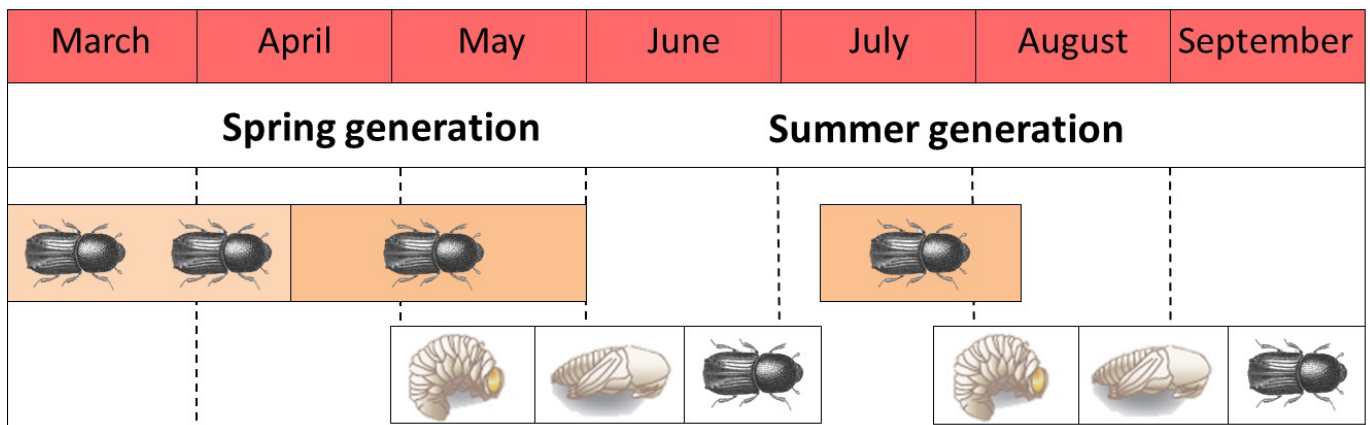


Agricultura

Vol 12, No 1-2

December 2015



Editor-in-Chief

Dejan ŠKORJANC, University of Maribor, Slovenia

Editorial Board

Adriano CIANI
University of Perugia, Italy

Aleš GASPARIČ
Krka, Slovenia

Andrea ALBERA
Associazione Nazionale Allevatori Bovini
Razza Piemontese, Italy

Anton IVANČIČ
University of Maribor, Slovenia

Bao ENDONG
Nanjing Agricultural University, P.R.China

Bruno BIAVATI
University of Bologna, Italy

Ana POSPIŠIL
University of Zagreb, Croatia

Ernst RÜHL
Forschungsanstalt Geisenheim, Germany

Fabio CAPORALI
Università della Tuscia, Italy

Franč BAVEC
University of Maribor, Slovenia

Franci ČUŠ
University of Maribor, Slovenia

Thorsten HAASE
Universität Kassel, Germany

Frans SWANEPOEL
University of Pretoria, South Africa

Sylvie GRANGER
Département des Sciences et Techniques
Agronomiques, France

Hana WEINGARTL
National Center for Foreign Animal Disease,
Canadian Science Center for Animal and
Human Health, Canada

Igor VOJTIC
Veterinary Administration
of the Republic of Slovenia

Igor ŽIBERNA
University of Maribor, Slovenia

Irena RAJČAN
University of Guelph, Canada

Jean-François ELÉOUËT
Institut National de la Recherche
Agronomique, France

Jernej TURK
University of Maribor, Slovenia

Ljubinko STARČEVIČ
University of Novi Sad, Serbia

Marc ROUX
ENESAD, Département des Sciences et
Techniques Agronomiques, France

Milan HOČEVAR
University of Ljubljana, Slovenia

Amarendra Narayan MISRA
Center for Life Sciences, School of Natural Sciences,
Central University of Jharkhand, India

Nikolaj KARITHONOV
State Agraria University, Ukraine

Peter DOVČ
University of Ljubljana, Slovenia

Silvio KOŠUTIČ
University of Zagreb, Croatia

Sonja JOVANOVAČ
University of Osijek, Croatia

Stefan VOGEL
Universität für Bodenkultur, Austria

Ted PUTMAN
University of Alberta, Canada

Tito ŽIMBREK
University of Zagreb, Croatia

V. P. SKULACHEV
Moscow State University, Russia

Viktor JEJČIČ
Agriculture Institute of Slovenia, Slovenia

Vjekoslav PAR
University of Zagreb, Croatia

Zlatko ČMELIK
University of Zagreb, Croatia

Marjeta ČANDEK POTOKAR
Agricultural Institute of Slovenia
University of Maribor, Slovenia

Agricultura (ISSN 1580-8432). Copyright © 2015 by the University of Maribor, Faculty of Agriculture and Life Sciences, Slovenia. All rights reserved. Agricultura is devoted to the advancement of basic and applied knowledge related to agricultural sciences. Agricultura is indexed in AGRICOLA and CAB Abstracts Full Text Select. Agricultura (ISSN 1581-5439) in electronic version: www.agricultura-online.com. Publishing of the journal of Agricultura is financially supported by Slovenian Research Agency.

The Agricultura is published two times a year by University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 10, 2311 Hoče, Slovenia. All manuscripts submitted to the Agricultura must be addressed to the Editor-in-Chief, Pivola 10, 2311 Hoče, Slovenia (Telephone: +386 2 320 90 00; Fax: +386 2 616 11 58; E-mail: dejan.skorjanc@uni-mb.si).

Subscription Price (2015). The annual subscription price is 100 Euro. Single issues are available. Subscription must be prepaid at UJP Slovenska Bistrica, 2310 Slovenska Bistrica; Bank account: 01100-609126312

Please mail payment with your order to University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 10, 2311 Hoče, Slovenia, Tel.: +386 2 320 90 25 (Dr. Škorjanc), Fax: +386 2 616 11 58; e-mail: dejan.skorjanc@uni-mb.si. Inquiries concerning subscriptions, single copies, change of address should be addressed to Editor-in-Chief. There are no page charges for Agricultura. Authors would be charged for the cost of colour illustration only.

Copyright © 2015. All rights reserved. Agricultura is not responsible for statements or opinion printed in its publications: they represent the views of the authors or persons to whom they are credited and are not binding on the Agricultura as a whole. Trade names are sometimes mentioned in papers published in this journal. No endorsement of these products by the publisher is intended, nor any criticism of them. Reproduction requires the permission of the publisher. Other users should request permission of author(s) and notify the publisher.

The cover illustration is generally life-cycle of the spruce bark beetle (*I. typographus*). Courtesy of mateha FELICIJAN, Faculty of Agriculture and Life Sciences, University of Maribor, Slovenia.

Agricultura

Contents

ARTICLES	<i>Page</i>
Business plan for a Zen garden <i>Maja ŽIBRAT, Karmen PAŽEK and Vesna WEINGERL</i>	1-7
Antioxidant defences of Norway spruce bark against bark beetles and its associated blue-stain fungus <i>Mateja FELICIJAN, Metka NOVAK, Nada KRAŠEVEC and Andreja URBANEK KRAJNC</i>	9-18
The accuracy of the germination rate of seeds based on image processing and artificial neural networks <i>Uroš ŠKRUBEJ, Črtomir ROZMAN and Denis STAJNKO</i>	19-24
Impact of various types of anti-hail nets on light exposure in orchards and quality parameters of apples– a review <i>Marinka BRGLEZ SEVER, Stanislav TOJNKO and Tatjana UNUK</i>	25-31
Sweet maize growth and yield response to organic and mineral fertilizers, N rates and soil water regimes <i>Franc BAVEC, Martina BAVEC, Silva GROBELNIK MLAKAR and Milojka FEKONJA</i>	33-40
Variations in leaf total protein, phenolic and thiol contents amongst old varieties of mulberry from the Gorizia region <i>Tina UGULIN, Tamas BAKONYI, Rebeka BERČIČ and Andreja URBANEK KRAJNC</i>	41-47

Business plan for a Zen garden

Maja ŽIBRAT, Karmen PAŽEK, Vesna WEINGERL*

University of Maribor, Faculty of Agriculture and Life sciences,
Pivola 10, SI-2311 Hoče, Slovenia

ABSTRACT

The placement of a theme park in the form of a Zen garden, as a business opportunity in the Slovenian rural area, is discussed. The design of the garden, with all the major points of a standard business plan, is accurately presented, with a description of the business, branch, and services, market analysis, marketing strategy, financial projections, and a plan of the work and activities. The financial aspect is presented as the amount of investment, net present value, and internal rate of return. The amount of investment is estimated at €14.891, which should be reimbursed within 4 years of operations. The estimated internal rate of return is estimated at 16.86%. Part of the study is the market analysis - conduction of a survey into knowledge of, and interest in, Zen and Zen gardens. The principles of landscape ecology are respected, as the Zen garden would be set in the woods and will blend seamlessly into the landscape.

