: 7.08 ± 0.46 mg l^{-1}; ethanol 80 %: 7.36 ± 0.37 mg l^{-1} (ethanol extracts); and 6.07 ± 0.19 mg l^{-1} (water extract) (Yu Xiao et al., 2014).

4 CONCLUSIONS

The impact of various submerged culture strategies (shaking regime, drying methods, oil supplements) on mycelium growth, EPS and IPS production, and antioxidant activity of *C. militaris* strains AG-1, PSJ-1 in submerged culture are presented. Submerged culture method drying methods and oils played an important role in mycelial biomass growth, EPS production, IPS production, and antioxidant activity of extracts. Submerged culture methods for optimal production of mycelial extracts with high levels of polysaccharides was achieved. The optimal initial pH for mycelial growth and extra-cellular polysaccharide production was 6.7. Of the five different types of oil sources tested, coconut oil, suet, and sunflower oil, all improved the dry mass of mycelia produced and also the production of both EPS and IPS. Specifically, maximum mycelium growth and extra-cellular polysaccharide concentrations were achieved in the PVC media containing 3.5 % coconut oil. Results from various original H_2O_2 radical scavenging activity, $ABTS^{\cdot+}$ radical activity, and lipid peroxidation assays revealed that extracts of mycelial from AG-1, PSJ-1 had significant antioxidant activity, and could represent a potential source of antioxidants of great importance for the treatment of disease. The strong antioxidant activity was related to the total flavonoid content (TFC), and total phenolic content (TPC). Future work on the identification, isolation and structural characteristics of the active components will be the target of our follow-up studies, as these compounds have the potential for use as phyto-therapeutic agents.

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Effect of Tasmanian blue gum (*Eucalyptus globulus* Labill.) leaf extract on cowpea weevil (*Callosobruchus maculatus* [Fabricius, 1775], Coleoptera: Chrysomelidae)

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Effect of Tasmanian blue gum (*Eucalyptus globulus* Labill.) leaf extract on cowpea weevil (*Callosobruchus maculatus* [Fabricius, 1775], Coleoptera: Chrysomelidae)

Abstract: A laboratory study was carried out to examine the efficacy of solvent extract of *Eucalyptus globulus* leaves in reducing post-harvest losses caused by cowpea weevil (*Callosobruchus maculatus*) on cowpea seed variety RSH 256. 450 g of *E. globulus* leaf powder were put in 1000 ml flask and 450 ml of n-hexane was used as the solvent. The extract was tested at dosages of 50, 100 and 150 µl 50 g^{-1} of cowpea grains on the weevil in stored cowpea. The different rates of treatment recorded significant differences ($p < 0.05$) in causing adult mortality compared to the untreated control. The different rates of treatment also recorded significant differences ($p < 0.05$) in emergence of F1 adults of each treatment compared to the control. The percentage masst loss and grain damage were also suppressed as a result of treatment with the plant material compared to the untreated control. However, among the treatments 150 µl 150 g^{-1} cowpea recorded the highest adult mortality rate and lowest emergence while control had the lowest mortality rate and the highest emergence of the insect. The rates of application were indicative of bioactive characteristics of the plant extract.

Key words: cowpea; *Callosobruchus maculatus*; *Eucalyptus globulus*; leaf exctracts; biopesticides

Vpliv listnega izvlečka modrega evkalipta (*Eucalyptus globulus* Labill.) na skladiščnega hrošča *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Chrysomelidae)

Izvleček: Za preučitev učinkovitosti izvlečkov listov modrega evkalipta (*Eucalyptus globulus*) na zmanšanje skladiščnih izgub, ki jih povzroča hrošč *Callosobruchus maculatus* na semenu kitajske vinje ('RSH 256') je bil izveden laboratorijski poskus. 450 g zmletih listov modrega evkalipta je bilo v 1000 ml steklenicah prelitih s 450 ml topila n-heksana. Vpliv izvlečka je bil preiskušen v odmerkih 50, 100 in 150 µl na 50 g semena kitajske vinje, naseljenega s hroščem. Različni odmerki so povzročili značilne razlike ($p < 0,05$) v smrtnosti hrošča v primerjavi s kontrolo. Različni odmerki so povzročili tudi značilne razlike v pojavljanju ($p < 0,05$) odraslih hroščev F1 rodu v primerjavi s kontrolo. Tudi odstotka izgube mase in poškodovanosti zrnja sta se zmanjšala kot posledica nanosa izvlečka listov modrega evkalipta v primerjavi s kontrolo. Med obravnavanji je imela uporaba 150 µl izvlečka listov modrega evkalipta na 150 g semena kitajske vinje največji vpliv na smrtnost škodljivca in zmanjšanje pojavljanja imagov, medtem ko je bila njihova smrtnost v kontroli najmanjša, pojavljanje pa največje. Uporabljeni odmerki izvlečkov dokazujejo bioaktivne učinke modrega evkalipta

Ključne besede: kitajska vinja; *Callosobruchus maculatus*; *Eucalyptus globulus*; listni izvlečki; bioinsekticidi

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1 INTRODUCTION

Cowpea (*Vigna unguiculata* [L.] Walp.) is an edible legume crop in many parts of the world especially in tropical and subtropical regions. Cowpea belongs to the family Fabaceae (Singh, 2002). Cowpea is the most economically important indigenous African legume and is of vital importance to the livelihood of several millions of people in West and Central Africa (Langyntuo et al., 2003).

Cowpea plays a critical role in the lives of millions of people in Africa and other parts of the developing world, where it is a major source of dietary protein that nutritionally complements staple low-protein cereal and tuber crops, and is a valuable and dependable commodity that produces income for farmers and traders (Singh, 2002; Langyintuo et al., 2003).

Cowpea is a drought tolerant and short warm-weather crop well adapted to the drier regions where other food legumes do not perform well. It requires annual rainfall of about 750-1100 mm (Hall et al., 2002; Hall 2004). As much as 1000 kg ha^{-1} of dry grain has been produced in a Sahelian environment with only 181 mm of rainfall and high evaporative demand (Hall and Petal, 1985).

Several diseases, insect pests, nematodes, and parasitic weeds cause significant losses in cowpea yield on field and in storage (Iheanacho et al., 2000). Cowpea is susceptible to a number of fungal, bacterial and viral diseases such as leaf spot, ashy stem blight, bacterial blight, black-eyed cowpea mosaic potyvirus (BICMV), cowpea aphid-borne mosaic potyvirus (CABVC), and cowpea mosaic comovirus (CPMV) (Shaw, 1988). All of these factors, singly or combined are responsible for the low grain yield obtained from the cowpea fields (Singh, 2002). Control by one or two applications of insecticide is invariably necessary. Infestation of insect pest on cowpea for commercial production will lead to downgrading of grain (Monfankye, 2014).

There has however been serious concerns about the long-term negative effect of continued or excessive use of synthetic formulations in insect pest control (Praveen & Dhandapani, 2001). The work therefore aims at evaluating the effect of *Eucalyptus globulus* Labill. leaf extract on cowpea weevil (*Callosobruchus maculatus* [Fabricius,1775]).

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SITE

This study was conducted in the Department of Crop Protection laboratory, and Ir. Leo Vande Mierop Biotechnology laboratory of the University of Ilorin. The extraction process was carried out in the Central Research and Diagnostic Laboratory, Tanke, Ilorin, Nigeria.

2.2 SOURCE AND PREPARATION OF SEEDS

The cowpea seed variety RSH 256 used was obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria. The seeds are white in colour with a maturity period of 60 days. The seeds were wrapped in a polyethylene bag and kept in the freezer compartment of a refrigerator in the Ir. Leo Vande Mierop Biotechnology laboratory, University of Ilorin, to kill immature stages of insects. The seeds were removed 10 days after freezing and then spread on a laboratory desk to thaw.

2.3 INSECT CULTURE

A culture of cowpea weevil, *C. maculatus,* was prepared at laboratory ambient temperature (30 ± 3°C) and relative humidity (68 ± 3%0), respectively. The weevils were collected from already existing stock in the Crop Protection laboratory, University of Ilorin, Nigeria. Fifty (50) unsexed adults of *C. maculatus* were picked with the aid of hair brush to infest cowpea seeds in a transparent plastic container which was covered with muslin cloth held tightly by perforated lid to ensure aeration and prevent escape of the insects. Freshly emerged adults were used for the study.

2.4 APPARATUS AND EQUIPMENT

The apparatus, equipment and materials used include: transparent plastic containers, cowpea seeds, water, n-hexane, measuring cylinder, conical flasks, *Eucalyptus globulus* leaves, muslin cloth, a pair of scissors, a razor blade, paper tape, hair brush, digital weighing balance, spatula, micro-pipette (0-200 µl), Soxhlet apparatus, a plastic sieve, electric blender, rubber band, laboratory desk, refrigerator, foil paper and beakers.

2.5 COLLECTION AND PLANT IDENTIFICATION

Mature leaves of *Eucalyptus globulus* were collected from the parent plant at the University of located in the southern Guinea Savannah agro-ecological zone of Nigeria between latitude 8°25'N and longitude 4°67'E in

the morning at sunrise. The leaves were taken to Plant Biology Department of the University for for proper identification.

2.5.1 Processing of the plant leaves

Fresh mature leaves of *E. globulus* were air-dried, ground with the aid of electric blender, sieved by using a 2 mm sieve to obtain uniform particles. The powder was stored in a vial under room condition until required for use.

2.5.2 Extraction process

Extraction from the *E. globulus* leaf powder was done using the following methods:

Cold extraction, and Soxhlet extraction

Cold extraction:

450 g of *E. globulus* leaf powder were put in 1000 ml flask and 450 ml of n-hexane was used as the solvent. Mixture of the powder and the solvent was shaken thoroughly and allowed to settle for 4 days. The flask was covered with paper sellotape. The extract was separated from the sediment into a 1000 ml beaker using a sieve and then placed on laboratory worktable for excess solvent to evaporate. The concentrated extract was kept in a vial until required for use in evaluating the insecticidal potential of the plant material.

Soxhlet extraction:

150 g of *E. globulus* leaf powder was wrapped in a muslin cloth and tied with rubber band before introducing it into the thimble of a Soxhlet extractor. 300 ml of n-hexane was introduced into a round bottom flask. The electric cooker served as source of heat which lasted for 6-7 h. The excess solvent was allowed to evaporate in order to concentrate the extract. The extract remaining after evaporation was kept in a vial until required for use in the determination of bioactive components of the plant material.

2.5.3 Determination of bioactive components of *E. globulus* leaves using n-hexane extract

Plant material consisted of leaves was put into distillation apparatus using Soxhlet apparatus. Mass of plant material was taken before loading in the Soxhlet apparatus and water is heated so that the steam passes through the plant material vaporizing the volatile compounds. The vapour flows through a coil where they condense back to liquid which is then collected in the receiving vessel.

2.5.4 Experimental procedure

13 g of the sticky extract of *E. globulus* leaves was dissolved in 4 ml of n-hexane. The mixture was stirred with the aid of spatula. Different volumes of the concentration i.e., 50, 100 and 150 µl, were measured using a micro pipette. Aqueous extract of *E. globulus* leaves was added to 50 g cowpea seeds in transparent plastic containers at three levels, 50, 100 and 150 µl using a micro-pipette. Each treatment was replicated three times including the untreated control. Ten freshly emerged adults of *C. maculatus* from the stock culture were added to the treated cowpea seeds in each plastic container and covered with muslin cloth held tightly with rubber band. The effect of the plant extract on the survival of adult *C. maculatus* was assessed by recording adult mortality on daily basis.

2.6 DATA COLLECTION

Data were collected on various parameters including adult mortality, total number of emerged progeny (adults), number and weight of damaged seeds (seeds with holes) and undamaged seeds (seeds without holes) and percentage seed weight loss, and average number of seeds per 50 g in a container. Counts of the emerged weevils commenced 23 days after infestation (DAI) and continued at intervals of 48 h until 100 % adult emergence was recorded.

The percentage seed mass loss was computed following the method of Haines (1991) as follows:

$$\frac{\text{Initial mass} - \text{Final mass}}{\text{Initial mass}} \times 100$$

2.6.1 Data analysis

Data were subjected to analysis of variance using Genstat 12th edition and means were separated using Duncan`s Multiple Range test (DMRT) at 5% level of significance

3 RESULT AND DISCUSSION

The results of the study revealed that the various treatments used in the experiment had effects on mortality increased with increase of dosages of extract.

The *E. globulus* leaf extract caused adult mortality of *C. maculatus* at the high and low rates when compared to the control, which was indicative of bioactive characteristics of the plant part. This is in agreement with the report of Oluma and Garba (2002) that *E. globulus* has potential as source of insecticide for protection of stored grains against attack by *C. maculatus.* They concluded that *E. globulus* extract had toxicity against adults of *C. maculatus.* The extract used in this study could have caused insect mortality due to their physical action on respiration through blockage of the spiracles of the *C. maculatus*. The differences in the adult mortality could be attributed to the active ingredients of *E. globulus* extract. The shortcomings of the use of synthetic chemicals which include development of resistance by insects, adverse effect on non-target species, pollution of the environment including soil, water and air and hazard of residues necessitated the evolution of natural insecticides of plant origin (Deedat, 1994).

The *E. globulus* leaf extracts reduced progeny emergence of *C. maculatus* in treated cowpea seeds (Table 2). This could be attributed to the adult mortality already observed (Table 1) and the inhibition of oviposition as well as the remarkably high reduction in survival to adulthood of mature stages of *C. maculatus* compared to the control. This result corroborates that of Okonkwo and Ewete (1999) in pepper fruit, Ogunwolu and Idowu (1994) in root powders of *Zanthoxylum* spp. and neem seed.

The *E. globulus* leaf extract was also observed to have effects in reducing the damage on cowpea seeds by *C. maculatus* (Table 3). Damage on cowpea seeds may have been reduced as a result of the extracts acting as a deterrent to *C. maculatus*, keeping them from infesting and damaging the seeds. A similar trend of eucalyptus oil activity in preventing grain damaged to cowpea by *C. maculatus* was observed by Longe (2011).

The study reveals that *E. globulus* leaf extract could be very effective for use as biopesticides for protecting cowpea seeds from *C. maculatus* infestation and damage. It has been reported by the pest management specialists that botanicals are not known to leave any residue in any crop they are used to protect and the protective ability of essential oils could be attributed to interspecific insect responses to oil constituents (Enan, 2001).

The use of natural toxicants from plants as insecticides had been existent since the ancient times (Bohinc et al., 2013) and is investigated in recent times (Bohinc et al., 2020) The natural insecticides which require low cost to prepare, are readily available, environmentally and ecologically friendly are best suited for use in the storage of produce.

Table 1: Mean adult mortality of *C. maculatus* exposed to *E. globulus* leaf extract (x n out of 10)

	Adult mortality of *C. maculatus* (DAT)							
E. globulus leaf extract (µl)	1	2	3	4	5	6	7	8
50	1.33^{b}	3.33^{b}	4.33^{b}	6.33^{b}	7.67^{b}	b 8.67^{b}	9.33^{b}	10.00
100	3.00^{a}	5.67^{a}	7.67^{a}	9.33^{a}	9.67^{a}	a 10.00^{a}	10.00^{a}	10.00
150	3.33^{a}	6.00^{a}	8.00^{a}	9.67^{a}	10.00^{a}	10.00^{a}	10.00^{a}	10.00
Control	0.00^{b}	0.00^{b}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	00.00
S.E.M	0.55	0.80	0.94	0.41	0.24	0.33	0.17	Ns

Values with the same superscript in the same column are not significantly different at 5 % level of significance using Duncan`s multiple range test.
DAT: Days after treatment

Table 2: Effect of cowpea seeds treated with *E. globulus* leaf extract on progeny emergence of *Callosobruchus maculatus*

	Progeny emergence of *C. maculatus* (DAT)						
E. globulus leaf extract (µl)	23	25	27	29	31	33	35
50	2.00^{a}	3.00^{ab}	2.33^{a}	3.67^{ab}	4.67^{a}	3.33^{a}	1.00^{a}
100	0.33^{b}	3.00^{ab}	3.33^{a}	2.67^{b}	3.33^{a}	1.33a	0.00^{b}
150	0.33^{b}	1.33^{b}	2.00^{a}	2.00^{b}	2.67^{a}	0.33a	0.00^{b}
Control	2.67^{a}	4.33a	4.33^{a}	6.00^{a}	4.00^{a}	2.00^{a}	0.00^{ab}
S.E.M	0.41	0.62	0.82	0.80	0.76	0.87	0.289

Values with the same superscript in the same column are not significantly different at 5 % level of significance using Duncan`s multiple range test
DAT: Days after treatment

Table 3: Effect of cowpea seeds treated with *Eucalyptus globulus* leaf extract on *Callosobruchus maculatus* activity

	Callosobruchus maculatus infestation				
E. globulus leaf extract (µl)	% Seed mass loss	Mass of damaged seeds	Mass of undamaged seeds	Number of damaged seeds	Number of undamaged seeds
50	18.3^{b}	17.8^{a}	23.1^{ab}	100.3^{ab}	108.3^{a}
100	15.9^{b}	15.8^{a}	26.2^{a}	86.3^{b}	130.0^{a}
150	12.3^{b}	15.8^{a}	22.7^{ab}	77.3^{b}	136.0^{a}
0	31.4^{a}	20.0^{a}	16.0^{b}	131.3^{a}	74.7^{b}
S.E.M	2.52	3.01	2.54	10.01	10.28

Values with the same superscript in the same column are not significantly different at 5 % level of significance using Duncan`s multiple range test.

4 CONCLUSION

Botanical control is said to be the best pest control of stored grain pests because it is biodegradable, environmentally friendly and does not leave toxic residues. Therefore, plant extracts can be another source of insecticides/pesticides against stored grain pests. The result from this research shows that *Eucalyptus globulus* have insecticidal potential for protecting stored cowpea against *C. maculatus. The E. globulus extract prove to be effective on adult mortality. Furthermore, 150* µl was the most effective and this was shown in the suppression of progeny emergence. Thus farmers can use *E. globulus extract in place of expensive synthetic pesticides used against the cowpea weevil.*

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Analiza fizikalnih lastnosti šotnega substrata

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Analiza fizikalnih lastnosti šotnega substrata

Izvleček: Šotni substrat je glavni substrat, ki se v rastlinski pridelavi uporablja predvsem za vzgojo sadik vrtnin in okrasnih rastlin. Šotni substrat ima dobre vodo zadrževalne lastnosti, majhno maso, nizek pH ter nima bolezni in škodljivcev. Predvsem vodo zadrževalne lastnosti so pomembne za optimizacijo namakanja in s tem porabo vode pri gojenju rastlin. Preverili smo vodo zadrževalne lastnosti neuporabljenega in rabljenega šotnega substrata ter različne mešanice substratov (šota z dodatki) in pojav vodoodbojnosti, saj le-ta vpliva na sprejem vode v substrat. Analizirane mešanice neuporabljenega šotnega substrata z dodatki so zadržale med 3,4 % in 18,4 % več vode kot rabljene substratne mešanice. Dnevne izgube vode so pri rabljenih substratnih mešanicah, ob izhodiščno manjši vsebnosti vode ob polnem nasičenju, manjše. Pri neuporabljenem šotnem substratu je bila pri tenziji med 10 in 33 kPa količina vode med 25 in 32 %. Točka venenja (TV) za neuporabljen šotni substrat je med 300 in 1500 kPa oziroma med 15 in 18 %. Šotni substrat ima širok interval poljske kapacitete ter hiter prehod iz območja poljske kapacitete do točke venenja (med 7 in 10 %). Ob sušenju vodoodbojnost tako neuporabljenega kot rabljenega šotnega substrata narašča.