Key words: theme parks, sustainable tourism, investment, financial indicators, landscape ecology

INTRODUCTION

The Government of the Republic of Slovenia defines tourism as one of the most important economic and strategic sectors, as it creates new jobs and has a highly positive impact on balanced regional development. From the Slovenian Tourism Development Strategy (2012-2016), which is the basic document for planning of tourism development at the national level, we can see that the designers of Slovenian tourism have, in recent years, reached a consensus that all tourism development must become sustainable (Ministry of Economic Development and Technology 2012).

Sustainable tourism development does not deplete natural resources, and does not pollute the environment, while respecting culture and human and ethical values. We can talk about soft tourism, which is the most suitable for rural areas. At the same time, sustainable development focuses not only on environmental issues, but emphasises development that meets the needs of the present without compromising the ability of future generations. Sustainable development links the concern for the capacity of natural systems with the social challenges facing humanity. Tourism is one of the fundamental pillars of balanced economic, social, ethical,

and environmental development – these are the components that comprise sustainable development. Thus, tourism is becoming an extremely important sector of both the global and the local economy. The sector is expected to grow constantly, both in qualitative and quantitative terms.

Any development of tourism cannot happen without affecting the development of the entire region. When we attract tourists with one attraction, they will visit by the way also other attractions, they will use public transport, eat in local restaurants and sleep in nearby hotels, which brings benefit to all actors and hence the entire region. However, there may cause also negative effects, such as increased traffic and thus greater noise and air pollution.

Nowadays, “relaxed tourism” is booming as people are in a constant hurry and are under stress, so there is a growing demand for relaxation techniques and a need for places where they can find peace. In the north-east of Slovenia alone, there are 7 thermal spas, over 25 tourist farms, and many other activities which attract tourists who want on-site facilities.

The landscape is a functional set of ecosystems and their environment, which is namely open, but able to be to a certain extent self-regulating. Landscape ecology is the study of the composition, structure and function of landscapes. A

*Correspondence to:

E-mail: vesna.weingerl@um.si

landscape is not necessarily defined by its size; rather, it is defined by an interacting mosaic of elements (e.g. ecosystems) relevant to some phenomenon under consideration (at any scale). Thus, a landscape is simply an area of land (at any scale) containing an interesting pattern that affects and is affected by an ecological process of interest (Turner et al. 2001, Turner, 2005).

Zen is the way of thinking, striving for inner peace and acceptance of other people and things as they are. To live in the present, disregarding the past and do not worry about the future. During the development of the Zen, various teachers used their own methods and approaches and that's why Zen parts for Japanese and Chinese. In addition, Zen parts of the course also due to the geographical division of the two countries. The Buddhist concept is completely enclosed in a Zen only that this concept arose the legend of Siddhartha Gautama, nowadays known as Gautama Buddha. The great masters of Zen and Buddhism may precisely explain the similarities and differences. However, if you live according to the principles of Zen, you live also according to the principles of Buddhism and vice versa. The Japanese and the Chinese, who were the originators of Zen thinking, created special gardens, called Zen gardens, which use many natural materials, such as sand, stone, rocks, and bamboo. Simple lines are used that do not impose any form, so each visitor experiences the garden in his own way. Zen garden it can be used as a tool to facilitate the achievement of the Zen, or just for a walk, relax, enjoying the nature, or an idea how to arrange the home garden. In Zen gardens there are created simple lines that do not impose any form in order to cleanse the mind of the observer for the understanding of superfluous elements, and allow the viewer to easily achieve deeper insights (Žibrat 2015).

Creating a business plan is necessary in order to cover the economic aspect. The concept of "economy" encompasses the entirety of the economic sciences, which are experienced very rapid changes due to the diversity of research. The subject of the economy is in every human activity in which we are confronted with the problem of limited resources with which to achieve the stated objectives. The task of economics is to ultimately achieve maximum efficiency, or minimisation of costs (Žnidaršič Krajnc, 1995).

By definition, investment means "increasing, or at least maintaining the value of capital". The process of investment lies in the realization of the path from the idea or concept to the market. Property is a lever of investment, which requires a continued increase or maintaining of the value. When investing, the proceeds or output should always be higher than the input (Vuk 2001).

The aim of our study was to accurately present the plan and the company that we want to establish, and to assess the financial feasibility of the investment, which was estimated on the basis of the assessment of net present value and internal rate of return. Another important part of the study was the market research - the survey into the knowledge of, and interest in, Zen and Zen gardens.

MATERIALS AND METHODS

Net present value and internal rate of return

The preferred approach to the evaluation of investment projects is a dynamic method known as net present value analysis (NPV) (Pažek and Rozman 2008).

The two most important criteria for the selection of investment projects are net present value (NPV) and internal rate of return (IRR). Most often, projects are evaluated equally according to both criteria, but sometimes these two criteria offer different estimates. In theory, the concept of NPV is better, but IRR offers more results, and thereby solves the problem of the evaluation of more complex and multilayered projects. New equations of NPV contain all possible solutions for IRR as a component. The analysis confirms the theory that NPV is a better concept, but IRR remains a compulsory core part of the corresponding NPV.

The NPV estimates the effectiveness of investment in the present. NPV reduces the expected cash income from investments for the sum, which depends on three factors: the riskiness of investments, the expected inflation, and the desire of investors to get their money back upon withdrawal from the project. If the cash inflows exceed the amount of the investment, then NPV is positive, and the project is financially attractive for investors (Kierulff 2008).

The rule for an investment decision on the basis of NPV is that the investment can be taken if NPV is greater than 0, and rejected if the NPV is less than 0. If the NPV is equal to zero, the decision maker could decide to be indifferent. From the alternative investment options, we choose the one with the highest NPV. The investment is acceptable only in the case when there is no alternative investment, which would give higher value yields for the same cost (Čebokli 2011). For an investment of t periods, the formula is as follows:

$$NPV_t = -I + \sum_{i=1}^n \frac{TR - TC}{(1+r)^i}$$

Where: NPV_t - Net Present Value (€), I - investment costs (€), TR - total revenue (€), TC - total costs (€), r - interest rate (%), t - time - number of years

The disadvantage of the NPV method is the fact that it does not take into account the value of creation of opportunities. Sometimes an investment that is inherently uneconomically feasible can create opportunities, which enable new investments for the company in favorable market conditions (Buckley 1998).

An associated concept is the IRR, which does not serve with nominal values, but rather with a discount rate, at which $NPV = 0$ and represents the maximum interest rate that would be transferred to an investment in the case of financing by credit.

Landscape ecology

Landscape ecology is a sub-discipline of ecology, which examines how patterns in space affect the spatial processes. It covers both the impact of biological processes on the landscape structure and the influence of the landscape structure on the wealth and distribution of organisms in space. The main concern of landscape ecology is the study of large-scale spatial heterogeneity, due to both natural and anthropogenic influences, and the effects of this heterogeneity on ecological processes and species persistence.

Setting a Zen garden in the woods is certainly an interference with the landscape, so the principles of landscape ecology need to be respected. Trees which absolutely have to be felled for the purpose of the garden arrangements will be used in the garden, to create simple benches, sculptures, and a variety of decorative accessories. For the purpose of the garden arrangements, it will also be necessary to fully clean the forest floor, which will destroy all undergrowth. To gain the required space, it will be necessary to remove the majority of young trees. However, it will not be necessary to discard them; they will be transplanted to the periphery, or used as an integral part of the garden. Some of the forest undergrowth plants - for example, various ferns and sedges - will be transplanted and arranged for the purpose of the systematic design of the garden.

Animals will not be driven out from the area of the garden. The assumption is that that they will eventually leave the area of their own accord, due to the noise. The area of the entire forest is much larger than that of the garden, so the animals will have the opportunity to withdraw to a quieter part of the forest.

A small market selling local produce, such as sausages, honey, pumpkin seed oil, teas, natural soaps, essential oils and so on, is planned to be in front of the garden. This will allow nearby residents to earn additional income, and, at the same time, will encourage the development of complementary activities in the region. Stands will be offered at a token rate (€2.00 per day).

The impact of tourism

Tourist activities are linked to the natural and cultural environment. An important part of Slovenian tourism is its abundant opportunities for recreation in an attractive natural environment with special and interesting elements. The expansion of different forms of recreation is increasing, due to people's desire to withdraw from urban centres and to enjoy active leisure pursuits in a healthy environment.

Increased interest in leisure activities in a natural environment brings with it a number of problems, including disturbance of the wildlife. This is known as environmental wildlife stress, and is caused by an increase in visitors and by the construction and maintenance of recreational facilities and infrastructures. Animals react to visual and auditory stimuli, as well as smells. In general, wildlife is better able to tolerate cyclical and repetitive stimuli than those which are unpredictable. For example, a motorway, which produces a constant background noise, is less disruptive to animals than a construction site which will produce sudden outbursts of noise at random times (Kolar 1999).

The development of tourism in a locality is not possible if it does not take into account the local population. It is important that local people see the value the development will add to the area. In order to gain their cooperation, local people should be integrated into the preparation of the development strategy, listened to, and, if possible, their opinions, suggestions and ideas should be taken into account. In fact, the local population has a much greater sense of, and the best vision for, their needs for the development.

Any development of tourism cannot happen without affecting the development of the entire region. When we attract tourists with one attraction, they will visit by the way also other attractions, they will use public transport, eat in local restaurants and sleep in nearby hotels, which brings benefit to all actors and hence the entire region. However, there may cause also negative effects, such as increased traffic and thus greater noise and air pollution.

Table 1: The estimated amount of investment required to build a Zen garden

	Amount	Unit	Price		Together (€)
Purchase of land	4,000	m ²	1.00	€/m ²	4,000.00
Paths (gravel)	30	m ³	9.00	€/m ³	270.00
Benches	15	pcs	80.00	€/pcs	1,200.00
Waste bins	10	pcs	30.00	€/pcs	300.00
Sand	15	m ³	17.00	€/m ³	255.00
Rocks	20	pcs	20.00	€/pcs	400.00
Vegetation	100	pcs	7.00	€/pcs	700.00
Machine work	50	h	30.00	€/h	1,500.00
Handwork	100	h	5.66	€/h	565.70
Website	1	pcs	500.00	€	500.00
Promotional material	2,000	pcs	0.10	€/pcs	200.00
Stands	5		500.00	€/stand	2,500.00
Project documentation					1,500.00
Other					1,000.00
				Total	14,890.70

Business plan

A business plan is a detailed and measured description of the business or business idea. It is urgently needed if the entrepreneur wishes to obtain financial support from banks, to apply for financial aid from the various funds, or to expect cooperation from other companies. It must include real and measurable objectives, including a description of the business and branches, research and market analysis, marketing plan, financial analysis, risk assessment, and schedule. Financial projections are the most important element of any plan, and are formed after detailed analysis of the business, costs, and revenues. If the company already exists, the projections are based on results from previous years; if not, on forecasts for the future, usually for 3-5 years (Vidic 1999).

Zen garden

When designing a Zen garden, it is reasonable to bear feng shui in mind. Feng shui has been configured as a system that understands the multi-layered nature of space, together with its subtle energy levels. This is known as the school of forms, which talks about how energy flows through the landscape. The basic principle of the school of forms is that the landscape is a living being, which pervades the life energy “qi”, and has the ability to regulate the flow of energy to be steady and balanced, with just the right level of intensity (Kryžanowski 2012).

In western countries, research has accumulated on the therapeutic effects of nature since the 1970s. The gap between eastern and western countries on the topics of therapeutic landscapes and healing gardens bridges the common knowledge that green public spaces are beneficial to people's physical, mental and social health, by providing spaces for therapeutic activities and contemplation, which relieves stresses and encourages social communication (Jiang 2014).

Zen gardens seem to be an ideal place for meditation, as they are designed to defuse and concentrate thoughts. Therefore, the garden will have a lot of landscaped corners, which will offer visitors some privacy.

Zen gardening is an art form with a profound spiritual significance. Zen gardens are known as ‘arid gardens’ or ‘stone gardens’. In their own language, the Japanese call them ‘karesansui’, which literally means ‘water of dry mountains’.

Many Zen gardens are designed with the aim of symbolic communication of Buddhist concepts; it is possible to

Table 2: Total revenue on an annual basis

	Price		Amount		Total	
Entrance fee	3.50	€	6,500	Visitors	22,750.00	€
Stands rental (5)	10	€/day	262	Days	2,620.00	€
Total annual revenue					25,370.00	€

experience ‘Zen’ in them, thanks to the particular vulnerability of nature to its most basic elements, which can still carry the whole message. For instance, the stone symbolizes a mountain, sand or curves in the sand the water, the piece of wood a tree, the forest a boat, and so on. The essence of such symbolism of communication is to cleanse the mind of the observer of all superfluous elements, and allow him to easily concentrate on his insight.

Table 3: Total operating costs of the Zen garden

	Nr	Net salary	Gross salary	Total per year	
Salaries of employees	1	995.64	1,521.38	18,256.60	€
Other costs					
Maintenance				1,200.00	€
Advertising				500.00	€
Total annual cost				19,956.60	€

Zen gardens sometimes include live plants in limited quantities, mostly moss, but always in proportion to the largely static and inanimate nature of such a garden. This is primarily a reflection of the Buddhist evaluation of the relative rarity of life and the cycle of birth and impermanence, which illustrates the change of seasons by the impact on the living nature in the garden. Many Zen gardens are positioned in such a way that each garden ‘borrows’ from its neighbourhood as part of its design. The purpose of such borrowing of the background is the optical zoom of the garden area and its logical connection with the surroundings. Planned gardens also fuse to the surroundings, so that they do not unduly interfere with the existing nature.

RESULTS AND DISCUSSION

Investment costs

The investment is the largest financial outlay, but it is absolutely necessary. Table 1 shows the costs and total value of the investment. The highest cost is the purchase of land, amounting to €4,000.00.

Zen garden is planned in north-eastern Slovenia in the Pomurje region, where property prices are generally lower due to a lower standard of the whole region. We have assumed the approximate average price for forest land in the market €1/m².

Part of the investment budget will be needed for machine work, buying stands, project documentation, and other aspects.

Annual cash flow

Cash flow is the movement of cash in and out during the period under analysis. This must be determined in order to

ensure or assess the current solvency of the company. The annual cash flow (ACF) is the most important parameter when calculating the net present value. ACF was calculated as the difference between total revenue (TR) and total cost (TC). Table 2 presents the total revenues. The garden would be financed from ticket sales and the rental of stands.

The total costs, represented in Table 3, amount to €19,956.60. The biggest costs would be staff salaries. Initially, only one person will be employed at an average Slovenian salary for the month of June 2014, i.e. €1,521.38 gross. (SAOP 2014)

Other costs would be maintenance costs and advertising, which together account for €1,700.00 per year.

$$\text{ACF} = \text{€}25,370.00 - \text{€}19,956.60$$

$$\text{ACF} = \text{€}5,413.44$$

Break-even point of the business and break-even price of tickets

The profitability threshold or break-even point is the moment when a company achieves a volume of business whereby it has neither a profit nor a loss. At such a volume, the company revenue sales just cover all costs. Above the break-even price, the profit increases; below it, losses increase. A calculation of the break-even point of the volume of ticket sales, comprising the number of visitors and break-even price of tickets, is presented in Table 4.

Table 4: Break-even point in business volume

Nr. of visitors	Entrance fee	Revenues	Expenses	Profit
6,500	€3.50	€25,370.00	€19,956.56	€5,413.44
6,000	€3.50	€23,620.00	€19,956.56	€3,663.44
5,500	€3.50	€21,870.00	€19,956.56	€1,913.44
5,000	€3.50	€20,120.00	€19,956.56	€163.44
4,955	€3.50	€19,962.50	€19,956.56	€594
4,954	€3.50	€19,959.00	€19,956.56	€2.44
4,953	€3.50	€19,955.50	€19,956.56	€-1.06

With a planned entrance fee of €3.50, and an expected number of visitors of 6,500, the company would have a profit of €5,413.44. By reducing the number of visitors, the profit decreases. The minimum number of visitors with which the company would still operate positively, is 4,954; the profit would be €2.44.

By reducing the amount of the entrance fee, profit drops sharply.

The minimum ticket price, where the company would still operate positively, under the assumption that the expected number of visitors is achieved, is €2.70 (Table 5).

Table 5: Break-even price of tickets

Nr. of visitors	Entrance fee	Revenues	Expenses	Profit
6,500	€3.50	€25,370.00	€19,956.56	€5,413.44
6,500	€3.30	€24,070.00	€19,956.56	€4,113.44
6,500	€3.00	€22,120.00	€19,956.56	€2,163.44
6,500	€2.80	€20,820.00	€19,956.56	€863.44
6,500	€2.70	€20,170.00	€19,956.56	€213.44
6,500	€2.60	€19,520.00	€19,956.56	€-436.56

Net present value and internal rate of return

Evaluations of the net present value and internal rate of return are shown in Tables 6 and 7. The net present value was estimated by taking into account interest rates of 8.00%. Positive business would begin in the 4th year of operations; estimated NPV will by then be €3,039.30.

The maximum interest rate which can be managed with the investment is estimated at 16.86%; estimated NPV will then be €0.69.

Table 6: Assessment of the net present value after the 5th year of business

Year	Interest rate - 8.00 %	NPV (€)
1	5,012.44	-9,878.26
2	4,641.15	-5,237.11
3	4,297.36	-939.74
4	3,979.04	3,039.30
5	3,684.30	6,723.59

Table 7: Assessment of the internal rate of return

Year	Interest rate - 16.86%	NPV (€)
1	4,632.41	-10,258.29
2	3,964.07	-6,294.22
3	3,392.15	-2,902.06
4	2,902.75	0.69

The survey

Among 136 respondents, there were 105 women and 31 men with an average age of 30-40 years, mostly from the countryside. Results showed that 52.2% of respondents preferred to spend their free time in natural surroundings. 36.8% of respondents were familiar with Zen and interested in the topic; 31.6% of respondents had not heard of Zen or a Zen garden, but they were interested in learning about it. 86.8% of respondents would visit a Zen garden if it was within a radius of 20-40 km. 26.5% of respondents did not mind how far away the attraction would be. 51.5% would visit a Zen garden in the springtime; 29.4% in the summer.