Ključne besede: šota; fizikalne lastnosti substratov; vodoodbojnost; substrat; tenzija; vsebnost vode

Evaluation of soil physical properties of peat substrate

Abstract: Peat substrate is the main substrate for plant production, mainly for the cultivation of vegetable seedlings and ornamental plants. Peat has good water retention properties, low mass, low pH and is free from diseases and pests. The water retention properties are particularly important for optimizing irrigation and thus water consumption in plant production. We investigated the water retention properties of unused and used peat substrate and various mixtures with additives, as well as the occurrence of water repellency, as this influences water absorption into the substrate. Unused peat substrate and different mixtures retained between 3.4 % and 18.4 % more water than the used substrate. The daily water losses are lower for the used substrates that initially contain lower water content at full saturation. At tensions between 10 and 33 kPa, the unused peat substrate contained between 25 % and 32 % water. The wilting point (WP) for unused peat substrate (tension between 300 and 1500 kPa) was between 15 and 18 %. Peat substrate has a wide interval of field capacity and the transition from the field capacity to wilting point is fast (change in water content between 7 % and 10 %). After drying, the water repellency of both unused and used peat substrates increased.

Key words: peat; physical properties of substrates; water repellency; substrate; tension; water content

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1 UVOD

Substrat, kot je šota, se v rastlinski pridelavi pogosto uporablja zaradi dobrih vodo zadrževalnih lastnosti, strukturne stabilnosti, nizkega pH (enostavno prilagajanje potrebam rastlin), odsotnosti patogenov, plevelov in majhne mikrobne aktivnosti (Raviv in Lieth, 1979). Fizikalno kemijske lastnosti, kot so nasipna gostota, vodo zadrževalne lastnosti, pH, kationska izmenjalna kapaciteta in vsebnost hranil, nihajo med substrati, zato imajo lahko občuten vpliv na rast in razvoj rastlin ter njihovo preskrbo s hranili in vodo (Grover in Baldock, 2013). Posledično vsak substrat ni primeren generalno za vse tehnologije vzgoje rastlin v hortikulturi.

Šota je eden izmed najbolj razširjenih substratov na svetu (77 % do 80 % substratov za rastlinsko pridelavo vsebuje šoto) (Handrek in Black, 2002). Šota nastane z delno razgradnjo šotnih in ostalih mahov ter drugih močvirskih rastlin, predvsem trav, šašev, ločkov in drugih vrst, ki rastejo v močvirnatih tleh, kjer je nizek pH in so anaerobne razmere (ni prisotnosti kisika) (Grover in Baldock, 2013). Glede na stopnjo razgradnje šote in vodne razmere, v katerih nastane, ločimo svetlo in temno šoto. Na območjih, kjer je debel organski horizont in plitva mineralna plast, nastaja svetla ali bela šota (do 30 cm globine), medtem ko temna šota nastaja od 30 cm do 200 cm globine. Z globino se spreminjajo fizikalne in kemijske lastnosti šote. Temna šota ima v primerjavi z belo šoto večjo poroznost in večjo zadrževalno sposobnost vode (zadrži 15-kratno količino vode svoje mase, svetle le 4 do 5-kratno količino). Svetla šota izgubi ob ponovni navlažitvi med 70 do 90 % volumna, temna šota pa med 10 in 25 % volumna. Slaba stran uporabe šote je, da se zaradi pridobivanja uničuje močvirske ekosisteme. Obnavljanje šotišč je prepočasno za naše potrebe, saj 1 meter šote nastaja 1000 let. Iz šotišč se sprosti ogromno ujetega CO_2, kar pospešuje klimatske spremembe (Schwärzel in sod., 2002).

Šota ima majhno vsebnost hranil, nizek pH , majhno volumsko gostoto in dobro zračnost. Zaradi teh lastnosti je kot rastni substrat primerna za gojenje mnogih rastlinskih vrst. Šota je neobnovljiv vir, tako se v zadnjih desetletjih iščemo primerne nadomestke, kot so npr. kamena volna, vermikulit, perlit, kokosova vlakna, lesna vlakna. Z novimi substrati in mešanicami želimo najti substrate brez ali z zelo majhno vodoodbojnostjo, boljšimi vodo zadrževalnimi lastnostmi in katerih raba ima majhen ekološkega vpliv (Giancarlo, 2015).

Fizikalne lastnosti substratov (šote) so pomembne pri namakanju rastlin v kmetijski pridelavi, zato smo analizirali fizikalne lastnosti šote in šotnih mešanic, z namenom določitve obrokov namakanja ter boljšega razumevanja obnašanja substrata v procesu osuševanja. Nadalje so nas zanimale morebitne spremembe fizikalnih lastnosti ob ponovni uporabi šotnega substrata in mešanic.

2 MATERIALI IN METODE

2.1 ZASNOVA POSKUSA

Analizirali smo lastnosti neuporabljenega in uporabljenega šotnega substrata (Klasmann N3), ki je sestavljen iz severno nemške črne šote in baltske bele šote. Namenjen je gojenju sadik tako v zelenjadarstvu kot v okrasnem vrtnarstvu (Humko, 2020). Za analizo neuporabljenega šotnega substrata smo odprli novo vrečo šotnega substrata. Za uporabljen šotni substrat smo uporabili šotni substrat iz lončnega poskusa s čiliji (Zamljen in sod., 2020. Uporabljen šotni substrat smo otresli iz koreninskega sistema rastlin in ga presejali skozi 4mm sito, da je bilo v njem čim manj rastlinskih ostankov (korenin). Vse vzorce smo pospravili v papirnate vrečke. Izenačen neuporabljen in uporabljen šotni substrat z enako vlažnostjo smo zatehtali v plastične lončke (Φ 12 cm) s prostornino 0,571 litra. Poleg neuporabljenega in uporabljenega šotnega substrata smo za analizo pripravili tudi mešanice šotnega substrata in različnih dodatkov (kamena volna, vermikulit in agrogel). Kombinacije substrata in dodatkov so bile (i) čista šota (kontrola), (ii) šota položena na 2 cm disk kamene volne, (iii) mešanica 80 volumskih odstotkov (%) šote in 20 % kameno volnatih kosmov, (iv) mešanica 80 % šote in 20% vermikulita ter (v) šota z dodanim agrogelom (0,25 %). Mešanice substratov so bile tehtane v plastičnih lončkih (φ = 12 cm) s prostornino 0,571 litra. Mešanice substratov smo napolnili v petih ponovitvah, jih navlažili do nasičenja in stehtali. Spremljali smo, kako hitro voda odteče ali izhlapi iz substrata. Lonce s substrati (neuporabljen in uporabljen) smo sušili na konstantni temperaturi (25 °C) do konstante mase. Vsak substrat se je dvakrat dnevno stehtalo. Iz podatkov smo izračunali maso ob polnem nasičenju in povprečno dnevno izgubo vode iz substrata ob postopnem izsuševanju.

2.2 LASTNOSTI ŠOTNIH SUBSTRATOV IN DODATKOV

Kamena volna se v hortikulturi uporablja v obliki plošč ali kock. Na tržišču je dostopna tudi v obliki kosmov, ki se uporabljajo predvsem kot dodatek substra-

tom. Naredijo jo iz mešanice treh kamnin (60 % diabaz, 20 % apnenec in 20 % koks), ki jo raztopijo pri 1600 °C (Raviv in Lieth, 2008). Pri ohlajanju se tvorijo tanka vlakna. Kamena volna je lahek material, z majhno gostoto 0,05–0,1 g cm^{-3}. Poroznost kamene volne je med 92 % in 98 % (Smith, 1987, 1998; Kipp in sod., 2000). Velika dostopnost vode pri majhnih tenzijah (pod 5 kPa) omogoča rastlini dobro preskrbljenost z vodo (Kipp in sod., 2000). Sposobnost zadrževanja vode pri kameni volni je specifična, saj se vsebnost vode naglo zmanjša, že pri tenzijah nad 5 kPa. Značilno za plošče iz kamene volne je izjemno omočeno dno (le 4 % zraka) in suho površje plošče (Smith, 1987).

Vermikulit je naravni mineral, vsebuje vodo med plastmi. Substrat vermikulit se segreje na 1000 °C, da se voda upari in poveča delež por, kar daje substratu značilno obliko, majhno maso ter veliko poroznost. Gostota delcev je 0,9 g cm^{-3} in nasipno gostoto med 0,07 in 1 g cm^{-3}. Dobro zadržuje vlago (do 70 % lastnega volumna). Pogosto se ga uporablja kot dodatek drugim substratom. Vermikulit se proizvaja v različnih velikostih delcev, najpogosteje v velikostih 0–2 mm, 2–4 mm in 4–8 mm. Občutno izboljša zračno vodni režim v substratu. Ker gre za kamnino, je okoljsko sprejemljiv. Ima nevtralen pH med 7,0 in 7,5. Zelo lahko se drobi (občutljiv na stiskanje), zato ni primeren za večkratno uporabo. Odlično se meša z ostalimi substrati in je pogosto uporabljen v vrtnarski pridelavi sadik (Raviv in Lieth, 2008).

Agrogel je brezbarven polimer, ki nase lahko veže več 100-kratno količino vode, zato je primeren za uporabo v več panogah kmetijstva. Je dobro obstojen in sposoben večkratnega navlaženja in sušenja (Raviv in Lieth, 2008).

2.3 DOLOČITEV VODO ZADRŽEVALNIH LASTNOSTI ŠOTE

Za določitev vodo zadrževalnih lastnosti šote smo uporabili tri metode: 1) Richardovo tlačno komoro, 2) neposredno merjenje vodnega potenciala v substratu s tenziometrom in 3) metodo izhlapevanja.

2.3.1 Richardova tlačna komora

Vzorec substrata smo položili v tlačno posodo sestavljeno iz debelega kovinskega ovoja, v kateri nastavimo želen nadtlak (International …, 2019). Vzorci so bili položeni na keramično membrano, oziroma ploščo, skozi katero lahko prehaja tekočina. Odvečna tekočina je iztekla iz posode skozi posebno cevko iz nerjavečega jekla. Vzorec vsebuje samo vodo, ki je vezana pri dotičnem tlaku, ko iz cevke ne izteka več voda (Richards, 1941). Vzorce substrata smo zatehtali v plastične obročke, položene na z vodo nasičeno keramično ploščo. Plošče smo prelili z vodo in jih pustili 24 do 48 ur tako, da so bili vzorci substrata popolnoma nasičeni z vodo. Vzorce smo vstavili v tlačno komoro, nastavili nadtlak in počakali, da se je vzpostavilo ravnovesje med nadtlakom in silo, s katero je voda vezana na substrat. Nadtlaki so bili 0,02; 0,1; 0,33; 1; 3; 5 ter 15 barov. Vzorce smo stehtali, sušili 48 ur na 55 °C in ponovno stehtali. Iz razlike v masi vzorcev pred in po sušenju smo izračunali, koliko vode je vzorec zadržal pri določenem nadtlaku.

2.3.2 Merjenje vodnega potenciala s tenziometrom

Vodni potencial (Ψ) ali tenzijo šotnega substrata smo merili neposredno s tenziometri (Soilmoisture Equipment, Santa Barbara, US, območje delovanja od 0 do 100 kPa, z natančnostjo 1 kPa) (Vaz in sod., 2013; Montesano in sod., 2015) in prikazuje silo vezave vode v porah substrata, oziroma kakšno silo rastlina potrebuje, da lahko vodo absorbira iz substrata (Schindler in sod., 2015). Večje tenzije pomenijo rastlinam težje dostopno vodo, saj je vezana z večjimi tlaki. Hkrati smo beležili tenzijo s tenziometrom in maso lonca, ki smo ju sušili v pečici na 25 °C na 12 ur. Iz podatkov smo izrisali krivuljo odvisnosti vsebnosti vode od tenzije.

2.3.3 Metoda izhlapevanja

Vodo zadrževalne lastnosti substrata smo določili z metodo izhlapevanja (Schindler in sod., 2010, Bezerra-Coelho in sod., 2018). Cilindre iz nerjavečega jekla (5 cm visoki in 8 cm široki) smo napolnili z vzorci šote z gostoto 1,05 g cm^{-3}. Vzorce smo nasičili z vodo tako, da smo jih postavili na z vodo navlaženo posodo. V vzorce smo vstavili tenziometre v središče vzorca. Površina šote je bila izpostavljena naravnemu izhlapevanju. Spremljali smo maso in tenzijo vsakih 10 minut, nekaj zaporednih dni. Hidravlični gradient je bil izračunan na podlagi tenzij izmerjenih med meritvami. Posamezne točke na krivulji vodo zadrževalnih lastnosti so bile izračunane na podlagi izgube vode na volumen vzorca (Schindler in sod., 2015). Po tej metodi nenasičeno prevodnost substrata določimo z merjenjem vodne tenzije s pomočjo majhnih tenziometrov in programa Hyprop fit (Schindler in sod. 2010), ter uporabo pretvorbene funkcije tal (pedotransfer function). Model (HYPROP-FIT software [Meter/UMS

GmbH, Germany]), po katerem smo izračunali krivuljo vodo zadrževalnih lastnosti, je bil model po Brooks in Corey (1964).

2.4 VODOODBOJNOST

Za preizkus vodoodbojnostnih lastnosti šotnega substrata smo uporabili neuporabljen šotni substrat in metodo WDPT (Water Drop Penetration Time) (Letey, 1969; Leelamanie in sod., 2008; Saldanha Vogelmann in sod., 2015). Metoda WDPT je enostavna, saj se jo lahko izvede na terenu ali v laboratoriju in se jo lahko izvede na porušenih ali neporušenih vzorcih. Prednost metode je v tem, da vzorcev ni potrebno predhodno obdelati, s čimer bi spremenili lastnosti površine vzorca (Ritsema in Dekker, 1994). Na vzorec substrata ali tal kanemo tri kapljice bi-destilirane vode ter merimo čas vpijanja s štoparico. S časom se kontaktni kot med substratom in kapljico zmanjša, kar omogoča kapljici prodor v substrat (Greiffenhagen in sod., 2006). Meritve smo izvajali med sušenjem na vsakih 7 ur. Substrat se je sušil v pečici na 50 °C. Po sušenju smo substrat ohladili v eksikatorju in stehtali maso substrata. Opravili smo pet ponovitev. Količino vode v substratu smo izračunali kot volumske odstotke vode.

2.5 STATISTIČNA OBDELAVA PODATKOV

Povprečja in standardne napake so bili izračunani s pomočjo programa R (Team R.D.C., 2008). Kjer je enosmerna analiza variance (ANOVA) potrdila statistično značilne razlike, smo naredili Tukey-test. Stopnja značilnosti je bila $\alpha \leq 0,05$.

3 REZULTATI Z DISKUSIJO

3.1 MASA SUHEGA IN NEUPORABLJENEGA SUBSTRATA PRED IN PO UPORABI

V Preglednici 1 so podatki za mase neuporabljenega in uporabljenega substrata ob konstantnem sušenju pri 25 °C. Za suhi substrat so podatki iz zaključka poskusa. Neuporabljen šotni substrat ima v povprečju maso 341,9 g. Pri kombinaciji s neuporabljenim substratom imata najmanjšo maso po nasičenju mešanici šota in 2 cm disk kamene volne in mešanica šote in agrogela (303,7 g in 308 g in), največjo maso pa šota z dodanimi 20 % vol kosmi kamene volne (372,7 g). Največja razlika med maso neuporabljenega vlažnega substrata in suhega substrata je pri šoti z 20 vol % kosmov in 20 vol % vermikulita (196,5 g in 173,5 g). Najmanjšo maso doseže suha mešanica šote in 2 cm diska kamene volne (135,8 g).

Pri uporabljenem substratu je največ vode zadržala kombinacija substrata s šoto in kosmi kamene volne (307 g), najmanj pa šota z agrogelom (286,1 g). Dva do trikratna razlika je prisotna pri masi neuporabljenega in uporabljenega suhega substrata. Med uporabljenimi posušenimi substrati ima najmanjšo maso substrat z agrogelom in 2 cm diskom kamene volne (58 g in 58,5 g). Največje razlike med maso uporabljenega vlažnega in suhega substrata je pri 2 cm disku (235,9 g) in kosmih (240,8 g). Razlike v masi med neuporabljenim substratom (suhim) in uporabljenim substratom (suhim) so posledica razgradnje šote med pridelavo rastlin. Zmanjšana masa uporabljenega substrata je posledica zmanjšane poroznosti, predvsem na račun zapolnitve por s koreninami in sesedanja šote ter kamene volne (Raviv in Lieth, 2008).

3.2 IZGUBA VODE

Izgube vode v šoti ter mešanicah šote z dodatki so predstavljeni na Slikah 1A in 1B. Na Sliki 1A so podatki o izhlapevanju vode iz neuporabljenih substratov. Izhodiščno se v neuporabljenem substratu zadrži več vode kot v ponovno uporabljenem, saj neuporabljen substrat pri

Preglednica 1: Povprečne mase (s standardno napako) neuporabljenega in uporabljenega substrata po nasičenju (po zaključku sušenja na 25 °C). Črke od a do d predstavljajo statistično značilne razlike med obravnavanji.
Table 1: Mass (average + standard error) of unused and used substrate after saturation (after drying at 25°C). Letters a to d represent statistically significant differences among treatments.

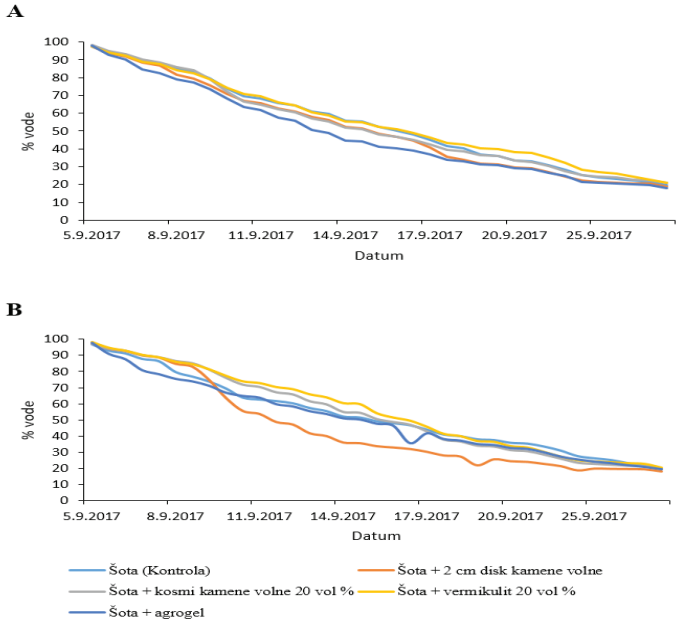
Substrat	Neuporabljen		Uporabljen	
	Vlažen (g)	Suh (g)	Vlažen (g)	Suh (g)
Šota (Kontrola)	341,9 ± 6,5 ab	180,9 ± 2,3 a	289,4 ± 3,1 b	62,4 ± 0,9 bc
Šota + 2 cm disk kamene volne	303,7 ± 5,3 b	135,8 ± 3,9 c	294,4 ± 5,8 ab	58,5 ± 0,8 cd
Šota + kosmi kamene volne 20 vol %	372,7 ± 1,92 a	176,2 ± 1,4 a	307,0 ± 3,8 a	66,2 ± 1,0 ab
Šota + vermikulit 20 vol %	340,9 ± 7,4 ab	167,3 ± 4,7 ab	294,1 ± 3,5 ab	68,1 ± 1,3 a
Šota + agrogel	308,0 ± 7,5 b	152,7 ± 10,4 bc	286,1 ± 1,2 b	58,0 ± 0,4 d

kontroli zadrži 16,2 %, pri 2 cm disku 3,4 %, pri kosmih 18,4 %, pri vermikulitu 12,9 % in agrogelu 7,3 % več vode kot pri uporabljenem substratu v kombinaciji s temi dodatki. Največ vode je zadržala mešanica 80 % neuporabljene šote in 20 % kosmov iz kamene volne (358 ml), najmanj pa šota z dodanim agrogelom (302 ml) in 2 cm diskom kamene volne (298 ml). Kamena volna izboljša zračnost substratnih mešanic in poveča sposobnost zadrževanja vode v substratu. Vermikulit izboljša poroznost substrata in njegovo sposobnost zadrževanja vode (Raviv in Lieth, 2008; Beyl in Trigiano, 2015).

Izgube vode med neuporabljenimi substrati so enakomerne. Uporabljen substrat, ki je bil ponovno navlažen, zadrži manj vode kot neuporabljen substrat. Največ vode zadrži šotni substrat s kosmi kamene volne (298 ml). Najmanj vode lahko zadrži substrat z dodanim agrogelom in klasičen šotni substrat brez dodatkov (oba 280 ml). Zanimivo se obnaša šotni substrat z 2 cm diskom kamene volne, saj v začetku zadržuje vodo podobno kot ostale mešanice, po treh dneh pa se je vsebnost vode strmo zmanjšala, do najnižje točke med vsemi substrati. Največja razhajanja med neuporabljenimi in že uporabljenimi substrati se pojavijo, ko izhlapi ali odteče 24 % vode iz substrata, štiri dni po začetku izsuševanja substrata. Razlike v sposobnosti zadrževanja vode so bolj izrazite pri že uporabljenih substratih, saj dodatki pridejo bolj do izraza (Diara et al., 2012). Iz podatkov lahko razberemo, da je priporočljivo namakanje sadik v rastlinjakih v več manjših obrokih in rednih intervalih (Ismail in sod., 2007; Raviv in Blom, 2001). Če substrate pustimo preveč dolgo nenamakane, se ti izsušijo in izgubijo del sposobnosti zadrževanja vode. Dodajanje kamene volne šoti izboljša poroznost mešanice in s tem poveča kapaciteto za vodo (Kim in Jeong, 2003). Podobne rezultate smo dobili tudi v naši raziskavi, kjer ima šota z dodatkom kosmov kamene volne boljšo sposobnost zadrževanja vode kot šota brez dodane kamene volne.

Povprečna dnevna izguba vode v substratih je predstavljena v preglednici 2. Šotni substrat (kontrola) dnevno izgubi 13,08 g mase (vode) pri neuporabljenem substratu. Uporabljen šotni substrat dnevno izgubi 11,67 ml vode. Največje dnevne izgube vode ima mešanica šote in kamenо volnatih kosmov (14,95 ml/dan), najmanjše pa šota na 2 cm disku kamene volne (12,30 ml/ dan). Kamena volna ima rahlo vezano vodo, ki lahko hitro izhlapi, zato je šota služila kot prepreka pred izhlapevanjem vode, posledično je imela ta kombinacija najmanjše izgube vode.

Med uporabljenimi substrati največ vode izgubi šota na 2 cm disku kamene volne (12,73 ml/dan), najmanj pa šota z vermikulitom (10,89 ml/dan). Pri vseh substratih je uporabljen substrat dnevno izgubil manj vode kot neuporabljen substrat, razen pri šoti na 2 cm disku kamene volne, kjer je uporabljen substrat dnevno izgubil več vode (0,44 ml/dan). Kljub manjši dnevni izgubi vode uporabljenega substrata je imel uporabljen substrat slabše vodo zadrževalne lastnosti, saj je vseh pet substratov zadržalo pod 300 ml vode, medtem ko so neuporabljeni



Slika 1: Izgube vode neuporabljenega šotnega substrata in mešanic (A) in izgube vode uporabljenega šotnega substrata in mešanic (B).
Figure 1: Water losses of unused peat substrate and mixtures (A) and water losses of used peat substrate and mixtures (B)

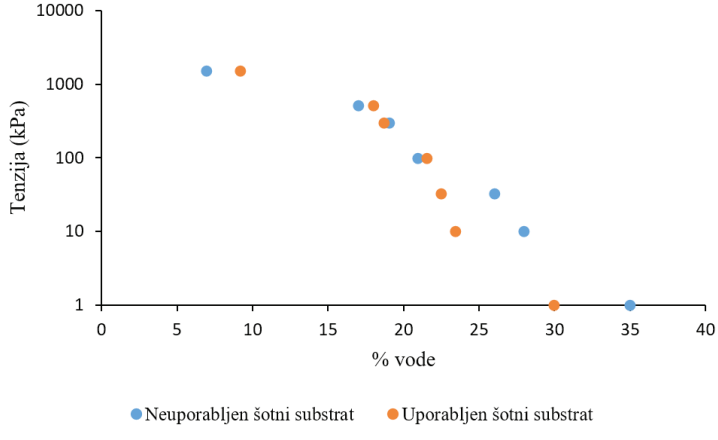
Preglednica 2: Povprečna izguba (s standardno napako) vode v ml na dan pri različnih mešanicah šotnih substratov. Črke od a do c predstavljajo statistično značilne razlike med obravnavanji.
Table 2: Average water loss (with standard error) in ml per day in different mixtures of peat substrates. Letters a to c represent statistical significant differences among treatments.

Substrat	Neuporabljen (ml/dan)	Uporabljen (ml/dan)
Šota (kontrola)	13,08 ± 0,76 bc	11,67 ± 0,06 ab
Šota + 2 cm disk kamene volne	12,30 ± 0,70 c	12,73 ± 0,63 a
Šota + kosmi kamene volne 20 vol %	14,95 ± 0,19 a	11,89 ± 0,22 ab
Šota + vermikulit 20 vol %	13,14 ± 0,79 bc	10,89 ± 0,08 b
Šota + agrogel	13,36 ± 0,81 ab	12,00 ± 0,40 ab

substrati zadržali nad 300 ml vode. Največja razlika pri izgubi vode je bila pri mešanici šote in kameno volnatih kosmov (3,06 ml/dan) med neuporabljenim in uporabljenim substratom. Za najboljšo mešanico se je izkazala kombinacija neuporabljene šote z dodanimi kosmi kamene volne, ki je izkazala največjo sposobnost zadrževanja vode. Kljub največji dnevni izgubi vode od vseh obravnavanih mešanic v enem tednu v povprečju še vedno ohrani največjo količino vode v primerjavi z drugimi substrati. Najslabša mešanica za daljše obdobje nenamakanja je mešanica šote in agrogela, saj ima najmanjšo sposobnost zadrževanja vode ter drugo največjo dnevno izgubo vode. Substratne mešanice s kameno volno imajo večje izgube vode predvsem na račun slabo vezane vode v kameni volni (Donnersa in sod., 2019). Zaradi velike poroznosti vermikulita in s tem dobre kapacitete za vodo, katera je dobro vezana, dnevne izgube zaradi sušenja niso tako očitne, kot pri drugih materialih (Raviv in Lieth, 2008). Podobno se je izkazalo v naši raziskavi, kjer je mešanica šote in vermikulita imela najmanjše dnevne izgube vode.

3.3 VODO ZADRŽEVALNE LASTNOSTI ŠOTE

V analizi smo preverili vodo zadrževalne lastnosti s tremi metodami – dvema laboratorijskima (Richardova tlačna komora, 3.3.1. in metoda izhlapevanja, 3.3.3.) in metodo, ki jo lahko uporabljamo na terenu oziroma rastlinjaku (metoda s tenziometrom, 3.3.2). Prednost tega pristopa je v širini intervala, ki smo ga zajeli, saj smo analizirali vodozadrževalne lastnosti tekom celotnega intervala rastlinam dostopne vode (Richardova tlačna komora, 3.3.1). Hkrati smo upoštevali strukturo, ki se vzpostavi v šotnem substratu, ko ga uporabimo za gojenje v loncih (metoda s tenziometrom, 3.3.2 in metoda izhlapevanja, 3.3.3.). Česar v poskusu nismo preverili, so mikrobiološke lastnosti substratov, katere v tleh lahko vplivajo na oblikovanje koloidnih delcev in s



Slika 2: Razmerje med vsebnostjo vode v šotnem substratu (%), in silo vezave (kPa), izmerjeno z Richardovo tlačno membrano. Vrednosti na y osi so logaritmirane.
Figure 2: Peat water content (%) and peat water tension (kPa), measured with Richard's pressure plate. The values on the y axis are in logarithmic scale.

tem vplivajo na sposobnost zadrževanja vode (Tecon in Or, 2017).

3.3.1 Richardova tlačna komora

Neuporabljen šotni substrat ima večjo kapaciteto za vodo oziroma večji % vode kot uporabljen substrat (Slika 2). Neuporabljen šotni substrat zadrži 5 % več vode pri 1 kPa kot uporabljen šotni substrat. Pri obeh šotnih substratih se delež vode enakomerno zmanjšuje do 100 kPa oziroma enega bara. Med 100 kPa in 300 kPa oziroma med 1 in 3 bari je % vode enak pri obeh substratih. Volumen vode se zmanjšuje pri obeh substratih enako. Pri vrednostih nad 500 kPa uporabljen šotni substrat zadrži večji delež vode kot neuporabljen substrat (za 2,25 % pri 1500 kPa oziroma 15 barih). Med uporabljenim in neuporabljenim šotnim substratom so očitne razlike v količini dostopne vode, uporabljeni šotni substrat ima ožji interval rastlinam dostopne vode (med območjem poljske kapacitete in točko venenja). V uporabljenem šotnem substratu se zmanjša poroznost, kar je lahko posledica starih korenin (Urrestarazu in sod., 2008).

3.3.2 Merjenje vodnega potenciala s tenziometrom

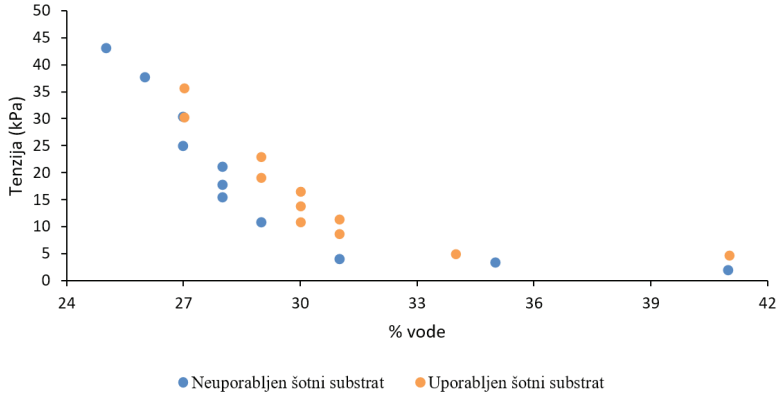
Pri uporabljenem šotnem substratu smo s tenziometrom izmerili večje tenzije ob postopnem sušenju substrata kot pri neuporabljenem substrat (Slika 3). Med neuporabljenim in uporabljenim šotnim substratom se razlike večajo, ko iz substrata izhlapi, odteče ali porabi med 30 in 35 % vode. Pri našem poskusu je bilo to med 500 in 600 ml vode. Bolj, ko se šota suši (večja izguba vode), bolj je vidna razlika med uporabljenim in neuporabljenim substratom.

Pri uporabljenem šotnem substratu je krivulja izgube vode manj strma kot pri neuporabljenem šotnem substratu, kjer krivulja po določeni izgubi vode začne hitro naraščati. Pri tenzijah med 0 in 5 kPa je vsebnost vode v obeh šotnih substratih podobna. Uporabljen šotni substrat zadrži nekoliko več vode kot neuporabljen šotni substrat. Razlike se pojavijo pri tenzijah nad 10 kPa. Za rastlino to pomeni, da je voda lahko dostopna do neke točke, nato pa se začne njena dostopnost hitro zmanjševati, kar lahko povzroči stres za rastline in njihov propad. V praksi to pomeni, da morajo pridelovalci količino vode vzdrževati nad to točko, kar je v primeru analiziranega substrata 10k. Namakanje v rastlinski pridelavi je priporočljivo v območju poljske kapacitete, ki je pri šoti pri tenziji med 10 in 30 kPa (Raviv in Lieth, 1979). V tem območju tenzije se pojavijo velike razlike med uporabljenim in neuporabljenim šotnim substratom, saj neuporabljeni šotni substrat zadrži več vode pri manjši tenziji kot šotni uporabljen substrat.

3.3.3 Metoda izhlapevanja

Pri šotnih substratih je območje poljske kapacitete (PK) med 10 in 33 kPa oziroma med 25 in 32 %. Optimalno območje namakanja za rastline je med 25 in 35 % (Handrek in Black, 2002). Točka venenja (TV) za šotni substrat je med 300 in 1500 kPa oziroma med 18 in 15 %. Točka poljske kapacitete je največja količina vode, ki jo tla lahko zadržijo, točka venenja pa je količina vode v tleh, pri kateri rastline trajno uvenijo in si ne opomorejo, četudi jih zalijemo (Pintar, 2006).

Voda, vezana z manjšo silo od poljske kapacitete (za



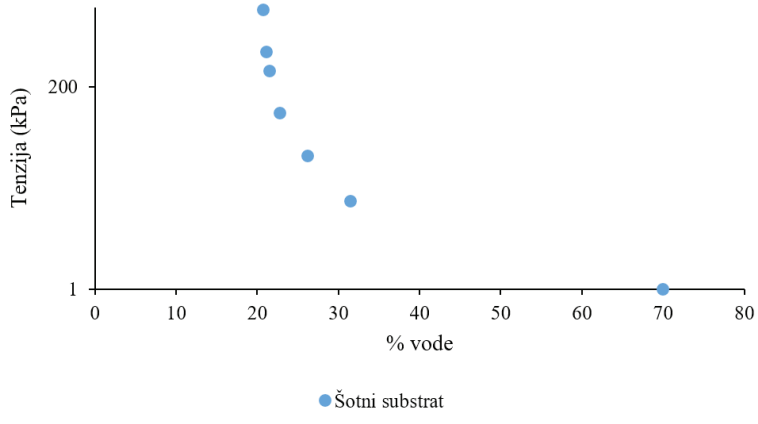
Slika 3: Razmerje med vsebnostjo vode v šotnem substratu (%) in silo vezave (kPa), izmerjeno s tenziometrom.
Figure 3: Peat water content (%) and peat water tension (kPa), measured with tensiometer.

mineralna tla je to med 10 in 33 kPa), prehitro odteče iz substrata in rastlini ni dostopna. Voda, ki je vezana s silo večjo od točke venenja (1500 kPa), je prav tako nedostopna rastlini (Pintar in Zupanc, 2017). Iz Slike 4 lahko razberemo, da je voda v šotnem substratu dobro razpoložljiva do meje 200 kPa, nato hitro postane nedostopna za rastlino, kar vodi v venenje listov zaradi padca turgorja v celicah. Daljše obdobje suše, kjer je dostopnost vode težja za rastlino (> 1500 kPa), lahko pripelje do propada celotne rastline. Pri šotnih substratih je prehod iz območja poljske kapacitete substrata do točke venenja hiter, razlika je 7 do 10 %, kar lahko pridelovalcu povzroči preglavice, če namakanje ni dosledno. V rastlinjakih se lahko zgodi prehod še hitreje, saj so temperature v rastlinjakih poleti lahko mnogo višje. Al-Mahdouri in sod. (2013) poročajo o povprečnih temperaturah zraka med 83 in 93 °C glede na različne kritine ter temperature tal med 104,8 °C in 119,5 °C, če se rastlinjakov ustrezno ne zrači. Z ustreznim namakanjem oziroma režimom namakanja lahko šotni substrat vzdržujemo v območju poljske kapacitete. Priporočljivo je namakanje v več manjših obrokih, saj tako lažje vzdržujemo konstanto količino vode v substratu, saj je voda v območju poljske kapacitete najlažje dostopna za rastlino (Zamljen in sod., 2020). Če namakamo v večjih količinah in manj pogosto, presežemo vodo zadrževalne sposobnosti substrata v loncu in voda odteče iz substrata. Voda, ki odteče, za rastlino ni več dostopna razen, če jo zadržimo s pomočjo podstavkov v primeru gojenja rastlin v posodah. Naši rezultati so pokazali, da lahko z mešanjem šote z drugimi tipi substratov občutno izboljšamo vodo zadrževalne sposobnosti šote.

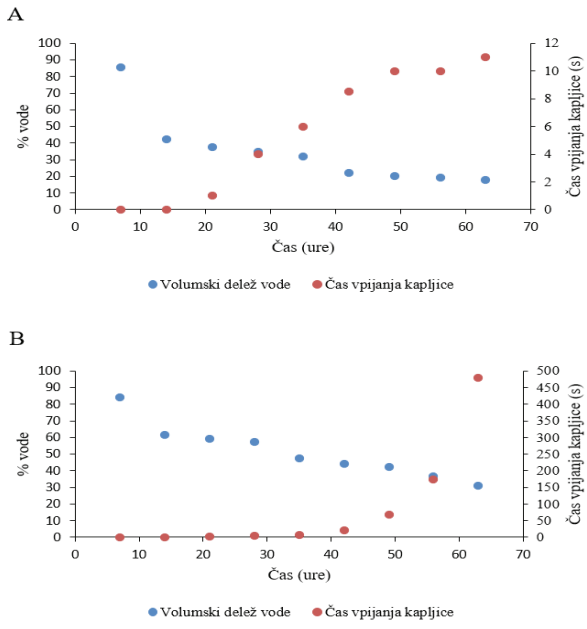
3.4 VODOODBOJNOST

Na Sliki 5 so podatki za čas vpijanja kapljice neuporabljenega šotnega substrata in rabljenega šotnega substrata. Šotni substrat smo sušili do konstantne mase, tri dni, dokler ni bil popolnoma suh. Hkrati smo na šotni substrat dodajali kapljice vode in spremljali, koliko časa potrebujejo, da se vpijejo. Masa šotnega substrata se s časom zmanjšuje na račun izgube vode. Bolj ko je šotni substrat suh, težje vpije vodo oziroma kapljice vode (Raviv in Lieth, 2008). Popolnoma navlažen šotni substrat nima vodoodbojnih lastnosti (čas vpijanja kapljice je nič sekund). Pri osuševanju popolnoma navlaženega neuporabljenega substrata se je pri 42 % čas vpijanja kapljice podaljšal na eno sekundo, pri uporabljenem šotni substratu se je čas vpijanja kapljice podaljšal že pri 59 %. Ko količina vode v šoti doseže kritično vrednost, postane šota hidrofobna, kar pomeni težji sprejem vode in ponovno navlažitev šotni substrata (Perdana in sod., 2018). Podobno se je izkazalo tudi v našem primeru. Čas vpijanja kapljice v šotni substrat začne naraščati, ko je v neuporabljenem šotni substratu med 35 % in 40 % vode. Za vsakih 10 do 20 ml izhlapene vode iz šotnega substrata se čas vpijanja kapljice poveča med 2 in 3 sekunde. Popolnoma suh šotni substrat potrebuje 15 sekund, da vpije kapljico, kar v primerjavi s peščenimi tlemi ni veliko (Oostindie in sod., 2013; Dekker in sod., 2001).