68.4% of respondents intended to visit a Zen garden out of curiosity. In terms of the entrance fee, the majority (45.4%) were ready to pay €2-3; 30.1% were willing to pay €4-5. 80% of respondents already had a local market nearby, but only about 30% attended it. 89.7% of respondents preferred the possibility of purchasing local products or crops when visiting local attractions (Žibrat 2014).

CONCLUSIONS

The aim of the study was to accurately present the branch and the company that we want to establish, and to assess the financial viability of the investment, which was estimated on the basis of the calculation of net present value and internal rate of return.

The survey was included for the market research reasons. It was necessary to know whether the people are interested to see the Zen garden, whether they are willing to pay an entrance fee and how much. The results of the market research were positive.

Investment in the construction of a Zen garden with corresponding activities was financially justified. The investment of €14,890.70 would be repaid in the 4th year of operations, with a NPV of €3,039.30. In the case of financing by credit, investment may carry a 16.86% discount rate, which will encourage investors.

The investment would provide employees with a monthly income at the average Slovenian salary. As of June 2014, this was €1,521.38 gross; with the estimated visitor numbers, such a figure can be achieved. Moreover, the estimated investment clearly shows positive economic indicators; it is necessary to point out other benefits that tourism brings to the region. Development of tourism brings jobs and encourages investments in infrastructure and the preservation of natural and cultural heritage, which in turn raises the living standard of local people. Without investments, it is impossible to develop any branch. Alternatively, we may also expect impacts based solely on Zen garden, such as increasing interest in meditation, Zen, and/or yoga which is scientifically proven to have a positive impact on the quality of life of people who are engaged in this, as well as for the broader neighbourhood.

REFERENCES

1. Buckley A. International Investment Value Creation and Appraisal – A Real Options Approach. Handelshojskolens Forlag, Copenhagen Business School Press, Netherland, 1998, 272p.
2. Čebokli Z. Investicije. <http://www.akc.si/investicije.php> (16. june 2014).
3. Jiang S. Therapeutic landscapes and healing gardens: A review of Chinese literature in relation to the studies in western countries, *Front. Architec. Res.* 2014;3(2):141–153.
4. Kolar B. Ekologija živali in varstvo okolja divjadi. Ljubljana, Lovska zveza Slovenije. Slovenia. 1999, 225p.
5. Kierulff H. 2008. Business Horizons - MIRR: A better measure. *Bus. Horizons*, 2008;51(4):321–329.

6. Kryžanowski Š. Feng shui: filozofija prostora in psihologija bivanja. Ljubljana, Mladinska knjiga. Slovenia, 2012, 352p.
7. Ministry of Economic Development and Technology. 2012. Strategy of development of Slovenian tourism (2012-2016). http://www.mgrt.gov.si/fileadmin/mgrt.gov.si/pageuploads/turizem/Turizem-strategije_politike/Strategija_turizem_sprejeto_7.6.2012.pdf (23 May 2014)
8. Pažek K., Rozman Č. The real options approach for assessment of business opportunities in spelt processing. *Agricultura*, 2008; 6: 13-17.
9. SAOP d.o.o. <http://www.saop.si/poslovne-informacije/podatki-za-obracun-in-opomniki/povprecne-in-minimalne-place/> (3 September 2014).
10. Turner M.G, Gardner R.H., O'Neill R.V. Landscape Ecology in Theory and Practice: Pattern and Process. Springer, New York. USA. 2001, 406p.
11. Turner M.G. Landscape ecology: what is the state of the science? *Ann. Rev. Ecol. Evol. Syst.* 2005;36:319–44.
12. Vidic F. Kako razviti uspešno podjetje. Gae College. Ljubljana, Slovenia. 1999.
13. Vuk D. Gospodarjenje s tehničnimi sredstvi 2. Investicijski management. Kranj, Moderna organizacija. Slovenia, 2001, 144p.
14. Žnidaršič Krajnc A. Ekonomika podjetja. Postojna, Slovenia. 1994.
15. Žibrat M. The survey: <http://www.mojaanketa.si/anketa/453511944/> (2014)
16. Žibrat M. Poslovni načrt za zen vrt / The business plan for a Zen garden; diplomsko delo. Maribor. 2015. 37 p: <https://dk.um.si/IzpisGradiva.php?id=48000>(17. september 2015).

Poslovni načrt za Zen vrt

IZVLEČEK

Diskutirana je umestitev tematskega parka - zen vrta, kot poslovna priložnost na območju slovenskega podeželja. Natančno je predstavljena zasnova vrta z vsemi glavnimi sklopi standardnega poslovnega načrta: opis podjetja in panoge, opis storitve, analiza trga, strategija trženja, finančne projekcije ter načrt dela in aktivnosti. Finančni vidik je predstavljen z oceno finančnih parametrov kot so višina investicije, neto sedanja vrednost in interna stopnja donosa. Višina investicije projekta je ocenjena na 14.890,70 €, povrnila naj bi se v 4 letih poslovanja. Ocenjena interna stopnja donosa ob povratku investicije je 16,86 %. Del raziskave predstavlja analiza trga - izvedba ankete o poznavanju in zanimanju za zen in zen vrt. Pri načrtovanju zen vrta so upoštevani principi krajinske ekologije, saj postavitev vrta v gozdovsekakor vpliva na krajino.

Antioxidant defences of Norway spruce bark against bark beetles and its associated blue-stain fungus

Mateja FELICIJAN ^{1*}, Metka NOVAK ², Nada KRAŠEVEC ³, Andreja URBANEK KRAJNC ¹

¹University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 10, 2311 Hoče, Slovenia

²Karolinska Institutet, Stockholm, Sweden

³National Institute of Chemistry, Ljubljana, Slovenia

SUMMARY

Bark beetles and their fungal associates are integral parts of forest ecosystems, the European spruce bark beetle (*Ips typographus* Linnaeus, 1758) and the associated pathogenic blue stain fungus *Ceratocystis polonica* (SIEM.) C. MOREAU, are the most devastating pests regarding Norway spruce [*Picea abies* (L.) H. KARST.]. Bark beetles commonly inhabit weakened and felled trees as well as vital trees. They cause physiological disorders in trees by destroying a phloem and cambium or interrupt the transpiration flow in the xylem. Conifers have a wide range of effective defence mechanisms that are based on the inner bark anatomy and physiological state of the tree. The basic function of bark defences is to protect the nutrient- and energy-rich phloem, the vital meristematic region of the vascular cambium, and the transpiration flow in the sapwood. The main area of defence mechanisms is secondary phloem, which is physically and chemically protected by polyphenolic parenchyma (PP) cells, sclerenchyma, calcium oxalate crystals and resin ducts. Conifer trunk pest resistance includes constitutive, inducible defences and acquired resistance. Both constitutive and inducible defences may deter beetle invasion, impede fungal growth and close entrance wounds. During a successful attack, systemic acquired resistance (SAR) becomes effective and represents a third defence strategy. It gradually develops throughout the plant and provides a systemic change within the whole tree's metabolism, which is maintained over a longer period of time. The broad range of defence mechanisms that contribute to the activation and utilisation of SAR, includes antioxidants and antioxidant enzymes, which are generally linked to the actions of reactive oxygen species (ROS). The presented review discusses the current knowledge on the antioxidant defence strategies of spruce inner bark against the bark beetle (*Ips typographus*) and associated blue stain fungus (*Ceratocystis polonica*).

Key words: antioxidants, ascorbate-glutathione system, blue-stain fungus *Ceratocystis polonica* (SIEM.) C. MOREAU, Norway spruce (*Picea abies* (L.) H. KARST.), phenolics, systemic acquired resistance (SAR)

THE BIOLOGY BEHIND THE ASSOCIATION OF SPRUCE BARK BEETLE WITH BLUE-STAIN FUNGI

Over their long lifetimes, conifers are targets of numerous attacks by different pests such as insects, other herbivores, fungi, and bacteria. They pave their way towards nutrient-rich phloem through the bark. Phloem and cambium represent a relatively small part of a tree's trunk, which could be even

faster and more easily damaged and destroyed than other tissues (Franceschi et al. 2005).

The majority of bark beetle species are considered as rather harmless species in their native ranges, colonising mainly weakened or dead trees and thereby represent an important ecological factor in forest rejuvenation (Müller and Job 2009, Smith et al. 2011, Novak et al. 2014). However, these species pose potential risks in the case of significant increase in abundance of populations and within changing or new environments. They should not be ignored when evaluating

*Correspondence to:

E-mail: mateja.felicijan@um.si

risks and threats to forest ecosystems or when determining quarantine measures for pests and pathogens. Forest pest insects and their associated micro-organisms are capable of movement through national boundaries and have a potential impact on forest-dependent industries (Christiansen and Bakke 1988, Linnakoski et al. 2012, Sallé et al. 2005, Raffa et al. 2008, Faccoli 2009, Smith et al. 2011).