Vodoodbojnost pri rabljenem šotnem substratu začne naraščati pri 59 %. Z zmanjševanjem vsebnosti vode rabljen šotni substrat pridobiva vse večje vodoodbojnostne lastnosti, ki se kažejo kot eksponentna rast. Pri 30 % navlaženosti šotnega substrata je čas vpijanja



Slika 4: Razmerje med vsebnostjo vode v šotnem substratu (%) in silo vezave (kPa), izmerjeno z metodo izhlapevanja. Vrednosti na y osi so logaritmirane.
Figure 4: Peat water content (%) and peat water tension (kPa), measured with evaporation method. The values on the y axis are in logarithmic scale.



Slika 5: Čas vpijanja vodne kapljice (s) glede na količino vode (%) pri neuporabljenem (A) in uporabljenem (B) šotnem substratu tekom meritev (ure).
Figure 5: Water drop penetration time (s) and water content (%) for unused (A) and used (B) peat substrate during the experiment (hrs).

kapljice 480 sekund oziroma 8 minut. Glavni razlog za povečanje hidrofobnih lastnosti rabljenega šotnega substrata je v povečanem deležu organske mase predvsem zaradi ostankov korenin (Wu in sod., 2020). Pri mineralnih tleh (glinenih tleh) se vodoodbojnostne lastnosti začnejo kazati, ko se vsebnost vode zmanjša pod 41 %, med tem ko se pri močno organskih tleh vodoodbojnostne lastnosti kažejo tudi pri popolnoma nasičenih tleh (Rasa in sod., 2007). Razmerje med časom vpijanja kapljice in maso šotnega substrata je obratno sorazmerno. Bolj kot je šotni substrat suh, daljši je čas vpijanja kapljice in obratno, bolj kot je vlažen šotni substrat, krajši je čas vpijanja kapljice. Kapljico izsušena tla lahko vpijejo v manj kot 10 minutah ali pa za vpijanje potrebujejo več kot 6 ur (Oostindie in sod., 2013).

4 SKLEPI

Šota je izjemno uporaben substrat za gojenje vrtnin. Pridelovalec mora rastline dosledno namakati ter vzdrževati konstantno količino vode v substratu. Prehod iz poljske kapacitete (50 do 70 % vode v substratu) v točko venenja (7 do 10 % vode v substratu) je zelo hiter in se lahko zgodi v nekaj urah. Šota ni primerna za daljše intervale namakanja, saj se v primeru izsušitve njene lastnosti spremenijo, pojavi se vodoodbojnost, substrat se skrči, zmanjša se poroznost. Suh šotni substrat je težje ponovno navlažiti. Prav tako uporabljen šotni substrat ne zadrži ob nasičenju enake količine vode kot neuporabljen substrat. Ponovna uporaba šotnega substrata ni priporočljiva, ker ima manjšo kapaciteto za vodo in hitreje pridobi vodoodbojne lastnosti.

Glede na sposobnost zadrževanja vode se je med najboljšimi mešanicami izkazala mešanica šote in 20 % kosmov kamene volne, saj zadrži največ vode. Najslabše se je med mešanicami substratov odrezala šota z diskom kamene volne in šota z dodanim agrogelom. Že rabljen substrat zadrži manj vode kot neuporabljeni substrat ne glede na mešanico. Šota na disku kamene volne izgubi veliko vode po treh dneh.

Šotni substrat je potrebno v rastlinski pridelavi namakati redno in v več manjših obrokih, da preprečimo izsušitev in vzdržujemo območje poljske kapacitete. Med preizkušenimi mešanicami bi bila za pridelovalce zanimiva uporaba 80 % šote in 20 % kosmov kamene volne, saj zadrži največ vode med vsemi preizkušenimi mešanicami. Ponovna uporaba že uporabljenih substratov ni priporočljiva, predvsem zaradi slabših vodo zadrževalnih lastnosti ter fitosanitarne vprašljivosti.

Raziskava dokazuje, da je šotni substrat zelo primeren za gojenje vrtnin ob ustreznem namakanju. Z različnimi dodatki lahko izboljšamo vodo zadrževalne lastnosti šotnega substrata, s čimer lahko zmanjšamo po-

rabo vode pri namakanju ter s tem zmanjšamo pritisk na naravne vire. V prihodnje želimo raziskavo nadgraditi s preizkušanjem mešanic v različnih razmerjih in kombinacijah dodatkov.

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Tradicionalne in molekularne metode za determinacijo ščitkarjev (Aleyrodidae)

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Tradicionalne in molekularne metode za determinacijo ščitkarjev (Aleyrodidae)

Izvleček: Ščitkarji (Aleyrodidae) so vrstno manj pestra skupina žuželk, ki obsega okoli 1500 vrst iz 160 rodov. Zaradi prehranjevanja z rastlinskimi sokovi so pomembni škodljivci kmetijskih in okrasnih rastlinskih vrst, kjer povzročajo neposredno in posredno škodo. Večina vrst izvira iz tropskih območij, pri nas pa so najpogostejši v rastlinjakih. Z vse pogostejšim vnosom ščitkarjev na nova območja in njihovih invazivnim delovanjem, je determinacija teh žuželk pomemben sestavni del varstva rastlin. Identifikacija ščitkarjev v stadiju odraslega osebka je zahtevna in problematična. Za morfološko determinacijo in taksonomsko določitev je primerna le razvojna stopnja puparija, zato so določevalni ključi redki in pomanjkljivi. Oblika puparija je odvisna od gostiteljskih rastlin in abiotskih vplivov, zato sta nabiranje in nadaljnja determinacija pogosto težavni. Razvoj modernih metod determinacije in genetska diagnostika je omogočila natančnejšo determinacijo, preučitev filogenetskih povezav med vrstami ščitkarjev in pripravo načrtov za biotično zatiranje teh škodljivcev. Najpogosteje se v modernem pristopu determinacije ščitkarjev uporabljajo računalniški programi za analizo fotografije, molekularne metode izolacije DNA in sekvenciranje.

Ključne besede: morfološka determinacija; mtCOI; programska oprema proposed sistem; ščitkarji; škodljivci

Traditional and molecular methods for the identification of whitefly (Aleyrodidae) species

Abstract: Whiteflies (Aleyrodidae) is small group of insects (Insecta) that comprises around 1500 species from 160 genera. Whiteflies damage important cultivated and ornamental plants by sucking plants juice. Most of the species are from tropical area, in our region they are the most common in greenhouses. Because worldwide transport, whiteflies become invasive all around the world. The identification of whiteflies species in adult stage is problematic. Morphological differentiation of pupae is one of the better methods for determining identity of species, but it may vary depending on the host plant on which they develop which can lead to misidentifications and erroneous naming of new species. The application of genetic diagnostics under the umbrella of classical taxonomy was imperative for successful development and delivery of the biological control program, phylogenetics and plans for biological control. The most common modern techniques for whiteflies determination are computer programs for photography analysis, molecular methods with DNA isolation and sequencing.

Key words: morphological determination; *mtCOI*; proposed system; whitefly; pests

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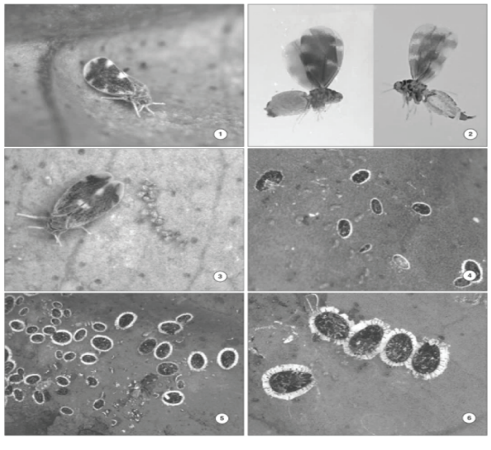
1 UVOD

Ščitkarje (Aleyrodidae) (slika 1) uvrščamo v podred prsokljuncev (Sternorrhyncha), red polkrilcev (Hemiptera) in razred žuželk (Insecta). Družina ščitkarjev (Aleyrodidae) obsega dve poddružini. Poddružina Aleurodicinae je endemična na območju centralne in južne Amerike in je glede na kompleksno zgradbo žilnega sistema kril taksonomsko primitivna. Zapletena zgradba žilnega sistema kril je lahko posledica velikosti predstavnikov poddružine Aleurodicinae (večji od 2 mm), saj so le-ti precej večji od poddružine Aleyrodinae. Poddružina Aleyrodinae je globalno razširjena in ima večje število vrst (Byrne in Bellows Jr., 1991). Do danes je v svetu opisanih več kot 1500 vrst ščitkarjev (Hodges in Evans, 2005), ki jih uvrščamo v 161 rodov. Odrasli osebki so veliki 2–3 mm in spominjajo na manjše molje, saj imajo zaradi voščenega poprha telo bele barve. Okončine so dobro razvite. Na glavi so členjene antene s sedmimi členi in ustni aparat za sesanje in bodenje (Perring in sod., 2018). Nimfe in odrasli osebki imajo pogosto vzorce različnih oblik, ki so strokovnjakom v pomoč pri determinaciji vrst (Botha in sod., 2000).

Ščitkarji se razmnožujejo spolno, le redko tudi partenogenetsko. Samice jajčeca oddajo na površje različnih rastlinskih organov, pogosto na spodnjo stran listov, nekatere samice predstavnikov poddružine Aleurodicinae pa odlagajo jajčeca tudi na plodove in zgornjo ali spodnjo stran listov. Jajčeca odložijo v polpravilnem ali pravilnem krogu, saj so medtem prisesane na stalno mesto. Ličinke prve stopnje so mobilne, gibljejo se na krajše razdalje. Na trajno mesto se prisesajo po prvi levitvi, ki ga do tretje stopnje ne zapustijo. Zadnja stopnja ličink so pupariji; na njihovi morfološki zgradbi je temeljila sistematika celotne družine (Gill, 1990). Ščitkarji so polifagni ali oligofagni. Prehranjujejo se s sesanjem floemskega soka, pri čemer bodalo vstavijo v tkivo rastlin. Zadržujejo se na toplih in vlažnih prostorih. Številne vrste so gospodarsko pomembne (Milevoj, 2003).

Ščitkarji so pomembni škodljivci, saj imajo na kmetijske rastlinske vrste neposredni in posredni vpliv. Med hranjenjem z rastlinskimi sokovi vbrizgavajo v zdrave rastline številne toksine (Watson, 2007), ki povzročijo venenje, slabšo rast in odmiranje rastlin (Botha in sod., 2000). Nimfe povzročijo fiziološke spremembe vegetativnih delov, kot so nepravilno zorenje plodov in sivenje listov (Hoddle, 2004). Nekatere vrste ščitkarjev so tudi vektorji prenosa gospodarsko pomembnih virusov (Trdan, 2015). Medena rosa ščitkarjev je medij gliv sajavosti (*Capnodium* spp.) (Byrne in Bellows Jr., 1991). Največjo gospodarsko škodo in izgubo pridelka povzročata tobakov ščitkar (*Bemisia tabaci* [Gennadius, 1889]) in rastlinjakov ščitkar (*Trialeudores vaporariorum* Westwood, 1856). V Sloveniji se na kapusnicah pogosto pojavlja in posredno povzroča škodo tudi kapusov ščitkar (*Aleyrodes proletella* [Linnaeus, 1758]) (Trdan in sod., 2003). Na območju Evrope ščitkarji povzročajo škodo na zelenjadnicah in okrasnih rastlinah, gojenih v rastlinjakih, kjer lahko povzročijo tudi do 50 % uničenja rastlin. So tudi škodljivci citrusov, različnih drugih sadnih dreves in grmov (Šimala in sod., 2015).

Sistematika poddružin ščitkarjev temelji na morfološki determinaciji četrte stopnje nimfe, imenovane puparij (Martin in sod., 2000). Za puparij je značilna fenotipska plastičnost, kot odgovor na zgradbo lista rastline in abiotskih dejavnikov okolja (Guershon in Gerling,



Slika 1: 1-Ščitkar *Aleurocanthus spiniferus*; 2-levo odrasla samica, desno odrasel samec; 3-ovalna, ledvičasto oblikovana jajčeca; 4-jajčeca in mlade nimfe v 1. in 2. stopnji; 5-odrasle nimfe v 3. in 4. stopnji; 6-pupariji (Radonjić in sod., 2014).
Figure 1: 1-*Aleurocanthus spiniferus*; 2-female left, male right; 3-elongate-oval to kidney-shaped eggs; 4-eggs and young nymphs - 1st and 2nd instars; 5-lder nymphs 3rd and 4th instars and sooty mould; 6-4th instars - puparia (Radonjić in sod., 2014).

2001). Zato je determinacija pogosto otežena. Morfološka determinacija odraslih osebkov je mogoča le za poddružino Aleurodicinae, saj določevalni ključi za ostali poddružini še niso enotni in v uporabi (Ghahari in sod., 2009). Moderne molekularne tehnike omogočajo natančnejšo determinacijo vrst ščitkarjev (Oliveira in sod., 2000; Calvert in sod., 2001; Shatters in sod., 2009). Za filogenetske povezave in determinacijo se uporablja mitohondrijska DNK (mDNK), saj pojasnjuje polimorfizem med bližnjimi vrstami (Brown in sod., 1979; Lunt in sod., 1996). Akumulacija sprememb nukleotidov in polimorfizem vplivata na izgube ali dodajanja restrikcijskih mest, brez bistvene spremembe v velikosti genoma (Hebert in sod., 2003). Spremembe znotraj mitohondrijskega gena citokrom oksidaze I (*COI*), so primeren kazalnik za uporabo DNK barkodiranega sistema determinacije na ravni določanja vrst (Hebert in sod., 2003; Kress in Erickson, 2008). Osnova molekularnega pristopa določanja ščitkarjev so specifični PCR začetni oligonukleotidi, ki se prilegajo *COI* genu, njegovo pomnoževanje in razrez z restrikcijskimi encimi (polimorfizem dolžin restrikcijskih fragmentov) (Vidigal in sod., 2002; Caldeira in sod., 2003; Thyssen in sod., 2005). Znane *COI* sekvence (preko 150 000) so zbrane v GenBank in The Barcode of Life Data System (BOLD) zbirkah ter so temelj za taksonomijo in filogenijo žuželk (Kwong in sod., 2012; Ptaszynska in sod., 2012; Smith in sod., 2012).

2 MORFOLOŠKA IDENTIFIKACIJA

Odrasli osebki ščitkarjev iz družine Aleyrodidae so podobni manjšim moljem, zato so jih v prvih morfoloških študijah mnogokrat zamenjevali s pritlikavimi listnimi zavrtači (Nepticulidae). Jajčeca so na spodnji strani listov gostiteljih rastlin na dolgih ali kratkih pecljih. Površje jajčec je pogosto gladko ali v obliki satovja. Številne vrste ležejo jajčeca v polkrožnih ali krožnih vrstah. Larvalne stopnje so štiri, pogosto je četrta larvalna stopnja puparij. Prva larvalna stopnja ima dobro razvite okončine in antene, je blede do prosojne barve in edina mobilna larvalna stopnja, ki lahko izbere stalno mesto za razvoj nadaljnjih larvalnih stopenj. Pri številnih vrstah je za puparij značilno izločanje voskastih sekretov iz papil ali enostavnih oziroma sestavljenih por. Odrasli osebek zapusti zadnjo larvalno stopnjo na območju zadka iz odprtine v obliki črke T.

Pri mnogih vrstah se odrasli osebki začnejo prehranjevati takoj po izleganju, še pred aktivacijo kril. Oba spola odraslih osebkov imata dva para membranskih kril brez prečnih žil. Spolni dimorfizem se kaže le na območju razlik v genitalijah, številu ventralnih abdominalnih voščenih ploščic, anten in manjšemu telesu samcev. Nekatere vrste imajo več kot en rod letno. Morfološka determinacija poteka s preučevanjem juvenilnih osebkov v različnih stopnjah razvoja pod mikroskopom. Determinacija specifičnih značilnosti je vezana na število, velikost in lokacijo por, odprtin, papil ter set (Perring in sod., 1993).

Identifikacija se začne z zbiranjem osebkov iz listov gostiteljskih rastlin s pomočjo lupe. Liste rastlin je potrebno posušiti in shraniti do njihove preparacije. Identifikacija in klasifikacija zbranih žuželk na ravni vrste se določi na podlagi morfoloških lastnosti odraslih žuželk in ličink, pri čemer so v uporabi klasične identifikacijske metode, kot so morfološki ključi. Ličinke in puparije ščitkarjev se določi z mikroskopiranjem v kanadskem balzamu po prilagojenih metodah Wilkey (1962) ali Watson in Chandler (1999). Standardizirani postopek po Wilkey (1962) je sledeč: vzorce potopimo v 10 % kalijev hidroksid (KOH), kjer jih lahko shranjujemo od 12 do 24 ur. Nato jih vzamemo iz KOH in jih za 10–15 min potopimo v destilirano vodo. V destilirano vodo dodamo eno ali dve kapljici kontrastnega barvila. Vzorce pustimo v raztopini 15 min. Osebke nato vzamemo iz barvila in jih potopimo v 75 % etanol (EtOH) za 10 min, kar razbarva vse nesklerotizirane regije. Osebke iz 75 % EtOH prenesemo v 95 % etanol (EtOH) za 10–15 min. Vzorce potopimo v olje nageljnovih žbic za 30 min ali več. Na objektno stekelce nanesemo kanadski balzam in vanj položimo vzorec, ki ga označimo in pokrijemo z drugim objektnim stekelcem. Vzorce sušimo v sušilni napravi tri tedne pri 35 °C (Hodges in Evans, 2005). Za identifikacijo so v uporabi določevalni ključi Takahashi (1952; 1954), Mound (1966), Habib in Farag (1970), Martin (1985; 1978; 1999), Bink-Moenen in Gerling (1990), Mifsud (1995) in Martin in sod. (2000). Natančna identifikacija se izvaja s stereomikroskopom in svetlobnim mikroskopom.

3 MODERNE TEHNIKE IDENTIFIKACIJE ŠČITKARJEV

3.1 PROGRAMSKA OPREMA PROPOSED SISTEM

Za identifikacijo bolezni in škodljivcev na rastlinah in njihovih škodljivcev so v uporabi številni moderni pristopi zaznavanja, prepoznavanja in taksonomskega uvrščanja posameznih vrst. Z razvojem tehnologije in računalniških sistemov, so se razvili tudi programi za prepoznavanje škodljivcev in obdelavo velike količine podatkov. Računalniški program, imenovan Proposed system, je programska oprema za detekcijo in klasifikacijo bolezenskih znamenj na listih rastlin ali rastlinskih škodljivcev. Shema programske opreme sestoji iz štirih ključnih korakov. Prvi korak je izdelava barvne strukture

Table 1: Vrste ščitkarjev, njihova razširjenost in gostitelji (Martin in sod., 2000)
Table 1: Whiteflies, their extent and hosts (Martin et. al., 2000)

Vrsta	Metoda determinacije	Osnovni ključ	Razširjenost	Gostitelj	Reference
Acaudaleyrodes rachipora (Singh, 1931)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Kreta, Ciper, Egipt, Izrael, Jordanija, Portugalska, Rodos, Španija, Sirija, Turčija	Lesne rastline, občasni škodljivci na citrusih (*Citrus* sp.), granatnem jabolku (Punica granatum L.), gvajava (*Psidium guajava* L.)	Singh, 1931
Aleurocanthus zizyphi (Priesner & Hosny, 1934)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Egipt, Izrael, Jordanija	Lalob (*Balanites aegyptiaca* (L.) Delile), *Terminalia laxiflora* Engl. & Diels, *Phyllanthus mullerianus* Ktze, *Dalbergia* sp., *Detarium microcarpum* Guill. & Perr., kana (*Lawsonia inermis* L.), *Ficus capensis* Forssk., *Ochna afzelii* Hoffm., *Ziziphus spinachristi* L., *Paullinia pinnata* L.	Priesner in Hosny, 1943
Aleurochiton acerinus (Haupt, 1934)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Avstrija, Bolgarija, Češka, Slovaška, Anglija, Francija, Nemčija, Madžarska, Italija, Poljska, Romunija, Sardinija, Sicilija, Slovenija, Hrvaška, Srbija, Črna Gora, Bosna in Hercegovina, Kosovo, Severna Makedonija	Maklen (*Acer campestre* L.)	Haupt, 1934
Aleurochiton aceris (Modeer, 1778)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Avstrija, Bolgarija, Češka, Slovaška, Danska, Anglija, Finska, Francija, Nemčija, Madžarska, Italija, Litva, Nizozemska, Norveška, Poljska, Romunija, Švedska, Švica, Slovenija, Hrvaška, Srbija, Črna Gora, Bosna in Hercegovina, Kosovo, Severna Makedonija	Ostrolistni javor (*Acer platanoides* L.), *Acer tataricum* L.	Modeer, 1966
Aleurochiton pseudoplatani (Visnya, 1936)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Avstrija, Češka, Slovaška, Francija, Nemčija, Madžarska, Italija, Nizozemska, Poljska, Romunija, Sicilija, Švica	Trokrpi javor (*Acer monspessulanum* L.), italijanski javor (*Acer opalus* Mill.), beli javor (*Acer pseudoplatanus* L.)	Visnya, 1936
Aleuroclava similis (Takahashi, 1938)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Avstrija, češka, Slovaška, Finka, Nemčija, Nizozemska, Norveška, Poljska, Švedska	Bodika (*Ilex* spp.), *Pieris japonieum* (Thunb.) D. Don ex G. Don, rododendron (*Rhododendron* sp.), brusnica (*Vaccinium vitis-idaea* L.), *Eurya japonica* Thunb.	Takahashi, 1938
Aleurolobus marlatti (Quaintance, 1903)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Egipt, Jordanija, Malta, Sicilija,	Lesnate rastline	Quaintance, 1914
Aleurolobus olivinus (Silvestri, 1911)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Korzika, Kreta, Ciper, Francija Grčija, Izrael, Italija, Jordan, Majorka, Maroko, Portugalska, Sardinija, Španija, Sicilija, Sirija, Turčija	*Erica arborea* L., oljka (*Olea europaea* L.), ozkolistna zelenika (*Phillyrea angustifolia* L.), širokolistna zelenika (*Phillyrea latifolia* L.)	Silvestri, 1915
Aleurolobus wunni (Ryberg, 1938)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Avstrija, Bolgarija, Češka, Slovaška, Finska, Francija, Nemčija, Madžarska, Italija, Latvija, Litva, Poljska, Romunija, Švedska, Švica, Slovenija, Hrvaška, Srbija, Črna Gora, Severna Makedonija, Kosovo, Bosna in Hercegovina	Navadni kopitnik (*Asarum europaeum* L.), *Linnaea borealis* L., dišeči kovačnik (*Lonicera fragrantissima* Lindl. & Paxton), črno kosteničevje (*Lonicera nigra* L.), tatarsko kosteničevje (*Lonicera tatarica* L.), bisernik (*Symphoricarpos albus* (L.) S. F. Blake), *Symphoricarpos racemosus* Michx., *Phlomis* sp., grozdnata svetilka (*Cimicifuga* sp.) navadni srobot (*Clematis vitalba* L.), *Spiraea* sp.	Ryberg, 1978
Aleurotrachelus globulariae (Goux, 1942)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Francija, Izrael, Maroko	*Globularia alypum* L.	Goux, 1942

Nadaljevanje tabele 1
Continued table 1

Aleurotrachelus rhamnicola (Goux, 1940)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Korzika, Kreta, Francija, Grčija, Italija, Majorka, Malta, Maroko, Portugalska, Sicilija, Španija	Češmin (*Berberis* sp.), navadna jagodičnica (*Arbutus unedo* L.), hrast (*Quercus* sp.), marakuja (*Passiflora edulis* Sims.), navadni srobot, *Rhamnus alaternus* L., *Rhamnus* sp., navadna robida (*Rubus fruticosus* agg. L.), *Ampelopsis* sp.	Goux, 1996
Aleurotuba jelinekii (Frauenfeld, 1867)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Krf, Kreta, Anglija, Francija, Nemčija, Grčija, Italija, Maroko, Portugalska, Sicilija, Španija, Turčija, Slovenija, Hrvaška, Srbija, Črna Gora, Severna Makedonija, Kosovo, Bosna in Hercegovina	Nepravi lovor (*Viburnum tinus* L.), brogovita (*Viburnum* spp.), navadna jagodičnica, vednozeleni gornik (*Arctostaphylos uva-ursi* (L.) Spreng.), mirta (*Myrtus communis* L.)	Frauenfeld, 1867
Aleuroviggianus adanaensis (Bink-Moenen, 1992)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Izrael, Rodos, Sirija, Turčija	*Quercus calliprinos* Webb., hrast prnar (*Quercus coccifera* L.)	Bink Moenen, 1992
Aleuroviggianus graecus (Bink-Moenen, 1992)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Krf, Kreta	Hrast prnar	Bink Moenen, 1992
Aleuroviggianus halperini (Bink-Moenen, 1992)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Izrael, Rodos, Turčija	*Quercus calliprinos*, hrast prnar, *Quercus ithaburensis* Decne.	Bink Moenen, 1992
Aleuroviggianus polymorphus (Bink-Moenen, 1992)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Francija, Maroko, Španija	*Quercus coccifera, Quercus ilex* L., *Quercus rotundifolia* Lam., hrast plutovec (*Quercus suber* L.)	Bink Moenen, 1992
Aleuroviggianus zonalus (Bink-Moenen, 1992)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Albanija, Krf, Kreta, Kos, Rodos, Turčija	Hrast prnar	Bink Moenen, 1992
Aleyrodes asari (Schrank, 1801)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Albanija, Avstrija, Češka, Slovaška, Nemčija, Madžarska, Litva, Poljska, Romunija	Navadni kopitnik	Schrank, 1932
Aleyrodes elevatus (Silvestri, 1934)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Korzika, Francija, Izrael, Italija, Rodos, Sicilija, Španija, Turčija	Enoletni golšec (*Mercurialis annua* L.), figa (*Ficus carica* L.), navadna krišina (*Parietaria officinalis* L.)	Silvestri, 1934
Aleyrodes lonicerae (Walker, 1952)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Avstrija, Korzika, Češka, Slovaška, Danska, Anglija, Finska, Francija, Nemčija, Madžarska, Izrael, Italija, Maroko, Nizozemska, Norveška, Poljska, Romunija, Sicilija, Švedska, Švica, Turčija, Slovenija, Hrvaška, Srbija, Črna Gora, Bosna in Hercegovina, Kosovo, Severna Makedonija	Zelnate in lesne rastline iz družin kovačnikovke (Caprifoliaceae) in rožnice (Rosaceae)	Walker, 1852
Aleyrodes proletella (Linnaeus, 1758)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Vse evropske in mediteranske države	Večina zelnatih rastlin	Linnaeus, 1801
Aleyrodes singularis (Danzig, 1964)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Jordanija, Sirija, Izrael	*Canarina canariensis* (L.) Vatke., *Lactuca serriola* L., navadna škrbinka (*Sonchus oleraceus* (L.) Wall.), *Crambe* sp., mleček (*Euphorbia* spp.)	Danzig, 1964
Asterobemisia carpini (Koch, 1857)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Avstrija, Bolgarija, Češka, Slovaška, Danska, Anglija, Finska, Francija, Nemčija, Grčija, Madžarska, Italija, Nizozemska, Poljska, Romunija, Španija, Švedska, Slovenija, Hrvaška, Srbija, Črna Gora, Kosovo, Bosna in Hercegovina, Severna Makedonija	Drevesne in grmičaste vrste	Koch, 1857

Nadaljevanje tabele 1
Continued table 1

Asterobemisia obenbergeri (Zahradník, 1961)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Albanija, Bolgarija, Češka, Slovaška, Francija, Grčija, Madžarska, Poljska, Slovenija, Hrvaška, Srbija, Črna Gora, Bosna in Hercegovina, Kosovo, Severna Makedonija	Srčastolistna mračica (*Globularia cordifolia* L.), kraški šetraj (*Satureja montana* L.), timijan (*Thymus* spp.)	Zahradnik, 1978
Asterobemisia paveli (Zahradník, 1961)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Češka, Slovaška, Nemčija, Madžarska, Izrael, Romunija, Španija	Mleček (*Euphorbia* spp.), dlakava košeničica (*Genista pilosa* L.), *Daphne gnidium* L.	Zahradnik, 1978
Bemisia afer (Priesner & Hosny 1934)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Korzika, Egipt, Anglija, Francija, Grčija, Izrael, Italija, Malta, Rodos, Sicilija, Španija, Turčija	Polifagi	Priesner in Hosny, 1970
Bemisia tabaci (Gennadius, 1889)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Vse evropske in mediteranske države, večinoma s kontinentalnim podnebjem, predvsem v rastlinjakih	Ekstremno polifagni	Gennadius, 1936
Bulgarialeurodes cotesii (Maskell, 1896)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Bolgarija, Madžarska, Romunija, Turčija, Slovenija, Hrvaška, Srbija, Črna Gora, Bosna in Hercegovina, Kosovo, Severna Makedonija	Damaščanska vrtnica (*Rosa damascena* Mill.), vrtnica (*Rosa* sp.)	Maskell, 1960
Calluneyrodes callunae (Ossiannilsson, 1947)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Češka, Slovaška, Finska, Portugalska, Švedska	Jesenska vresa (*Calluna vulgaris* (L.) Hull.), vresa (*Calluna* sp.), *Erica arborea* L., resa (*Erica* sp.)	Ossiannilsson, 1961
Dialeurodes chittendeni (Laing, 1928)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Belgija, Češka, Slovaška, Danska, Anglija, Finska, Nemčija, Italija, Nizozemska, Švedska, Švica	Rododendron (*Rhododendron* spp.)	Laing, 1928
Dialeurodes citri (Ashmead, 1885)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Alžirija, Korzika, Egipt, Francija. Grčija, Izrael, Italija, Libanon, Malta, Maroko. Sardinija, sicilija, Španija. Tunizija. Turčija, Slovenija, Hrvaška, Srbija, Črna Gora, Bosna in Hercegovina, Kosovo, Severna Makedonija	Kritosemenke	Ashmead, 1916
Dialeurodes kirkaldyi (Kotinsky, 1907)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Ciper, Egipt, Izrael, Libanon, Portugalska, Sirija	Lesnate vrste	Kotinsky, 1914
Dialeurodes setiger (Goux, 1939)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Krf, Korzika, Francija, Italija, Maroko, Španija	Nepravi lovor, navadna jagodičnica	Goux, 1999
Dialeurolobus rhamni (Bink-Moenen, 1992)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Izrael, Turčija	Granatno jabolko, *Rhamnus palaestina* L.	Bink Moenen, 1992
Neopealius rubi (Takahashi, 1954)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Bolgarija, Finska, Francija, Madžarska, Poljska, Švedska, Turčija	Večina lesnatih rastlin	Takahashi, 1954
Parabemisia myricae (Kuwana 1927)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Kreta, Ciper, Egipt, Grčija, Izrael, Italija, Sardinija, Sicilija, Španija, Tunizija, Turčija	Večina lesnatih rastlin	Kuwana, 1952
Pealius azaleae (Baker & Moles, 1920)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Belgija, Anglija, Italija, Nizozemska, Škotska	Rododendron (*Rhododendron* spp.)	Baker in Moles, 1920
Pealius quercus (Signoret, 1868)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Finska, Francija, Nemčija, Madžarska, Irska, Litva,Nizozemska, Poljska, Romunija, Škotska, Švedska, Wales	Bukovke (Fagaceae), brezovke (Betulaceae)	Signoret, 1939
Simplaleurodes hemisphaerica (Goux, 1945)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Krf, Korzika, Kreta, Francija, Italija, Maroko, Španija	Zelenika (*Phillyrea* spp.)	Goux, 1945
Siphoninus immaculatus (Heeger, 1856)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Avstrija, Češka, Slovaška, Anglija, Nemčija, Madžarska, Irska, Italija, Švedska, Švica, Wales	Navadni bršljan (*Hedera helix* L.)	Heeger, 1940

Nadaljevanje tabele 1
Continued table 1

Siphoninus phillyreae (Haliday, 1835)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Avstrija, Češka, Slovaška, Anglija, Nemčija, Madžarska, Irska, Italija, Švedska, Švica, Wales	Navadni bršljan	Haliday, 1940
Tetraleurodes bicolor (Bink-Moenen, 1992)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Izrael, Turčija	Mirta	Bink Moenen, 1992
Tetraleurodes hederae (Goux, 1939)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Francija, Italija, Malta, Sicilija	Navadni bršljan	Goux, 1939
Tetraleurodes neemani (Bink-Moenen, 1992)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Ciper, Izrael, Libanon, Rodos, Sirija, Turčija	Nepravi lovor, *Arbutus andrachne* L., navadni lovor (*Laurus nobilis* L.), navadni jedikovec (*Cercis siliquastrum* L.), mirta, *Rhamnus alaternus*, limona (*Citrus limon* (L.) Osbeck), vinska trta (*Vitis* sp.)	Bink Moenen, 1992
Tetralicia ericae (Harrison, 1917)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Avstrija, Korzika, Krf, Kreta, Češka, Slovaška, Danska, Anglija, Francija, Nemčija, Italija, Majorka, Malta, Nizozemska, Portugalska, Škotska, Sicilija, Španija, Švedska, Švica, Wales	Resa (*Erica* spp.)	Harrison, 1917
Tetralicia iberiaca (Bink-Moenen, 1989)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Portugalska, Španija	*Erica arborea*, *Erica lusitanica* Rudolphi	Bink Moenen, 1989
Trialeurodes ericae (Bink-Moenen, 1976)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Korzika, Kreta, Anglija, Francija, Italija, Majorka, Nizozemska, Portugalska, Španija	Resa (*Erica* spp.)	Bink Moenen, 1976
Trialeurodes lauri (Signoret, 1862)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Francija, Grčija, Izrael, Italija, Malta, Sicilija, Turčija, Slovenija, Hrvaška, Srbija, Črna Gora, Bosna in Hercegovina, Kosovo, Severna Makedonija	*Arbutus andrachne*, navadni lovor	Signoret, 1947
Trialeurodes packardi (Morrill, 1903)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Madžarska	Navadni jagodnjak (*Fragaria vesea* L.)	Morrill, 1915
Trialeurodes ricini (Misra, 1924)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Egipt	Kritosemenke	Misra, 1931
Trialeurodes sardiniae (Rapisarda, 1986)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Sardinija	*Erica arborea*	Rapisarda, 1986
Trialeurodes vaporariorum (Westwood, 1856)	Morfološki ključ	Opis in skica; Martin in sod., 2000	Večina evropskih in mediteranskih držav	Ekstremno polifagni	Westwood, 1856

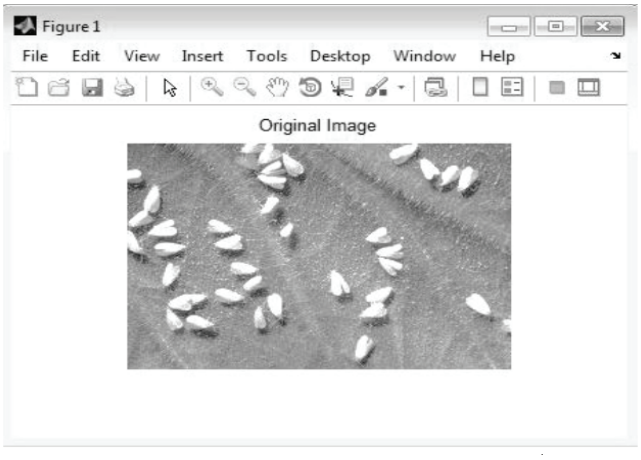
vhodne RGB fotografije, v drugem koraku se ustvarijo zelene slikovne točke v procesu segmentacije fotografije, tretji korak je statistična obdelava uporabnih segmentov fotografije in v četrtem koraku izbrani deli fotografije preidejo v proces klasifikacije. Uspešnost algoritmov pri detekciji rastlinskih bolezni je kar 94 %. Posebna pozornost je namenjena tudi prepoznavanju škodljivcev za namen determinacije in biotičnega varstva rastlin. Metoda temelji na barvnem hologramu, prepoznavanju robov in ekstrakciji pomembnih elementov (Uma Rani in Amsini, 2016).

3.1.1 Predpriprava fotografije za obdelavo

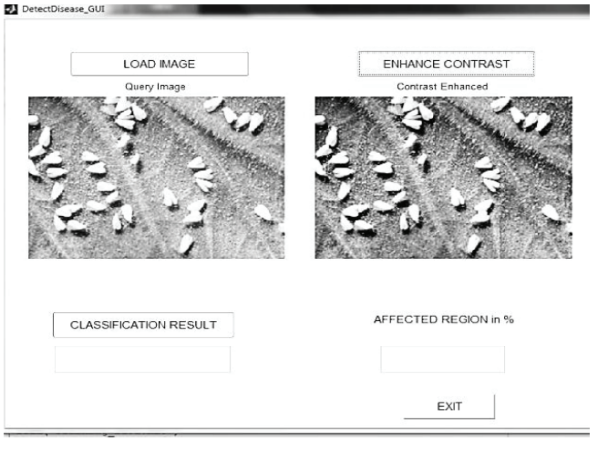
V sklopu priprave fotografije je potrebno posneti fotografijo poškodovanega lista rastline ali škodljivca in jo nato prenesti v program za predpripravo (slika 2), segmentacijo in SVM (angl. support vector machines) klasifikacijo. Raztezanje kontrasta (slika 3) je tehnika, ki izboljša kontrast vhodne fotografije, saj je potrebno pred izvedbo k-algoritma izboljšati kvaliteto fotografije (Uma Rani in Amsini, 2016).