One of the most destructive forest insects in Europe is the spruce bark beetle *Ips typographus* (Linnaeus 1758.) (Curculionidae, Scolytinae), which affects Norway spruce and is with its fungal associates, the pathogenic blue-stain fungus *Ceratocystis polonica* (SIEM.) C. MOREAU the integral parts of the forest ecosystems (Wermelinger 2004, Klepzig et al. 2009, Linnakoski et al. 2012, Novak et al. 2014). Their symbiotic relationship has been described as mutualistic (Whitney 1982). Although a number of studies have been devoted to resolving the nature of bark beetle-fungus interactions, these interactions remain poorly understood (Jurc 2011, Six and Wingfield 2011).

The fluctuations in bark beetles' populations are interactions between at least three main biological components: the host tree, bark beetles, and pathogen fungi (Novak 2014).

Normally has *Ips typographus* two (spring and summer) generations per year (Fig. 1). If global warming extends the growing season, a higher proportion of the second generation may reach the cold at the hardy adult stage and survive the winter (Holger et al. 2006). Bark beetles construct galleries under the bark within the phloem layers of woody plants, mostly in weakened or recently killed trees, where they lay eggs and their brood feed and develop during the winter (Six and Wingfield 2011).

Adult beetles form wounds through the bark into the phloem, as a source of food and the place of reproduction, to the cambium. Parental adults construct an initial egg gallery, which is continued by larvae into the larval galleries (Raffa et al. 2008). During the constructions of galleries, bark beetles serve as vectors for several types of micro-organisms that enter into the host tree. They benefit the fungus because it is transferred into a nutrient-rich environment, where competition is limited (Whitney 1982, Beaver 1989, Paine et al. 1997). Specific roles of fungal associates in the *Ips typographus* symbiotic system and some of its underlying molecular mechanisms have been the topics of considerable previous work (Krokene and Solheim 1998, Hammerbacher et al. 2013, Urbanek Krajnc et al. 2014).

Now it is known that within a symbiotic system bark beetles serve as vectors for fungi, which would otherwise be unable to reach a new host (Six and Wingfield 2011), alternatively fungi benefit the beetle as sources of nutrients, or they may weaken tree defences (Linnakoski et al. 2012). The concept that tree-killing bark beetles require fungal pathogens to overcome tree defences and to incur tree mortality has received the most attention over the years. This hypothesis, which is referred to as the classic paradigm, has formed the basis for the majority of research conducted on these interactions (Six and Wingfield 2011). Blue-stain fungi are thought to be primarily responsible (Johnson and Croteau 1987) or required (Whitney 1982) for mortality regarding conifers attacked by bark beetles.

Such conclusions are based on observations that:

- beetles are capable of vectoring or dispersing the fungi,
- beetles are rarely found in the absence of staining fungi,
- sapwood of beetle-killed trees is stained (Paine et al. 1997).

Although the mechanisms are not fully understood, a tree is killed as a result of the simultaneous activities and interactions of both organisms, rather than the successive actions of beetles and pathogen (Nebeker and Hodges 1993, Paine et al. 1997). On the other hand, Six and Wingfield (2011) suggest that fungal phytopathogenicity has a more important role for the fungi, rather than supporting the bark beetles in tree killing. It may be a factor helping the fungi to survive within a living tree.

DEFENCE STRATEGIES OF NORWAY SPRUCE BARK

Due to the variety of pests the conifers evolve complex anatomical and chemical defences against pathogen attack, which may deter beetle invasion, impede fungal growth, and seal entrance wounds (Rohde et al. 1996, Bonello et al. 2001, Baier et al. 2002, Wermelinger 2004, Franceschi et al. 2005). Bark anatomy and the physiological condition of a potential host tree are crucial for the success of an attack/infection. Until now, three types of tree's defence strategies have been described: constitutive, induced defences and systemic acquired resistance (Franceschi et al. 2005). Defence mechanisms are based on the bark anatomy and

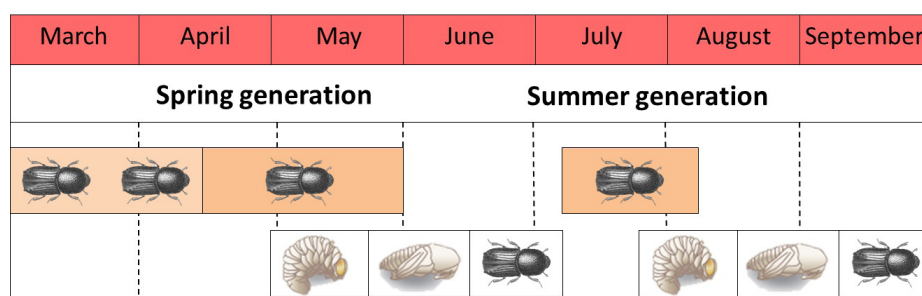


Fig.1: Generalised life-cycle of the spruce bark beetle (*I. typographus*), which normally has two generations per year - spring and summer generations. Bark beetles construct galleries, lay eggs and at the same time inoculate fungi into the bark phloem of woody plants(Six and Wingfield 2011).

its physiological condition. The main area of defence mechanisms is secondary phloem, which represents both physical and chemical protections (Hudgins and Franceschi 2004).

Constitutive and induced defence

An effective defence is provided by a multi-stage system, which runs as a series of different resistance mechanisms. Conifer trunk includes constitutive and inducible defences, which have attracted much attention over recent years. Most of the new knowledge has been obtained on resin-producing and resin-storing structures. Furthermore, quantitative and qualitative changes in the local metabolism have been studied around the entrance hole. These investigations have focused on phenolics, terpenoids and protein-based chemical defences (Brignolas et al. 1998, Evensen et al. 2000, Nagy et al. 2004, Wermelinger 2004, Franceschi et al. 2005, Erbilgin et al. 2006, Zeneli et al. 2006). Without prior attack the bark implements mechanical and chemical constitutive passive defence mechanisms. It involves initiation of a wound periderm, and based on bark anatomy also formation of cells with phenols, resin ducts, concentric layers of polyphenolic parenchyma (PP) cells, sclerenchyma and calcium oxalate crystals (Hudgins and Franceschi 2004, Franceschi et al. 2005, Hudgins et al. 2004, Krekling et al. 2004).

An invasion activates inducible defence systems, which include secondary resin production, synthesis of new phenolics, and protein-based chemical defences. The induced defence detects attacks and closes the initial wounds by the formation of resin ducts, which contribute to increased synthesis of terpenes and resin flow. The induced defence is more effective and represents a more sustainable method of defence, as is provided by qualitative and quantitative changes in the chemical compositions of the trees' metabolisms. Furthermore, induced defence increases the formations of new PP cells and phenolic compounds, which are toxic for invasive organisms (Hudgins and Franceschi 2004, Franceschi et al. 2005, Schmidt et al. 2005, Witzel and Martin 2008). Pathogens use mechanical force or release cell wall degrading enzymes to break down this barrier. At the cell wall, they also trigger pathogen-associated molecular patterns (PAMPs) either inadvertently or as a consequence of plant degradative enzymes (e.g. the release of chitin oligomers by plant chitinases). Plants, in turn, appear to sense these PAMPs and damage to their cell walls and activate a variety of defences, including the production of reactive oxygen species (ROS), the production and export of anti-microbial compounds and fortification of their cell walls. PAMPs also activate a localised and systemic acquired resistance (LAR and SAR), initiating the synthesis of pathogenesis-related proteins (PR proteins), installation of lignin and accumulation of phenols and toxins (Hématy and et al. 2009). In addition, sensing PAMPs may activate intracellular defences such as the salicylic acid pathway, perhaps priming the plant for the next stage of warfare.

Collectively, constitutive and inducible compounds may deter beetle invasion, impede fungal growth, and seal entrance wounds (Bonello et al. 2001, Nagy et al. 2004, Wermelinger

2004, Franceschi et al. 2005, Zeneli et al. 2006, Rodrigues et al. 2009).

SAR - systemic acquired resistance

The last and very important defence strategy is composed of a systemic acquired resistance (SAR), which becomes affective during successful attack and ensures faster and easier responses to attacks in the future (Evensen et al. 2000, Percival 2001, Nagy et al. 2004, Franceschi et al. 2005, Bonello et al. 2006, Witzel and Martin 2008). This mechanism gradually develops throughout the plant during pest colonisation, and provides a systemic change in the whole tree metabolism. This leads to the production of fewer carbohydrates but more proteins, which are needed for defence (Viiri et al. 2001, Wermelinger 2004). The broad range of defence mechanisms, which contribute to the appearance of SAR, include antioxidants (ascorbic acid, cysteine, glutathione, total phenols) and antioxidant enzymes (Foyer and Rennenberg 2000, Riedle-Bauer 2000, Urbanek Krajnc 2004, 2009), which are generally linked to the actions of ROS (Hayat et al. 2009). Although anti-oxidative defence systems are often used as stress indicators for the diagnosis of disturbances in forest trees (Foyer and Rennenberg 2000, Riedle-Bauer 2000, Tausz et al. 2003, 2004, Grill et al. 2001, Tegischer et al. 2002), to date only a few investigations have dealt with anti-oxidative system of attacked Norway spruce (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2014).

While several studies have demonstrated that the induction of phenolics occurs on a local scale (Evensen et al. 2000, Franceschi et al. 2000, 2005), several recent studies have also found evidence of the systemic induction of soluble low molecular weight phenolics (Bonello et al. 2001, 2006, Wallis et al. 2008). A SAR hypothesis postulated by Bonello et al. (2006) illustrated the interplay between SAR and induced susceptibility in trees against microbes and herbivores. The authors postulated that the time course of pathogen infection may have a bell-shaped effect on the strength of SAR that is similar to the spatial variations of constitutive secondary metabolites generated by resource availability. In the earliest stages of pathogen infection, SAR responses are predicted to rapidly and systemically increase concentrations of compounds involved in defence against pathogens and insects. However, if the pathogen is able to grow despite the deployment of localised defence responses, the infection will progress, and the tree will become increasingly stressed by the resulting resource limitations (e.g. reduction of sap flow, limited nutrient and water absorption, decreased carbon assimilation and growth). Consequently, a degradation of molecules involved in SAR would follow.

SAR has been actively studied in herbaceous plant species, and, over recent years, in woody plant species (Bonello et al. 2006, Eyles et al. 2009), and is fast emerging as an intriguing, eco-friendly concept for enhancing tree resistance. However, before applying SAR becomes possible, there is a need to increase our knowledge of the mechanisms of defence in forest trees. Over recent years, research has focused on studying the impact of exogenous methyl jasmonate (MJ) and SA treatment on the inductions of certain metabolites

which are involved in SAR. Many authors (Franceschi et al. 2002, Hudings and Franceschi 2004, Hudings et al. 2004, Schmidt et al. 2005, Erbilgin et al. 2006) have examined the effect of MJ treatment in combination with the induced resistance of Norway spruce, where the positive impacts have been observed of MJ treatment and bark beetle attacks or *Ceratocystis polonica* infection. They reported that MJ treatment stimulated the synthesis and accumulation of terpenes (Kozłowski et al. 1999, Franceschi et al. 2002, Martin et al. 2002, 2003, Fäldt et al. 2003, Hudgins and Franceschi 2004, Hudgins et al. 2004, Miller et al. 2005, Erbilgin et al. 2006, Zeneli et al. 2006).

In our previous field experiment (Urbanek Krajnc et al. 2011), 100 mM SA was applied to the bark sections of Norway spruce prior to being attacked by bark beetles, in order to study interactions with antioxidants and their significance for mediating stress-tolerance under natural conditions. SA-treatments significantly elevated the total SA levels over the whole sampling period. Total glutathione (tGSH) and total cysteine (tCys) increased, respectively, two weeks after treatment, in comparison with controls. One month after pheromone dispensers were placed on trees, an intensification of the ascorbate-glutathione system occurred within moderately-affected bark but to a greater extent after SA-treatment. Total SA levels within SA-treated moderately-affected trees remained at the control level until June. In contrast, strong attack was characterised by a successive increase in total SA and degradation of tGSH as well as total phenolics (tPH), a moderate increase in total ascorbate (tASC) and an oxidation of the ascorbate-glutathione pool within non-treated bark.

Furthermore, at the end of the three month experiment, the SA-treated bark had less entrance holes, and exhibited fewer and shorter maternal galleries than the control-bark. From this perspective, exogenous SA was successfully implicated as an activator of SAR in Norway spruce, providing tolerance against the complex interactive effects of bark beetle attack and environmental factors (Urbanek Krajnc et al. 2011).

OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE OF CONIFER METABOLISMS

Antioxidants play an important role in chemical defence as they prevent the oxidations of other substances such as inactivate oxygen compounds and inhibit the formations of free radicals (Brodnjak Vončina 2006). Antioxidants are classified into three groups: real antioxidants that bind free radicals; reducing agents, while the third group consists of antioxidant synergists (Kugler 2011).

Both biotic and abiotic stress factors trigger plant metabolism changes and mostly react with the reactive oxygen species (ROS; H_2O_2). Oxidative stress reflects an imbalance between the systemic manifestations of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox states of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell.

Antioxidants can slow down or even totally stop the oxidations of these molecules by hunting the free radicals and by removing oxidative damaged biomolecules (Kugler 2011, Veberič 2010, Harris and Hansen 2012). The antioxidants do not accumulate in the cytoplasm but in the vacuoles and other organelles such as plastids and mitochondria, where they are urgently needed as an effective "redox shuttle" (Kreft et al. 2000).

Phenolics

Among antioxidants phenolics represent a more important component of the inducible defence strategy regarding conifer bark. They range from simple, low molecular weight, single aromatic-ringed compounds to large and complex tannins and derived at polyphenols that provide different chemical and structural defence strategies.

In regard to the synthesis and accumulation of phenolic compounds, the barks of all conifer families have polyphenolic parenchyma cells (Krekling et al. 2000, Franceschi et al. 2000, 2005, Schmidt et al. 2005). Within their vacuoles, polyphenolic parenchyma cells contain variable amounts of phenolic bodies that are thought to serve as antifeedant and antifungal agents (Beckman 2000). Polyphenolic parenchyma cells are also major sites for storing starch and lipids (Krekling et al. 2000). In this capacity, they can be seen as targets for beetles and fungi, and constitutive phenolics can be hypothesised to protect the cells themselves, as well as prevent fungal penetration towards the cambial zone. In any case, the multiple layers of polyphenolic parenchyma cells providing physical and chemical resistance to penetration of the bark (Franceschi et al. 2000, 2005, Schmidt et al. 2005).

In our previous study on Norway spruce, the increase in total phenolics' concentrations two weeks after a bark beetle attack was recognized as an immediate inducible response to the attack (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2011). Additionally, phenolics accumulated, to a higher extent, in the control tissue, which was explained as a wound reaction induced by mechanical injury (Christiansen et al. 1999, Franceschi et al. 2005, Ralph et al. 2006, Urbanek Krajnc 2009). The increase in total phenolics during the initial bark beetle colonisation was accompanied by the degradations of total ascorbic acid and tGSH, as well as oxidation of the ascorbate-glutathione pool. These initial events pointed out that the antioxidant defence is a multi-tier system with a spatial and temporal component. The spatial component is determined by the positions of PP cells' concentric rings from the periderm surface to the cambial zone (Franceschi et al. 2005) and the temporal component consisted of seasonal, continuous and enhanced production of phenolics in the attacked tissue. However, at later sampling dates, the trees with moderate attack were characterised by increased and steady-states of the tPH concentration, whereas the strong attacks were shown by drastically decreased concentrations of tPH (Urbanek Krajnc 2009). As the PP cells are the primary sites of phenolic biosynthesis within the secondary phloem (Franceschi et al. 2000, 2002, 2005), an assumption can be made that the synthesis of phenolics is lacking when the phloem is damaged, by the establishment of a complete

breeding system.

The more important phenolics in the Norway spruce phloem are stilbenes, flavonoids, as well as tannins (e.g. Lieutier et al. 2003, Schmidt et al. 2005, Witzell and Martin 2008, Hammerbacher et al. 2011). In general, phenolics fulfil different defensive functions. Most of the phenolics contribute to resistance indirectly. For instance, some low molecular weight phenolic compounds may function as precursors for other defensive compounds (e.g. lignin; Bonello and Blodgett 2003), or they may confer resistance as a group, rather than as individual compounds (Wallis et al. 2008). Catechin is a building block of condensed tannins for which antimicrobial activity via protein precipitation and iron depletion has been suggested (Witzell and Martin 2008).

Significant quantities of stilbene in pine bark were found years ago (Sjöström 1993), while now it is known that pine bark extract contains several types of stilbene (the more important being astringin and isorapontin) and flavonoids. Changes in the concentrations of stilbenes and flavonoids in Norway spruce in response to injury or fungal infection are considered to be an active defence response (Brignolas et al. 1995, 1998, Viiri et al. 2001, Havsteen 2002, Schmidt et al. 2005, Witzell and Martin 2008, Li et al. 2012, Hammerbacher et al. 2011, 2013). Stilbenes are known to inhibit fungal growth by interfering with microtubule assembly (Woods et al. 1995, Adrian et al. 1997), disrupting plasma membranes and uncoupling electron transport within fungal spores and germ tubes (Pont and Pezet 2008, Adrian and Jeandet 2012). Stilbenes have also been shown to protect plants against oxidative stress (He et al. 2008), to deter herbivores (Torres et al. 2003), and to inhibit the growth of competing plants (Fiorentino et al. 2008).