3.1.2 K-vrednost grozdenje

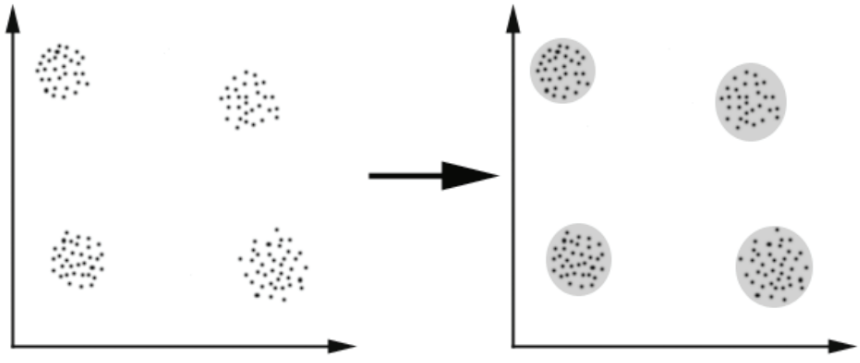
Segmentacija fotografije je klasifikacija območij na fotografiji v posamezne skupine. K-vrednostni algoritem omogoča grozdenje podatkov in segmentiranje primernih objektov od ozadja. K-vrednost grozdenje je metoda vektorske kvantizacije. Grozd je zbirka podobnih objektov, ki se razlikujejo od grozda ostalih objektov. Grozdenje (slika 4) je posledica



Slika 2: Vhodna fotografija škodljivca (Uma Rani in Amsini, 2016).
Figure 2: A sample leaf image with whiteflies (Uma Rani in Amsini, 2016).



Slika 3: Raztezanje kontrasta (Uma Rani in Amsini, 2016)
Figure 3: The leaf image after preprocessing (Uma Rani in Amsini, 2016).



Slika 4: Grozdenje medsebojno podobnih območij (Uma Rani in Amsini, 2016).
Figure 4: Clustering process diagram (Uma Rani in Amsini, 2016).

	Positive(+1)	Negative(-1)
Positive	True positive(TP)	False negative(FN)
Negative	False positive(FP)	True negative(TN)

Slika 5: Binarna klasifikacija pridobljenih podatkov (Uma Rani in Amsini, 2016).
Figure 5: SVM binary classification (Uma Rani in Amsini, 2016).

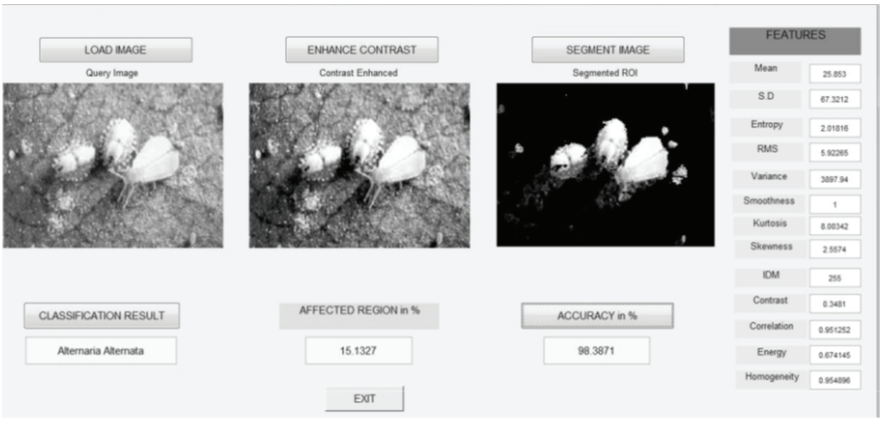
statistične analize, prepoznavanja in obdelave vhodne fotografije ter medsebojno podobnih območij. Zaradi enostavnosti in učinkovitosti je ena izmed prvih tehnik segmentacije fotografij. Za takšno obliko analize je potreben program Mat Lab. Kvaliteta analize se odraža v večjem številu grozdov. V kolikor je število grozdov manjše, je kvaliteta analize vprašljiva (Uma Rani in Amsini, 2016).

3.1.3 SVM klasifikator

SVM (angl. support vector machine) je močno orodje v binarni klasifikaciji, ki hitro generira pridobljene podatke. Regresijske in klasifikacijske analize so osnova za SVM klasifikator. Ocena klasifikatorja temelji na vhodnem območju, ki se generira kot »pravilno« in »nepravilno« (slika 5), kar predstavlja binarno klasifikacijo fotografije (Uma Rani in Amsini, 2016).

3.1.4 Natančnost ocene poškodovanega območja in parametri ocene

Škodljivci, kot so ščitkarji, so manjših velikosti in napadajo liste rastlin. Samice izležejo do 150 jajčec, okoli 25 na dan. Življenjski krog ščitkarjev traja 21–36 dni. Regija poškodovanega in napadenega lista ali škodljivca se tako izračuna s SVM klasifikatorjem. Parametri k-vrednosti klasifikacijske metode so povprečje, standardna deviacija, entropija, RMS, varianca, gladkost podlage, kontrast, korelacija in homogenost vzorca (Uma Rani in Amsini, 2016).



Slika 6: Segmentacija škodljivca (Uma Rani in Amsini, 2016).
Figure 6: Segmentation of leaf pest (Uma Rani in Amsini, 2016).

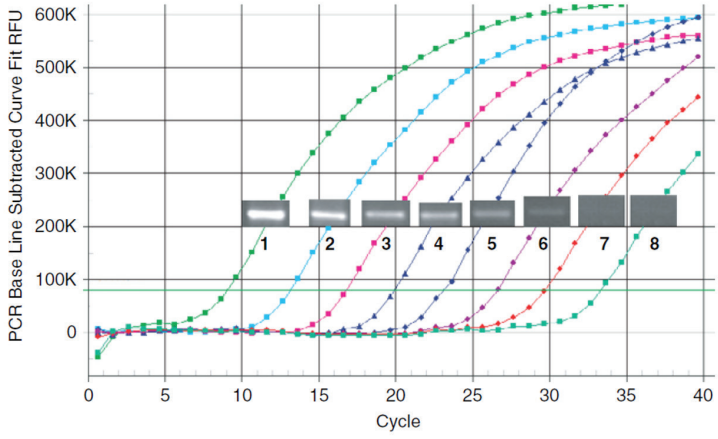
3.1.5 Rezultati

Vhodna fotografija nastane z digitalnim fotoaparatom in je popolnoma neobdelana, zato jo je potrebno pripraviti na statistično analizo s k-povprečjem barv in tehniko segmentacije. Nato lahko s SVM klasifikatorjem natančno izračunamo območje poškodovane regije ali škodljivca (slika 6) (Uma Rani in Amsini, 2016).

3.2 MOLEKULARNA IDENTIFIKACIJA

Zaradi dolgotrajnega in omejenega morfološkega določanja vrst ščitkarjev, kjer je za le-to ustrezna le stopnja puparija, so se razvile številne molekularne metode, kjer vzorec predstavlja odrasla žuželka ščitkarja. Identifikacija odraslih osebkov ščitkarjev je zapletena, pri čemer pa je omejena na gostiteljske rastline določene vrste, kar pogosto privede do zapletov pri prepoznavanju novih vrst. Za določanje vrst ščitkarjev je v uporabi analizna metoda na osnovi mitohondrijske citokrom oksidaze I (*COI*). Gen *COI* je eden izmed trinajstih genov, ki kodirajo proteine znotraj mitohondrijskega genoma. Gradi ga 512 aminokislin, ki so urejene iz konca 5‘ tRNA-Trp-Tyr-Cys in na 3‘ koncu tRNK-Cys (Hajibabaei in sod., 2007). Po sekvenciranju pridobljene rezultate vnesemo v bazo podatkov GenBank, EMBL ali BOLD. Informacije iz posamezne baze so omogočile določitev vrste ščitkarja (Ovalle in sod., 2014). Na osnovi pridobljenih sekvenc in znanih aminokislinskih zaporedij je mogoče izdelati taksonomsko drevo, iz katerega je razvidna filogenetska povezava posameznih vrst ščitkarjev. Izdelano taksonomsko drevo je pogosto v veliko pomoč morfološki determinaciji posamezne vrste (Ovalle in sod., 2014).

Pri nekaterih vrstah ščitkarjev je mogoče določiti tudi posamezne biotipe. Tobakov ščitkar je vrsta ščitkarja, za katero velja izrazita genetska raznolikost, ki pa je ni moč zaznati morfološko, zato je determinacija posameznega biotipa mogoča le z molekularno tehnologijo. Genetski polimorfizem so sprva proučevali na omenjeni vrsti na osnovi vzorcev esteraz (Brown in sod., 1995). Ta metoda ima še vedno veliko vrednost pri določanju biotipov, predvsem zaradi zgodovinskih povezav. Z namenom prepoznavanja posameznih biotipov vrste tobakov ščitkar so v uporabi številni biokemijski in molekularni markerji (Frohlich in sod., 1999), ki omogočajo prepoznavo polimorfizma znotraj vrste. Med številnimi nekodirajočimi in kodirajočimi regijami, ki predstavljajo molekulske markerje, ima *mtCOI* sekvenca največjo variabilnost pri posamezni vrsti, kar doprinese največje število informacij o raznolikosti posameznih biotipov znotraj vrste. Z napredkom molekularne tehnologije je v zadnjem obdobju v uporabi PCR v realnem času, to je implementacija 5‘ nukleaznega fluorogenega testa, znanega kot TaqMan PCR v realnem času. TaqMan analiza temelji na sekvenci specifično označene oligonukleotidne sonde, ki se pripne na tarčno DNK znanih začetnih oligonukleotidov. Metoda TaqMan temelji na hidrolizi sond. Pri podaljševanju verige se sonda odcepi od tarče, poročevalec se loči od dušilca, pri čemer nastane fluorescenca, ki se za vsak cikel prikazuje na zaslonu naprave PCR v realnem času. Fluorescenčni signal je enak količini proučevanega produkta v vzorcu. Prednosti uporabe TaqMan metode in analize PCR v realnem času je izredna občutljivost, natančnost, specifičnost in uporaba splošnih generičnih nastavitev za posamezni cikel. Rezultati so grafični prikaz občutljive detekcije biotipov znotraj vrste tobakov ščitkar (slika 7) (Papayiannis in sod., 2009).



Slika 7: Prikaz občutljivosti metode PCR v realnem času glede na RT-PCR za biotip B vrste tobakov ščitkar (Papayiannis in sod., 2009).
Figure 7: Sensitivity of the qPCR assay for B. tabaci biotype B detection compared with conventional PCR (Papayiannis in sod., 2009).

4 SKLEPI

V primerjavi z listnimi ušmi (Aphidoidea) in celotnim razredom žuželk, so ščitkarji vrstno manj pestra skupina. Eden izmed razlogov je kompleksna in nenatančna morfološka determinacija, pomanjkljivi in poddružinsko specifični določevalni ključi ter problematična zgodovina taksonomije. Pomanjkljiva sistematika in taksonomija temelji predvsem na morfološki determinaciji ščitkarjev le na podlagi značilnosti puparija, ki ima velik potencial filogenetske variabilnosti tudi znotraj vrst. Prav to je vodilo v večkratno opisovanje istih vrst (Martin in Mound, 2007). Zaradi omejene in otežene morfološke determinacije so se razvile številne moderne tehnike determinacije ščitkarjev, med katerimi sta najpogosteje v uporabi programska oprema Proposed system in molekularne analize specifičnih genov ter PCR v realnem času. Računalniško procesiranje fotografije je pomembna tehnika zgodnjega odkrivanja in prepoznavanja škodljivcev na rastlinah in pripomore k nadaljnjim ukrepom varstva rastlin (Uma Rani in Amsini, 2016). *COI* sekvenčna analiza je natančna in učinkovita metoda vrstne identifikacije ščitkarjev. Molekularne metode so pomemben element hitrega in natančnega določanja vrste ter uporabne pri identifikaciji domačih vrst in napovedi invazivnih vrst po vdoru na novo območje. Molekularna diagnostika omogoča identifikacijo intraspecifičnih sprememb znotraj vrste, spremljanje širjenja posamezne vrste, napoved pojava naravnih sovražnikov ščitkarjev in s tem načrtovanje biotičnega zatiranja tega škodljivca (Ovalle in sod., 2014). Primer kriptične vrste je *B. tabaci*, saj bioloških in genetskih variacij te vrste ni mogoče morfološko ločiti med seboj. Pri tem imajo pomembno vlogo molekularne metode, ki omogočajo določitev posameznih markerjev, na podlagi katerih lahko razlikujemo posamezne biotipe in polimorfizem vrste. Med številnimi nekodirajočimi in kodirajočimi markerji, se največja variabilnost kaže znotraj gena *mtCOI*, kar doprinese veliko količino informacij o razlikah znotraj vrste in posameznega biotipa (Papayiannis in sod., 2009). Pomembnost in natančnost molekularnih pristopov se izraža tudi pri določanju seznama karantenskih organizmov. Zaradi zgolj morfološke determinacije, ščitkarji pogosto niso uvrščeni na seznam karantenskih organizmov, saj je glede na določitev na območju zastopana le ena, neinvazivna vrsta. Molekularne metode so pogosto potrdile zastopanost dveh vrst na določenem območju, kjer je bila morfološko določena le ena vrsta, kar je omogočilo prepoznavanje in uvrstitev invazivnih vrst na seznam karantenskih organizmov (Malumphy in sod., 2009).

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Discovery and molecular characterisation of the first ambidensovirus in honey bees

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Discovery and molecular characterisation of the first ambidensovirus in honey bees

Abstract: Honey bees play a critical role in global food production as pollinators of numerous crops. Several stressors cause declines in populations of managed and wild bee species, such as habitat degradation, pesticide exposure and pathogens. Viruses act as key stressors and can infect a wide range of species. The majority of honey bee-infecting viruses are RNA viruses of the Picornavirales order. Although some ssDNA viruses are common in insects, such as densoviruses, they have not yet been found in honey bees. Densoviruses were however found in bumblebees and ants. Here, we show that densoviruses are indeed present in the transcriptome of the eastern honey bee (*Apis cerana*) from southern China. On the basis of non-structural and structural transcripts, we inferred the genome structure of the Apis densovirus. Phylogenetic analysis has shown that this novel Apis densovirus belongs to the *Scindoambidensovirus* genus in the Densovirinae subfamily. Apis densovirus possesses ambisense genome organisation and encodes three non-structural proteins and a split VP (capsid) protein. The availability of a nearly complete Apis densovirus genome may enable the analysis of its potential pathogenic impact on honey bees. Our findings can thus guide further research into the densoviruses in honey bees and bumblebees.

Key words: honey bees; densovirus; genome organisation; molecular characterisation

Odkritje in molekularna karakterizacija prvega ambidensovirusa pri čebelah

Izvleček: Čebele igrajo ključno vlogo v svetovni proizvodnji hrane kot opraševalci številnih poljščin. Številni stresorji povzročajo upad populacij gojenih in divjih vrst čebel, kot so degradacija habitata, izpostavljenost pesticidom in patogeni. Virusi delujejo kot glavni stresorji in lahko okužijo številne vrste. Večina virusov, ki okužijo čebele, so RNA virusi iz reda Picornavirales. Čeprav so nekateri ssDNA virusi pogosti pri žuželkah, na primer densovirusi, jih pri čebelah doslej še niso našli. Densovirusi pa so bili najdeni pri čmrljih in mravljah. Pokazali smo, da so densovirusi prisotni v transkriptomu azijskih čebel (*Apis cerana*) z južne Kitajske. Na osnovi nestrukturnih in strukturnih transkriptov smo ugotovili genomsko strukturo Apis densovirusa. Filogenetska analiza je pokazala, da novi Apis densovirus spada v rod *Scindoambidensovirus* v poddružini Densovirinae. Apis densovirus ima ambisense organizacijo genoma in kodira tri nestrukturne proteine in razcepljeni VP (kapsidni) protein. Dostopnost skoraj celotnega genoma Apis densovirusa bo omogočila analizo njihovega potencialno patogenega vpliva na čebele. Naše ugotovitve lahko privedejo do nadaljnjih raziskav densovirusov pri čebelah in čmrljih.

Ključne besede: čebele; densovirus; organizacija genoma; molekulska karakterizacija

1 INTRODUCTION

Honey bees (*Apis mellifera*) play a critical role in global food production as pollinators of numerous crops (Klein et al., 2007; Fürst et al., 2014). Several stressors cause declining populations of managed and wild bee species such as habitat degradation, pesticide exposure and pathogens (Goulson et al., 2015; Potts et al., 2010; Evans and Schwarz, 2011; McMenamin et al., 2016; McMenamin and Genersch, 2015). Viruses act as key stressors and can infect a wide range of species (Grozinger and Flenniken, 2019). Overt viral infections can result in a

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wide range of symptoms, including wing deformities, discoloration, hair loss, bloated abdomens, trembling, paralysis, and mortality (Chen and Siede, 2007). Honey bee populations have become increasingly susceptible to colony losses due to pathogenic viruses spread by parasitic Varroa mites (Martin et al., 2012).

The majority of honey bee-infecting viruses are RNA viruses of the Picornavirales order (Chen and Siede, 2007; Levitt et al., 2013; Brutscher et al., 2016; McMenamin and Flenniken, 2018; Beaurepaire et al., 2020). Common bee viruses include: the Dicistroviruses (Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV), and Black queen cell virus (BQCV)); the Iflaviruses (Deformed wing virus (DWV), Kakugo virus, *Varroa destructor* virus-1/DWV-B, Sacbrood virus (SBV), and Slow bee paralysis virus (SBPV)); and taxonomically unclassified viruses (Chronic bee paralysis virus (CBPV) and the Lake Sinai viruses (LSVs)) (reviewed in Chen and Siede, 2007 and Brutscher et al., 2016). Recently identified positive sense single-stranded RNA viruses (+ssRNA) viruses include Bee macula-like virus (BeeMLV) in the Tymoviridae family (Galbraith et al., 2018), *Apis mellifera* flavivirus and *Apis mellifera* nora virus 1 (Remnant et al., 2017). *Apis mellifera* rhabdovirus and bunyavirus were recently described (Remnant et al., 2017) and represent first bee-infecting negative sense single-stranded RNA viruses (-ssRNA).

Honey bees are infected by a small number of DNA viruses (Chen and Siede, 2007). Among double-stranded DNA viruses two honey bee-infecting viruses have been found. The *Apis mellifera* filamentous virus (AmFV) is from the Baculoviridae family and has been sequenced and characterized (Gauthier et al., 2015; Hartmann et al., 2015). The *Apis cerana* iridovirus from the Iridoviridae family has not yet been sequenced (Bailey et al., 1976; Bromenshenk et al., 2010; Tokarz et al., 2011). Very recently, a number of single-stranded DNA viruses (ssDNA) associated with *Apis mellifera* have been reported, belonging to circoviruses (Circoviridae) (Galbraith et al., 2018), genomoviruses (Genomoviridae) (Kraberger et al., 2019), CRESS DNA viruses (Cressdnaviricota) (Kraberger et al., 2019) and microviruses (Microviridae) that infect the honey bee bacterial community (Kraberger et al., 2019).

Although some ssDNA viruses are common in insects, such as densoviruses (Parvoviridae) (Cotmore et al., 2014; Pénzes et al., 2020), they have not yet been found in honey bees. Densoviruses were however found in bumblebees and ants (Schoonvaere et al., 2018; Valles et al., 2013). Here, we show that densoviruses are indeed present in the *Apis cerana* transcriptome from southern China. Genome organisation and phylogenetic analysis have shown that this novel Apis densovirus belongs to the *Scindoambidensovirus* genus in the Densovirinae subfamily. It is interesting that the Bombus and Apis densoviruses are not very similar and belong to different densoviral genera. Although the Bombus densovirus is also present endogenised in the *Bombus impatiens* genome, this was not the case for the Apis densovirus. The availability of a nearly complete Apis densovirus genome may enable the analysis of its potential pathogenic impact on honey bees. Our findings can thus guide further research into the densoviruses in honey bees.