Specific phenolic compounds in spruce inner bark against *Ceratocystis polonica* infection were previously interpreted by Urbanek Krajnc et al. (2014). Increases in tPH, catechin, astringin, isorhapontin and taxifolin were monitored. By analysing each single phenolic compound, on later sampling dates, it could be observed that within certain time shifts the phenolics followed the eco-physiological concept and basically fit with the temporal sequence of changes in tPH concentrations after the moderate bark beetle attack, previously reported on the spruce/bark beetle pathosystem (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2011). The initial decline in catechin was followed by an increase in catechin concentration until September, when taxifolin accumulated within the infected bark at significantly higher levels until June, and later the concentrations dropped toward the control levels. On the other hand, a slight accumulation of astringin observed in response to fungi infection, when the concentration at later sampling dates dropped. The same was for isorhapontin concentration, where a dramatic initial increase was observed, and later it simply diminished (Urbanek Krajnc et al. 2014).

Ascorbate-glutathione cycle

Besides phenolics, ascorbate–glutathione system plays an important role in plant metabolism and defence. The ascorbate–glutathione cycle is considered to be the main pathway for ROS removal, and both ascorbate and glutathione

are recognised as the heart of the redox hub within the cell (Foyer and Noctor 2012).

Amongst antioxidants, glutathione is a low molecular sulphur metabolite, which plays multiple roles within tree-environment interactions and defences (Grill et al. 2001, Tausz et al. 2004, Zhao et al. 2008, Noctor 2006, 2012). It functions as a reductant in the enzymatic detoxification of ROS within the glutathione-ascorbate cycle and as a thiol buffer in the protection of proteins via direct reaction with ROS or by the formation of mixed disulphides (Zhao et al. 2008). In this role it has been suggested as a general redox sensor and signalling agent in plant cells (Meyer and Hell 2005, Cameron and Pakrasi 2010, Noctor et al. 1998, 2012).

Owing to its redox-active thiol group, GSH has often been considered as playing an important role in plant defence against oxidative stress (Grant et al. 1996). Trees under stress seem to generally require and synthesise higher concentrations of glutathione (Gullner and Kömives 2001, Tausz et al. 2003, 2004). Glutathione synthesis depends on

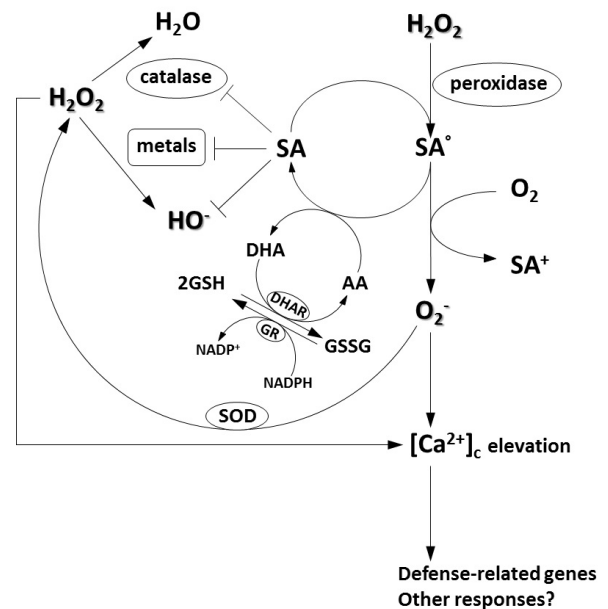


Fig. 2: A model for the mechanism of SA action within the generation of active oxygen species, which induces the expression of the defence-related genes via an increase in $[Ca^{2+}]_c$. SA and a trace of H_2O_2 are used for the SA \cdot -generating peroxidase reaction. Then the resultant SA \cdot reacts with O_2 to produce $O_2^{\cdot-}$ that triggers an increase in $[Ca^{2+}]_c$. The increased $[Ca^{2+}]_c$ may induce further physiological responses including the induction of PR genes. Ascorbate reconverts SA \cdot to SA yielding monodehydroascorbate. An excess of ascorbate may inhibit the SA \cdot -dependent generation of $O_2^{\cdot-}$. While the generation of $O_2^{\cdot-}$ is going on, SA inhibits the decomposition of H_2O_2 by catalase and a Fenton-type reaction. SA also lowers the $HO\cdot$ level by blocking the Fenton reaction and by directly trapping $HO\cdot$. Thus SA protects the cells from highly reactive $HO\cdot$, while producing the less reactive $O_2^{\cdot-}$ and H_2O_2 through a peroxidase-catalysed reaction, as the intermediate signals.

the distribution and cycling of sulphur in trees. Glutathione biosynthesis is restricted to the cytosol and the plastids, but mitochondria, which lack the capacity for glutathione biosynthesis, have been described as major sites of glutathione accumulations (Zechmann et al. 2007).

In all cells where GSH is found, besides reduced tripeptide form, the oxidised form of GSH (glutathione disulphide: GSSG) is also present in small quantities (< 20 %). While glutathione reductase (GR) uses NADPH to reduce GSSG to GSH, various free radicals and oxidants are able to oxidise GSH to GSSG (Fig. 2).

Both GSH and GSSG are considered as markers of oxidative stress in the plant, by increasing the antioxidant pool and as actors in cell signalling. Based on the current understanding of redox status and the roles of ROS, thiols, oxidisers and cellular antioxidants, scientists have redesigned a definition of oxidative stress (Harris and Hansen 2012), now it is based on changes in translation of thiol proteins, important for the control of the redox potential. Thiol redox couples such as GSH / GSSG, cysteine / cystine (Cys / cySS) form independent centres within the cells, which are linked to changes in the redox potential.

Glutathione is central to the regeneration of ascorbate within the ascorbate-glutathione cycle (Tausz et al. 2003, 2004). Glutathione, as an antioxidant, together with ascorbate, removes ROS, including hydrogen peroxide, superoxide and hydroxide radicals, which generate in cells as a result of oxidative stress (Xiang et al. 2001, Alscher 2006). In addition to being the most abundant water-soluble antioxidant in plant cells (Smirnoff and Wheeler 2000), ascorbic acid (AA) is also required for the re-conversion of SA, as ascorbate is highly reactive against phenoxyl radicals generated by peroxidases during oxidative stress (Fig. 2) (Kawano and Muto 2000, Smirnoff and Wheeler 2000). Together with glutathione, it is present in oxidation-reduction processes within the ascorbate-glutathione cycle. Dehydroascorbate (DHA) formed when ascorbate is oxidised, must be reduced again in order to prevent decreases in the total ascorbate pool (Likar and Regvar 2003, Šuštaršič 2012). At alkaline pH values, GSH rapidly reduces DHA to ascorbate in a non-enzymic reaction. In plant tissues this reaction is catalysed by dehydroascorbate reductase (DHAR) (Noctor et al. 1998). The ascorbate glutathione cycle is essential for the removal of ROS and connects the conversion of ascorbic acid and glutathione.

Ascorbic acid is due to the two hydroxyl groups being one of the stronger reductants in the cell and allows the detoxification of reactive oxygen species. In the cell metabolism AA operates in oxidation-reduction processes, in the ascorbate-glutathione chain, where it is reversibly oxidised to DHA. At this stage, the oxidised form of ascorbic acid is restored via glutathione, when the GSH is oxidised to GSSG, and at the same time increases the GSSG / GSH ratio and the activity of GR (Foyer and Noctor 2012).

Although the antioxidant response is relatively well-documented on the level of spruce needles (Tegischer et al. 2002, Tausz et al. 2004), there is little known about changes in the ascorbate-glutathione chain at the inner bark level during bark beetle and fungus infection. In two of our previous experiments, the roles of thiols and ascorbate in the spruce

inner bark were studied during the five month sampling period after the spruce inner bark was exposed to beetle attack and *Ceratocystis polonica* infection (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2014).

The initial reaction of Norway spruce to *Ceratocystis polonica* infection was characterised by a significantly more oxidised glutathione pool which was accompanied by higher GR activity. At the same time, the tCys dropped slightly below the levels of the non-infected samples and the cysteine redox state shifted towards a more oxidised value. Similarly, in one of the previous experiments, 2 weeks after the exposures of spruce trees to bark beetles slight but insignificant decreases were measured for tCys as well as tGSH, which were accompanied by a slightly more oxidised glutathione redox state (Urbanek Krajnc 2009). Until July, the infected inner bark was characterised by a gradual increase in tGSH, which was accompanied by a significantly increasing GR activity. Furthermore, within the infected inner bark the tCys remained elevated over the whole sampling period. Based on a previous experiment Urbanek Krajnc et al. (2009, 2014), concluded that the antioxidant shift within the *Ceratocystis polonica* infected bark indicated a successful defence reaction, which was characterised by a higher accumulation of thiols and a more reduced redox state. In September, the glutathione system reached a steady-state, and the GR activity and tCys concentrations remained increased. Consequently, thiols accumulated in May were accompanied by a significant degradation of tASC within the *Ceratocystis polonica* infected inner bark until July, followed by a significant accumulation in September. However, when comparing the tASC contents in the *Ceratocystis polonica* infected samples, the concentrations increased continuously from the spring onwards to September, which reflected increased pressure on the ascorbate–glutathione cycle (Urbanek Krajnc 2009, 2014).