2 MATERIALS AND METHODS

2.1 DISCOVERY OF THE APIS AMBIDENSOVIRUS IN PUBLIC TRANSCRIPTOMIC DATABASES

Sequence database searches were finished in July 2020. The protein queries were diverse densoviral NS1 and VP sequences. The database analysed was the Transcriptome Shotgun Assembly (TSA) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Comparisons were made using the TBLASTN program (Gertz et al., 2006), with the E-value cut-off set to 10^{-5} and default settings for other parameters. TBLASTN searching was restricted to different taxa (Protostomia, Hymenoptera, Apoidea and *Apis*). *Apis cerana* transcriptome (erroneously named *Apis mellifera carnica*) contains 52.177 contigs. Apis ambidensovirus sequences were compared to reference protein sequences of all parvoviruses. DNA sequences of the Apis ambidensovirus were translated with the Translate program (web.expasy.org/translate/).

2.2 ANALYSIS OF ENDOGENOUS VIRUS ELEMENTS

Endogenous copies of densoviruses were detected using the TBLASTN algorithm against hymenopteran genomes available in the Whole Genome Shotgun Database (WGS) and Sequence Read Archive (SRA) at the NCBI, using densoviral protein sequences as queries. The queries involved NS1, NS2, NS3 and VP protein sequences. Comparisons were made using the TBLASTN program (Gertz et al., 2006), with the E-value cut-off set to 10^{-5} and default settings for other parameters.

2.3 PREDICTION OF PROTEIN DOMAINS

In order to recognize potential protein domains in the protein sequences analysed, we used NCBI CDD database (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi),

by applying a cut-off E-value of 0.01. All Apis and Bombus densovirus proteins were compared against SMART (smart.embl-heidelberg.de), InterPro (www.ebi.ac.uk/interpro/) and Pfam (pfam.xfam.org) protein domain databases at default parameters.

2.4 PHYLOGENETIC ANALYSIS

To infer the phylogenetic relationships among densoviruses, we used their NS1 protein sequences. Key representatives of the densoviral lineages were included in the phylogenetic analysis. 24 protein sequences of the NS1 were aligned using MAFFT (Katoh and Standley, 2013). Phylogenetic trees were reconstructed using the maximum likelihood (ML) method. For phylogenetic reconstruction, we used IQ-TREE with the in-built automated test to choose the best substitution model for each tree (Trifinopoulos et al., 2016). Branch support was computed for all trees using 100 replicates of parametric bootstrap, and 1000 replicates of the approximate likelihood ratio test and ultrafast bootstrap. The iTOL online tool (http://itol.embl.de/) was used for phylogenetic tree annotation (Letunic and Bork, 2016).

3 RESULTS AND DISCUSSION

3.1 DISCOVERY OF THE ACTIVELY TRANSCRIBING DENSOVIRUS IN THE HONEY BEE TRANSCRIPTOME

Densoviruses are infecting diverse insect lineages (Cotmore et al., 2014; Penzes et al., 2020). Previous studies have found numerous endogenised densoviruses in insect genomes (Liu et al., 2011; Francois et al., 2016). Metatranscriptomic analyses of major invertebrate lineages have enabled the discovery of a very large number of novel RNA viruses (Shi et al., 2016). Recently, the metatranscriptomic analysis of diverse invertebrates has enabled the discovery of novel invertebrate DNA viruses (Porter et al., 2019). This methodology can identify actively transcribing DNA viruses in metatranscriptomic libraries. Here, we used this approach to find novel densoviruses in invertebrate transcriptomes at NCBI TSA database. We used both NS1 and VP proteins of diverse densoviruses as queries. A large number of novel densoviruses can be found in invertebrate transcriptomes; some are partial transcripts, while others represent separate NS and VP transcripts or nearly whole genomes. To our surprise, we found the first honey bee densovirus transcripts, with the size range between 1.9 and 2.6 Kb. These transcripts correspond either to the non-structural part of the densovirus genome (encoding NS proteins) or the structural part of the genome (encoding VP proteins). In the transcriptome of the eastern honey bee from China we found 8 VP transcripts (encoding a capsid protein) and 4 NS transcripts (Table 1). The size of the complete Apis densovirus VP protein is 760 amino acids, while the sizes of the NS3, NS2 and NS1 proteins are 177, 298 and at least 546 amino acids, respectively. Among NS transcripts only one encodes the complete set of NS3, NS2 and NS1 proteins (GALO01034698, 2215 bp long). NS1 protein is nearly complete, missing is only the C-terminal part (from 2 to 20 amino acids), depending on the most similar sequences that are quite divergent.

The most similar sequence to the NS1 protein of Apis densovirus is the ant *Solenopsis invicta* NS1; they are 49 %

Table 1: Apis densovirus transcripts

Transcript	NCBI accession number	Size of the transcript (in bp)	Presence of the intron
VP transcripts	GALO01020880	2454	no
	GALO01020879	2571	yes
	GALO01020878	2502	yes
	GALO01020884	2372	no
	GALO01020881	2489	yes
	GALO01020883	2420	yes
	GALO01020882	2425	no
	GALO01020885	2343	no
NS transcripts	GALO01034701	1921	no
	GALO01034700	1998	no
	GALO01034699	2138	no
	GALO01034698	2215	no

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acccgtttcgccgaggaatccggtatacacgcggcgatcggtaaagttggattggacgtt
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aagcagaccatcgaaaaattaacaggagttttgtacccatctgttccaggtaagatatga
 K  Q  T  I  E  K  L  T  G  V  L  Y  P  S  V  P
ctagaaaattgaaacctccaccagacgaaagaccgaactatgaatttttaaatgagggcc

aaaaacgttatgcgtgggaacaatataaattggcacgtgttcgcagggattaccgatcg
                                              G  D  Y  R  S
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Figure 1: Apis densovirus possess a typical scindoambidensoviral intron in the VP1 gene. The VP1 intron is 117 bp long (*italic*). Splicing recognition sites are bold and underlined.

identical. Apis NS2 protein has best match in the *S. invicta* NS2 protein; they are 37 % identical. Apis NS3 protein is however unique and has no orthologs. Apis VP protein is more divergent and shows only 31 % amino acid identity with the *Planococcus citri* VP1 protein. We checked the conserved protein domains in the encoded Apis densovirus proteins and all of them are typical for densoviruses. In Apis VP protein, we can see the Parvo_coat_N domain (N-terminal region of the parvovirus VP1 coat proteins) and the large Denso_VP4 domain (capsid protein VP4 – four different translation initiation sites of the densovirus capsid protein mRNA give rise to four viral proteins, VP1 to VP4). Parvo_coat_N domain indeed encodes a special parvoviral phospholipase A_2 (PLA_2) that is necessary for their infectivity (Zadori et al., 2001). It is conserved in Apis VP protein and encodes at least 34 amino acids, with the conserved active site of the PLA_2 and Ca^{2+}-binding loop. In the NS1 protein, the DNA helicase protein that is required for the initiation of viral DNA replication is encoded in a protein domain named Parvo_NS1 superfamily. No conserved protein domains could be found in the NS2 and NS3 proteins.

In some of the Apis densovirus VP transcripts, we found an intron that is 117 bp long (Fig. 1). The presence of introns in a VP gene is typical for the *Scindoambidensovirus* genus. Members of the *Scindoambidensovirus* genus are characterized by a split VP-encoding ORF, which gives rise to the VP1 minor capsid protein *via* a spliced transcript as well as another major capsid protein (VP2) containing a unique N-terminal region, which has not been observed in any other parvovirus to date. The name "Scindo" refers to this split VP gene (Penzes et al., 2020, Tijssen et al., 2016). The Apis VP1 protein is 275 amino acids long, while the VP2 is 506 amino acids long. The presence of the split VP-encoding ORF in Apis densovirus indicates that it is most likely the new representative of the *Scindoambidensovirus* genus.

3.2 APIS DENSOVIRUS IS A MEMBER OF THE *SCINDOAMBIDENSOVIRUS* GENUS

To infer the phylogenetic affinity of Apis densovirus and relationships among densoviruses, we used their

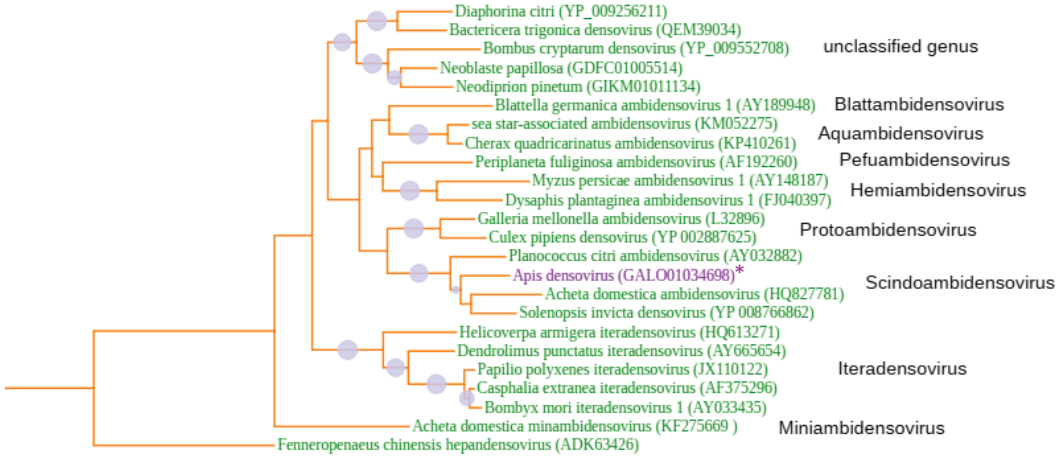

Figure 2: Maximum likelihood phylogeny of the densoviruses. The tree was inferred by IQTree program under a LG + F + I + G4 model from the NS1 proteins. Only bootstrap values larger than 80 % are shown as circles. The hepandensovirus was used to root this tree. Apis densovirus is shown in cyan color with asterisk.

NS1 protein sequences. Representatives of eight classified and one unclassified Densovirinae genera were included in the phylogenetic analysis. Best-fit model according to Bayesian information criterion was LG + F + I + G4. Tree was rooted with the hepandensovirus. Maximum likelihood phylogenetic analysis confirmed that Apis ambidensovirus is a new member of the *Scindoambidensovirus* genus (Fig. 2). On the other side, the bumblebee (*Bombus impatiens*) densovirus is a representative of a novel unclassified Densovirinae genus.

3.3 INFERRED GENOME ORGANISATION OF THE APIS AMBIDENSOVIRUS

Ambidensoviruses share a genomic characteristic: all of them exhibit antisense genome organisation. They maintain the division of the genome into separate nonstructural (NS3 to NS1) and structural (VP capsid) gene cassettes; these cassettes are inverted with respect to one another. In ambisense densovirus genomes, the nonstructural proteins (NS3 to NS1) are expressed from an ORF in the left half of the genome. The capsid proteins are translated from an ORF on the right hand side of the genome, but from an RNA generated in the opposite orientation (Mietzsch et al., 2019). Although we lack direct evidence for the Apis densovirus genome, we can simply infer its genome sequence from the available NS and VP transcripts. While the obtained genome sequence is not complete, it contains nearly 90 % sequence of the Apis densovirus. Missing are only terminal inverted repeats with promoters and the 50–100 bp in the center of the genome. The assembled partial Apis densovirus genome is currently 4786 bp long, while the expected complete genome size could be up to 5300 bp long (Fig. 3).

3.4 APIS DENSOVIRUS IS NOT ENDOGENISED

Previous studies of densoviruses in invertebrate genomes have found numerous endogenised densoviruses, some of them possessing complete genomes (Liu et al., 2011; Francois et al., 2016). We searched the available Apis genomes at the NCBI WGS and NCBI SRA databases for the presence of endogenised densoviruses. No endogenised densovirus sequences can be found in the available Apis genomes. The search for endogenised densoviruses in hymenopteran genomes showed that besides their presence in ant genomes they are also present in the bumblebee (*Bombus impatiens*) genome (AEQM02016195, 3848 bp long). This Bombus densovirus encodes intact NS1, NS2 and VP proteins (Fig. 4). It is most similar to the *Bombus cryptarum* and *Diaphorina citri* densoviruses but has very low level of similarity to the Apis densovirus (Fig. 2).

3.5 POTENTIAL IMPACT OF THE APIS DENSOVIRUS ON HONEY BEES

The relationship between densoviruses and their arthropod hosts ranges from mutualism (Xu et al., 2014) to severe pathology (Szelei et al., 2011), which is especially problematic in large insect rearing facilities (Tijssen et al., 2016; Schoonvaere et al., 2018). Densoviruses are highly pathogenic for insects at larval stages, which are infected through the ingestion of contaminated food

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NS3

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KEELPLQSKEKYEDEINIVVSTEYCLHSTLAIGINKWCCNNCIHTFLKNR
RKGTVFIHVSTVRSACRVSRAYKAICGNCAKPVQLKVYDAKGIKLDPIHF
CWNDSYTVTGNVYPPDGRPRIRKRYYR*

NS2

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QKDILATLFFFKDKNTVIDAFDTCAKNLAPTWVNSSYGSLKQITCTSSTI
APGQTDLVDAEFLTYPSFDDMFKRVCEEPSTSQNSTEQTTKVYFYTSLCR
NGKASEKFGLEEEYSDYLIKMKLYNGKICREQHPNYWLGKLKEVDISVPR
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NS1

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DRCIRHLRQESGSYMGKLFIWVVETDHLHIVHDCPWSNGSCRCRILDVPF
IRRHVQKSVRRTKYISELDRTDYEGILLYFIVSKWESEREIWIGRRIQRL
PDQDEIVQWQDLSRTASELLARETEGGGHIGPEGSSYNDPRGSDIYEEFD
GTSKKRRAIDDGPRRAKETKWTKILSKIQAVLTEFMPIPAVHVRDLLVGI
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NPFSYYHTREDSFQYLNRLLTYQLGGDTDIVREFLFNMKEWFNRKGWTGN
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KRFIVGNEISMEEGAKEDFKKLCEGTALNIRVKYQGDKIYKKTPVLLISN
SMLDICSDPAFKGIRLVTFTWNVAPFLRDSTLKPYPLAIFDLYNMYG

VP1

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RDHDLKYQEIQEKYEKGQIDKSSFVAEVKEADREAATRFAEESGIHAAIG
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VP2

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TDTQGYFDVIAEMEINTQYPVYRPHIENCITGEGDFYVMRAATDESVPTF
SGLYQV*

Figure 3: The nucleotide sequence of the Apis ambidensovirus genome with encoded NS and VP proteins. Partial Apis densovirus genome was reconstructed from the two transcripts (GALO01034698 and GALO01020879). This genome is 4786 bp long, the gap in the middle of the genome is from 6 to 60 bp long (or 2 to 20 amino acids long); missing are ITRs.

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ATTAAAAGAGTGGTACAAGGAAATTTTCGAGGAACAGCTTATTTGGTATGCGATCACGGAGACCACTATC
ACATTGTGCACGACTGCCATAGATCAGGGCAAAGATGTCGCTGTCATCGACTCGACGAAACCAGGAACGT
CTTCGGTCGAGCAGTGTCTGAACGAGTTGTTAGAGACAACATCTTCGACATCGAACATTGGATCAATCTC
GCAGAGTATTTCCAAAAAGACGAACGGCACCTTATCTACATGGAAGTCTGCGGGAGAGAAAGGACTGAAT
GTGTTCAAAATAGAAAAGTATTCGTTCAAGGAAGTGTCCAAGCTAGACAAGACGAAATGGTGGATGACAC
CGTCAGCAGTGAGAGCCCTATTCGTGACTTCCTCTCCTTTGGATCCTGTGGCGATACATGCAGACCAAGT
ACTGCTGCTGGGGACGAAGAGGTTGACCAAGCTGCCCGGAGTTCAGAAGGAGGAAAAACAACTAATGTCG
AAAAATACATACGAAAGTTTCTCACGTCTCCAATAACACATTTACTATCTACATCATATTGGATTAATTC
GAAGTACTATATGATCAATACACAATCTAATTGGTTTCAATGTGTTATGCGTAAGATATCTTTTTCATTT
AATCGTATGACTATATACGAATTATTTCAATACGCTAAACCACTGGATATGGATAAGCTGCTATATAGTA
GTCCTACGGAAATGATATTCGATTATTACTATGATATACGCACAAGTGTATACATTTTGGATGAGCTACT
ACGATTCCAGATGCAAGATGAAGATGAAATTGTATCATTCTTGGAAGTTCTATTAGCTGTCTTGGATAAA
TCAATACCGAAGAAGAATTGCTTACATATACAAGGACCACCATGTTCAGGGAAGAACCTATTCTTCGACT
GTGTGACATCGTTTTGTATTAATTGCGGACACTTGGGCAATTTCAACAAGTATAATTCTTTTCCGATGAT
GGATTGTATAGATAAACGTGTTATCATGTGGAATGAGCCTATCTTAGAAGTATCAGCACTCGAAACATTA
AAAATGGTATTCGGTGGAGATACTTGTCCTGTTAAAGTTAAATATCTCGGAGATAAGTTATTGCTTCGTA
CTCCGATTATCTGTTTATCGAATAATCAGCCATTTCCACAGGATGACGCATTTACCTGTCGTATGTTTAC
TTACCAATGGCAACAATGTAATGATTTAAAGAAAGTATCAAAAAAACCTCATCCTTTAGCTTTTCCTTAC
CTATTAATAAAATATAAGATATGGGAAGATGTAGAATTGAATGAGAAAGAAAAAGAATATTTATATTAAT
AAACATTTATTCAGCATAATATATGTGTACATTTAATTTACAGTATCCTGACCCATATTACTTAGTCTAT
CAAATTTATTTCCTAAATTACGACGAACAGGTCCCATCCTCTCATCATCCCTATATGCCATCTTATATTG
AGCTGCTCTTGGTGGATTTGGTGGAGTGAAGTCCTCTGTTTGTGTTGATCTTGTAGCAGGTGGTTCAGGT
GCTGGCATAGCAACCAATCCAGATAGTTGACAATATCCATTGTATGCTCTATTTCCAACCCATATAGTAG
TATCTGATGGAAATTGTACTGTGCCATTCTCCGAATAGTATGATCCATGATGTAGTTCAACATCTAATTC
AGCTTCAACTGCCCAATATGCAGATGTATTCTGGAATGTAGTACTATCCGATCCTGGAGTCATAGCTGGT
ACTGCCTGAATTCCTATATGTATTTGTGGTTGTGCTTTAAATGGGAATCCTCCATTCTGGAAATTATGTC
CACCTTTCTCCAAAAATTGTTCTACATTTGTTACGGGAGTAGAATTGTAATCATTTATTTTATCGCTTAT
ATCTGTAGTATGTGCTGCACCTAATGTTGTACTATGATCTCCATTCTTTATTACTTCTAATGCAAATGCT
CTAGTACGTGGACCACCATAATGTACATATCTTGTATGAACATTAGGAGGATTATAATAGGGTGCACTAA
TAGGAGCATGTTTACACTTATACGTATAATTAACTACAGGTTTTCCTATTGCTGTGTTTATTAGAAATTG
ATCTACAAATTGATCTTTACGCATCTGTCCGTGATTGTTAGTGAACACTGTTGCACCAGTCGTAGGTCCT
GGACTAACAAATGCTGCATATCCCACTGTATGTCTTGTGACTAGATTAGTATGTGAATCATCCGCTCTAT
AGTATTTGTTAATTATATCGCTAGCATTAATCAATGACATATTATTCGGTTTCATATTATCCATTGTTCC
ATATGTACAATTTACAATTTCTGTATTTACATTCAGTCCTACAGATACTAATCCTATAGGTACAAATTCG
TTAGTAGTATGTCCTGATGTTGTCCCTCCAAATTCAAACGCAGTACGAATACCGAGAGGTGTTACTTTAA
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TAAATCTGTAGGTATTAAAGCTAATGGTGTTGTATAATAATCATCGCTGTTATTATTTATTCTCTTTGTA
CATATTCCATAGCTGAAGAATATTCTATTCTTACGGAATGTAACAATAGATGGTTTAGGTGCAAGAGGTC
TAGGTATAGTAACAATGTGTCCTACAGTGTGACTACCACTACTACGTCCACTAGCACCTGATGGACCAGA
TGCTGCTAACACCTGATTATCATTCACACCATTTATACTTGGTACATCGAACTCCATGCCTTCTATATCG
TTAAACAATCCCAATAGACTATTATCTGATCCCTGACTACTGTCTGGTTGTTGTATTTGTTCTTCTTGTT
GTTCCTGTGGTACGTATTTACTTGTTCCTGGCTGATTTCTCTTTAATTCGTCCCAGGCGTCCTTTGAATG
GGCTTGTTGTATCTCTCGAAAGGATAATCCTGTCTCTCTACTAGTATCTGCTAATTGTCTCTGTATTTGT
GCGTACCTCTTTCTTTGTTCCAACTGTGCTGGTGTTGGTTGTCTTCTCTTCATATTAGGATATAACACTC
CTGTCAAGGATTCTACACCATACTTAGCTGCTAATCCTGCAGCACCCAAATAACCATGTGGTGATTGTAA
TTCCCAAAAATGTTTTATGGCGTCTCGATCTGCCTCTCTTATATCTTCCTCAGTCTTTGCACGATCATAC
AAATTATCATGTATTCTCGCTATTTCATCGTCTTCATCCACTGGTTCACCGTTCTTCAATTTATTCCCTG
GCCCTAAATAGCGATGGCCGTACCAATTCATACTGACTTATATCATCTCATCGCATGTCTTATATAGTCT
GAATAGATCGTATGGTAGGGTGCTCCGAGAATCACAGGTAAACTATTATGATCTATTCCATCCTTACT

Fig 4 continues on the next page

NS1 (70% aa id with *Bombus cryptarum* densovirus)
INTILEYLRHTSMGDIGELFCREDIPEEFETFVDQVIAGECENESNFGYI
AENRSNIGEASCSGNVPMEISERVYGFSEQGSAAYMVPPGESITATQRDA
AERRKLLRQIFLERFESQHRRNSVCHQIFSKRSIPGANAEIKRVVQGNFR
GTAYLVCDHGDHYHIVHDCHRSGQRCRCHRLDETRNVFGRAVSERVVRDN
IFDIEHWINLAEYFQKDERHLIYMEVCGRERTECVQNRKVFVQGSVQARQ
DEMVDDTVSSESPIRDFLSFGSCGDTCRPSTAAGDEEVDQAARSSEGGKT
TNVEKYIRKFLTSPITHLLSTSYWINSKYYMINTQSNWFQCVMRKISFSF
NRMTIYELFQYAKPLDMDKLLYSSPTEMIFDYYYDIRTSVYILDELLRFQ
MQDEDEIVSFLEVLLAVLDKSIPKKNCLHIQGPPCSGKNLFFDCVTSFCI
NCGHLGNFNKYNSFPMMDCIDKRVIMWNEPILEVSALETLKMVFGGDTCP
VKVKYLGDKLLLRTPIICLSNNQPFPQDDAFTCRMFTYQWQQCNDLKKVS
KKPHPLAFPYLLIKYKIWEDVELNEKEKEYLY*

NS2 (76% aa id with *Bombus cryptarum* densovirus)
MNQILDILPKTDLTLEKLHVLEMCLWRLAKEYMDSQNKDQLLIWFHRVKA
LQQHREMLLKDENCSGKYSWKDLRASIDATQSAIKFLVKEVSQELMLKLK
EWYKEIFEEQLIWYAITETTITLCTTAIDQGKDVAVIDSTKPGTSSVEQC
LNELLETTSSTSNIGSISQSISKKTNGTLSTWKSAGEKGLNVFKIEKYSF
KEVSKLDKTKWWMTPSAVRALFVTSSPLDPVAIHADQVLLLGTKRLTKLP
GVQKEEKQLMSKNTYESFSRLQ*

VP1 (59% aa id with *Bombus cryptarum* densovirus)
MNWYGHRYLGPGNKLKNGEPVDEDDEIARIHDNLYDRAKTEEDIREADRD
AIKHFWELQSPHGYLGAAGLAAKYGVESLTGVLYPNMKRRQPTPAQLEQR
KRYAQIQRQLADTSRETGLSFREIQQAHSKDAWDELKRNQPGTSKYVPQE
QQEEQIQQPDSSQGSDNSLLGLFNDIEGMEFDVPSINGVNDNQVLAASGP
SGASGRSSGSHTVGHIVTIPRPLAPKPSIVTFRKNRIFFSYGICTKRINN
NSDDYYTTPLALIPTDLVGFYLSPTEFRELPNNAWAKECRVKVTPLGIRT
AFEFGGTTSGHTTNEFVPIGLVSVGLNVNTEIVNCTYGTMDNMKPNNMSL
INASDIINKYYRADDSHTNLVTRHTVGYAAFVSPGPTTGATVFTNNHGQM
RKDQFVDQFLINTAIGKPVVNYTYKCKHAPISAPYYNPPNVHTRYVHYGG
PRTRAFALEVIKNGDHSTTLGAAHTTDISDKINDYNSTPVTNVEQFLEKG
GHNFQNGGFPFKAQPQIHIGIQAVPAMTPGSDSTTFQNTSAYWAVEAELD
VELHHGSYYSENGTVQFPSDTTIWVGNRAYNGYCQLSGLVAMPAPEPPAT
RSTQTEDFTPPNPPRAAQYKMAYRDDERMGPVRRNLGNKFDRLSNMGQDT
VN*

Figure 4: The nucleotide sequence of the endogenised *Bombus impatiens* densovirus genome with encoded NS and VP proteins. Partial ambisense *Bombus impatiens* densovirus genome was obtained from the NCBI WGS database (AEQM02016195). This genome is 3848 bp long, missing are ITRs.

(Tijssen et al., 2016). In honey bees, brood diseases are common and are caused by fungi, bacteria and viruses (Brutscher et al., 2016). Until now, among the honey bee-infecting viruses, only the Sacbrood virus was connected with the honey bee brood disease (Brutscher et al., 2016). The question to be resolved is whether densoviruses infecting pollinators (honey bees and bumblebees) exert any detectable pathological effects on them.

3.6 INFORMATION ABOUT THE ORIGIN OF THE EASTERN HONEY BEE TRANSCRIPTOME

The honey bee transcriptome in which we found the first Apis densovirus was erroneously classified as *Apis mellifera carnica* transcriptome (SRR922440, 52.177 contigs) and is still available under such name. It was produced from the whole heads of the workers by the researchers from the Yangzhou University, Jiangsan Province, China (Ji et al., 2014). The analysis of the SRR922440 transcriptome showed that 86 % of contigs have strong signals to the *Apis cerana* and only 1.2 % to the *Apis mellifera*. China, the largest producer of honey, introduced *A. mellifera* (diverse subspecies, including *carnica)* besides the native *A. cerana*. Since the erroneous classification of transcriptomes is misleading, we also investigated the origin of the SRR922440 transcriptome with complete mitochondrial genomes of *A. cerana* and *A. mellifera carnica*. This analysis demonstrated that the SRR922440 transcriptome originates from the *Apis cerana*, since numerous sequences show 99 % identity with the mitochondrial genome of *A. cerana*. The authors of this transcriptome (Ji et al., 2014)

provided some information about the origin of the *A. cerana* colonies: M and C colonies were unrelated local strains in the same *A. cerana* population and were bred in the Guandong Entomological Institute, Guangzhou (also known in the West as the Canton), southern China. All Apis densovirus transcripts were obtained from the C colony that was Varroa resistant. No information about the health status of the bees was provided in the original reference (Ji et al., 2014). We checked if these bees were infected with some RNA viruses, discovering only a full-length Sacbrood virus in this transcriptome (in the sequence GALO01042235). It should be noted that the additional *A. cerana* brain transcriptome is available at the NCBI TSA database (SRR361851), but shows the absence of the Apis densovirus. This transcriptome was produced by the researchers from the Fujian University: eastern honey bees originated from the Honey bee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi province, east China. The availability of diverse *A. cerana* transcriptomes therefore shows limited presence of Apis densovirus in southern China.

4 CONCLUSIONS

Viruses, especially RNA viruses, pose a major threat to the survival of honey bees. Combined with additional stressors, the consequences for honey bees and agriculture can be extremely severe. Here, we present the first densovirus in honey bees, which can pose a potential threat to them. Densoviruses are often associated with high mortality and great economic losses in commercially important infected insects, such as farmed crickets and silk moths. Given that densoviruses have also been detected in bumblebees, their potential pathogenicity could pose a serious threat to diverse pollinators (honey bees and bumblebees) and consequently to agriculture. The availability of the nucleotide sequence for the honey bee and and bumblebee ambidensovirus genomes, its transcripts, and all coding proteins provides a good starting point for more detailed studies of the pathogenicity of densoviruses in honey bees and bumblebees. Research on the effects of infection on the survival of honey bee colonies is also needed, as larvae are the most common victims of densoviruses in the majority of infected insects. We detected the densovirus only in eastern honey bees (*Apis cerana*) from southern China. Of course, this does not mean that this virus is not more widespread or that it lacks the potential to rapidly spread around the world. Dead larvae should be tested for the presence of the Apis densovirus. Research on densoviruses in diverse pollinators and their impact on the survival of honey bees and bumblebees is therefore urgently needed.

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V spomin izr. prof. dr. Juriju Poharju
(1951–2020)

Izr. prof. dr. Jurij Pohar je s svojim širokim opusom pomembno zaznamoval razvoj slovenske živinoreje, posebej akvakulture, populacijske genetike in trženja v živilstvu. S svojim delom je uspešno povezoval raziskovalno delo in praktično preverjanje znanstvenih konceptov. Tako je principe kvantitativne genetike uvajal v selekcijsko delo v akvakulturi, s svojim smislom za iskanje ustreznih tehničnih rešitev pa pomembno prispeval k razvoju ribogojnice na Pšati in številnih drugih akvakulturnih objektov po Sloveniji. Drugi del njegove kariere je zaznamovalo najprej poklicno delo na področju trženja, ki se mu je v zadnjem desetletju posvetil tudi raziskovalno.

Profesor Pohar je bil izjemen učitelj, ki je uspel študentom predstaviti kompleksne probleme v populacijski genetiki in genetiki kvantitativnih lastnosti na tak način, da so začutili željo po iskanju odgovorov na odprta vprašanja. Njegovo pedagoško delo na področju akvakulture je pritegnilo veliko študentov in jih navdušilo za to vejo živalske proizvodnje. Podiplomsko izobraževanje na področju mednarodnega trženja v začetku devetdesetih let na Danskem je pomenilo pomemben mejnik v njegovi karieri. Njegovo poklicno delo v trženjskih agencijah je vneslo svež duh v trženje živilskih izdelkov in povezovanje stroke z novimi trženjskimi prijemi. Svoje praktične izkušnje s tega področja je uspešno prenesel nazaj v akademsko sfero in obogatil pedagoški proces in raziskovalno delo na področju trženja v živilstvu.

Znanstveni opus prof. Poharja obsega več kot 70 znanstvenih in strokovnih člankov, prispevkov na konferencah in poglavij v monografijah. Bil je mentor več kot štiridesetim dodiplomskim študentom, trem magistrskim in dvema doktorskima študentoma. Njegov celotni ustvarjalni opus je prežet z željo po kompleksnem dojemanju sveta in problemov, ki odpira obzorja za iskanje novih rešitev. Profesor Pohar je bil širok, načelen, topel, vsestransko angažiran in spoštovan član akademske skupnosti, ki ji je skozi celotno obdobje svojega delovanja dajal prepoznaven osebni pečat.

Izr. prof. dr. Aleš Kuhar
Prof. dr. Peter Dovč

In memoriam Assoc. Prof. Dr. Jurij Pohar (1951–2020)

Assoc. Prof. dr. Jurij Pohar significantly marked the development of Slovenian animal science, especially aquaculture, population genetics, and marketing of food products. With his work, he successfully combined research work and practical verification of scientific concepts. Thus, he introduced the principles of quantitative genetics into selection work in aquaculture. With his sense of finding appropriate technical solutions, he importantly contributed to the development of the fish farm Pšata and many other aquaculture facilities in Slovenia. The second part of his career was marked first by his professional work in the field of marketing, and finally by research work in this field in the last decade.

Professor Pohar was an inspiring teacher who was able to present the complex problems in population genetics and genetics of quantitative traits to the students in a way that they were motivated to find the answers to open questions. His teaching in the field of aquaculture attracted many students and inspired them for this branch of animal production. Postgraduate education in international marketing in the early 1990s in Denmark was an important milestone in professor Pohar's career. His professional work in marketing agencies has brought a fresh spirit to food marketing and the food sector with new marketing approaches. He successfully transferred his practical experience in this field back to the academic sphere and enriched the pedagogical process and research work in the field of food marketing.

The scientific opus of prof. Pohar comprises more than 70 scientific and professional articles, conference papers, and chapters in monographs. He has mentored more than forty undergraduate students, three master's - and two doctoral students. His entire creative oeuvre is imbued with a desire for a complex perception of the world and problems, which opens wide horizons for finding new solutions. Professor Pohar was an open minded, principled, warm, versatile, and respected member of the academic community, giving her a recognizable personal mark throughout his career.

Assoc. Prof. Dr. Aleš Kuhar
Prof. Dr. Peter Dovč

Navodila avtorjem

UVOD

Acta agriculturae Slovenica je četrtletna odprtodostopna znanstvena revija z recenzentskim sistemom, ki jo izdaja Biotehniška fakulteta Univerze v Ljubljani. Revija sprejema izvirne in še neobjavljene znanstvene članke v slovenskem ali angleškem jeziku, ki se vsebinsko nanašajo na širše področje rastlinske pridelave in živalske prireje in predelave. Zajema sledeče teme: agronomija, hortikultura, biotehnologija, fiziologija rastlin in živali, pedologija, ekologija in okoljske študije, agrarna ekonomika in politika, razvoj podeželja, sociologija podeželja, genetika, mikrobiologija, imunologija, etologija, mlekarstvo, živilska tehnologija, prehrana, bioinformatika, informacijske znanosti in ostala področja, povezana s kmetijstvom. Pregledne znanstvene članke sprejemamo v objavo samo po poprejšnjem dogovoru z uredniškim odborom. Objavljamo tudi izbrane razširjene znanstvene prispevke s posvetovanj, vendar morajo taki prispevki zajeti najmanj 30 % dodatnih izvirnih vsebin, ki še niso bile objavljene. O tovrstni predhodni objavi mora avtor obvestiti uredniški odbor. Če je prispevek del diplomske naloge, magistrskega ali doktorskega dela, navedemo to in tudi mentorja na dnu prve strani. Avtorji omenjenih del morajo biti tudi soavtorji članka, ki podaja izsledke dela. Navedbe morajo biti v slovenskem in angleškem jeziku, kadar so prispevki v slovenščini. V primeru člankov v slovenščini so dolžni avtorji priskrbeti angleški prevod naslova, izvlečka s ključnimi besedami in naslovov slik in tabel. Uredništvo revije zagotovi prevode izbranih bibliografskih elementov (naslova, izvlečka, opomb in ključnih besed) v primeru tujih avtorjev. Prispevke sprejemamo skozi celo leto samo preko spleta v OJS sistemu.

POSTOPEK ODDAJE PRISPEVKOV

Avtorji lektorirane prispevke oddajo v elektronski obliki na spletni strani OJS Acta agriculturae Slovenica.

Author guidelines

INTRODUCTION

Acta agriculturae Slovenica is an open access peer-reviewed scientific journal published quarterly by the Biotechnical Faculty of the University of Ljubljana, Slovenia. The journal accepts original scientific articles from the fields of plant production (agronomy, horticulture, plant biotechnology, plant-related food-and-nutrition research, agricultural economics, information-science, ecology, environmental studies, plant physiology & ecology, rural development & sociology, soil sciences, genetics, microbiology, food processing) and animal production(genetics, microbiology, immunology, nutrition, physiology, ecology, ethology, dairy science, economics, bioinformatics, animal production and food processing, technology and information science) in the Slovenian or English languages. Review articles are published upon agreement with the editor. Reports presented at conferences that were not published entirely in the conference reports can be published. Extended versions of selected proceedings-papers can also be considered for acceptance, provided they include at least 30 % new original content, but the editorial board must be notified beforehand. If the paper is part of a BSc, MSc or PhD thesis, this should be indicated together with the name of the mentor at the bottom of the front page and will appear as footnote. Authors of mentioned thesises should be also coauthors of manuscript. Slovenian-language translation of selected bibliographic elements, for example the title, abstract, and key words, will be provided by the editorial board. Manuscripts are accepted throughout the year. Only online submissions are accepted..

SUBMISSION PROCESS

Manuscripts should be submitted to the Acta agriculturae Slovenica OJS site. Complete manuscripts in-

Oddajo kompleten članek (naslov, avtorji z njihovimi naslovi, označen mora biti korespondenčni avtor in naveden njegov elektronski naslov, besedilo z vsemi poglavji in vključenimi ilustracijami (slike, tabele, enačbe). Pred oddajo prispevka se mora avtor na spletni strani najprej prijaviti oziroma registrirati (priporočamo, da je to korespondenčni avtor), če prvič vstopa v sistem (potrebno je klikniti na Registracija in izpolniti obrazec za registracijo). Bodite pozorni, da na dnu registracijskega obrazca ne pozabite odkljukati potrditvenega polja »Avtor«, sicer oddaja prispevka ne bo mogoča.

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- dodatna pojasnila uredniku je mogoče vpisati v ustrezno polje.

Korak 2: Oddaja prispevka
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Korak 3: Vpis metapodatkov
- Podatki o avtorjih: ime, priimek, elektronski naslovi in ustanove vseh avtorjev v ustreznem vrstnem redu. Korespondenčni avtor mora biti posebej označen.
- Vpišite naslov in izvleček prispevka.
- Vpišite ključne besede (največ 8, ločeno s podpičjem) in označite jezik besedila.
- Vnesete lahko tudi podatke o financerjih.
- V ustrezno besedilno polje vnesite reference (med posameznimi referencami naj bo prazna vrstica).

Korak 4: Dodajanje morebitnih dodatnih datotek
- Za vsako dodatno naloženo datoteko je treba zagotoviti predvidene metapodatke.

Korak 5: Potrditev
- Potrebna je končna potrditev.

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