CONCLUSIONS

Our previous field experiments (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2011, 2014) demonstrated that the sequence of changes in the endogenous levels of antioxidant molecules within the affected Norway spruce inner bark strengthened the general eco-physiological stress-response concept as suggested by Larcher (2003) and Tausz et al. (2004).

The time-course analysis of the individual molecules enabled us to establish a relationship between salicylic acid, phenolics and the ascorbate–glutathione system in response to the complex interactive effects of bark beetle attack, infection by blue-stain fungus, and environmental factors (Urbanek Krajnc 2009, Urbanek Krajnc et al. 2011, 2014). Based on the ascorbate–glutathione concept of Foyer and Noctor (2011), phenolics within the concentric layers of PP cells can be viewed as a wheel of the bark defence mechanism, driven by the ascorbate–glutathione system, as a central part or redox hub of the wheel that integrates metabolic information and environmental stimuli to tune defence responses against pathogen and pests. The presented article reviews the current knowledge on physiological and biochemical defense

responses of Norway spruce defence against bark beetles and associated fungi and also allows predictions of antioxidative defence responses in other conifer pathosystems.

REFERENCES

- Adrian M, Jeandet P, Veneau J, Weston LA, Bessis R. Biological activity of resveratrol, a stilbenic compound from grapevines, against *Botrytis cinerea*, the causal agent for grey mould. *J. Chem. Ecol.* 1997;23:1689–1702.
- Adrian M and Jeandet P. Effects of resveratrol on the ultrastructure of *Botrytis cinerea* conidia and biological significance in plant/pathogen interactions. *Fitoterapia* 2012;83:1345–1350.
- Alscher RG. Biosynthesis and antioxidant function of glutathione in plants. *Physiol. Plantarum* 2006;77:457–464.
- Baier P, Führer E, Kirisits T, Rosner S. Defence reactions of Norway spruce against bark beetles and the associated fungus *Ceratocystis polonica* in secondary pure and mixed species stands. *For. Ecol. Manage.* 2002;159:73–86.
- Beaver RA. Insect-fungus relationship in the bark and ambrosia beetles. In: Wilding NM, Collins PMH and JFW (Eds). *Insect-Fungus Interactions*. San Diego, CA, Academic Press, 1989;119–143.
- Beckman CH. Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? *Phys. Mol. Plant Pathol.* 2000;57:101–110.
- Bonello P, Gordon TR, Storer AJ. Systemic induced resistance in Monterey pine. *For. Pathol.* 2001;31:99–106.
- Bonello P, Blodgett JT. *Pinus nigra*–*Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. *Physiol. Mol. Plant Pathol.* 2003;63:249–261.
- Bonello P, Gordon TR, Herms DA, Wood DL, Erbilgin N. Nature and ecological implications of pathogen-induced systemic resistance in conifers: A novel hypothesis. *Physiol. Mol. Plant Pathol.* 2006;68(4–6):95–104.
- Brignolas F, Lacroix B, Lieutier F, Sauvard D, Drouet A, Claudot AC, Yart A, Berryman AA, Christiansen E. Induced responses in phenolic metabolism in two Norway spruce clones after wounding and inoculations with *Ophiostoma polonicum*, a bark-beetle associated fungus. *Plant Physiol.* 1995;109(3):821–827.
- Brignolas F, Lieutier F, Sauvard D, Christiansen E, Berryman AA. Phenolic predictors for Norway spruce resistance to the bark beetle *Ips typographus* (Coleoptera: Scolytidae) and an associated fungus, *Ceratocystis polonica*. *Can. J. For. Res.* 1998;28:720–728.
- Brodnjak Vončina D. Antioksidanti Analizna kemija II, Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo. 2006.
- Cameron JC and Pakrasi HB. Essential role of glutathione in acclimation to environmental and redox perturbations in the cyanobacterium *synechocystis* sp. PCC 6803. *Plant Physiol.* 2010;154: 1672–1685.
- Christiansen E in Bakke A. The spruce bark beetle of Eurasia. In: Berryman AA (Ed). *Population ecology: theory and application*. New York, Plenum Press, 1988;479–503.
- Christiansen E, Krokene P, Berryman AA, Franceschi VR, Krekling T, Lieutier F, Lonneborg A, Solheim H. Mechanical injury and fungal infection induce acquired resistance in Norway spruce. *Tree Physiol.* 1999;19:399–403.
- Erbilgin N, Krokne P, Christian E, Zeneli G, Gershenson J. Exogenous application of methyl jasmonate elicits defenses in Norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*. *Oecologia* 2006;148:426–436.
- Evensen PC, Solheim H, Hoiland K, Stenersen J. Induced resistance of Norway spruce, variation of phenolic compounds and their effects on fungal pathogens. *Forest Pathol.* 2000;30:97–108.
- Eyles A, Bonello P, Ganley R, Mohammed C. Induced resistance to pests and pathogens in trees. *New Phytol.* 2009;185:893–908.
- Fäldt J, Martin D, Miller B, Rawat S, Böhlmann J. Traumatic resin defense in Norway spruce (*Picea abies*): methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. *Plant Mol. Biol.* 2003;51:119–133.
- Felicijan M. Časovna analiza vsebnosti kondenziranih taninov v lubju navadne smreke pri napadu podlubnikov. Univerza v Mariboru. Fakulteta za kmetijstvo in biosistemske vede. Diplomsko delo 2011.
- Foyer CH, Rennenberg H. Regulation of glutathione synthesis and its role in abiotic and biotic stress defence. In: Brunold C, Rennenberg H, De Kok LJ, Stulen I, Davidian J-C (Eds). *Sulfur nutrition and sulfur assimilation in higher plants*. Paul Haupt, Bern 2000;127–153.
- Foyer CH in Noctor G. Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol.* 2011;155:2–18.
- Franceschi VR, Krokene P, Krekling T, Christiansen E. Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (*Pinaceae*). *Am. J. Bot.* 2000;87:314–326.
- Franceschi VR, Krekling T, Christiansen E. Application of methyl jasmonate on *Picea abies* (*Pinaceae*) stems induces defense-related responses in phloem and xylem. *Am. J. Bot.* 2002;89(4):578–586, 602–610.
- Franceschi VR, Krokene P, Christiansen E, Krekling T. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New phytol.* 2005;167(2):353–376.
- Grant CM, MacIver FH, Dawes IW. Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* 1996;29(6):511–515.
- Grill D, Tausz M and De Kok LJ. Significance of glutathione in plant adaptation to the environment. In: De Kok LJ (Ed). *Handbook of Plant Ecophysiology*. Kluwer Academic Publishers, Dordrecht, 2001.
- Gullner G, Komives T. The role of glutathione and glu-

- tathione-related enzymes in plant-pathogen interaction. In: Grill D, Tausz M, De Kok LJ (Eds). Significance of glutathione to plant adaptation to the environment. Kluwer Academic Publishers, Dordrecht, Boston, London, 2001;207–239.
29. Hammerbacher A, Ralph SG, Bohlmann J, Fenning TM, Gershenzon J, Schmidt A. Biosynthesis of the major tetrahydroxystilbenes in spruce, astringin and isorhapontin, proceeds via resveratrol and is enhanced by fungal infection. *Plant Physiol.* 2011;157:876–890.
 30. Hammerbacher A, Schmidt A, Wadke N, Wright LP, Schneider B, Bohlmann J, Brand WA, Fenning TM, Gershenzon J, Paetz C. A common fungal associate of the spruce bark beetle metabolizes the stilbene defenses of Norway spruce. *Plant Physiol.* 2013;162:1324–1336.
 31. Harris C, Hansen JM. Oxidative stress, thiols, and redox profiles. *Methods Mol. Biol.* 2012;889:325–46.
 32. Hayat Q, Hayat S, Ifran M Ahmad A. Effect of exogenous salicylic acid under changing environment: A review. *Environ. Exp. Bot.* 2009;68:14–25.
 33. Havsteen BH. The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.* 2002;96(2–3):67–202.
 34. He S, Wu B, Pan YJ, Jiang LY. Stilbene oligomers from *Parthenocissus laetevirens*: isolation, biomimetic synthesis, absolute configuration, and implication of antioxidative defense system in the plant. *J. Org. Chem.* 2008;73(14):5233–5241.
 35. Holger L, Bjørn Ø and Krokene P Thresholds in the life cycle of the spruce bark beetle under climate change. *Int. J.* 2006;1–10.
 36. Hudings JW, Christiansen E, Franceschi VR. Induction of anatomically based defense responses in stems of diverse conifers by methyl jasmonate: a phylogenetic perspective. *Tree Physiol.* 2004;24:251–264.
 37. Hudings JW and Franceschi VR. Methyl jasmonate-induced ethylene production is responsible for conifer phloem defense responses and reprogramming of stem cambial zone for traumatic resin duct formation. *Plant Physiol.* 2004;135(4):2134–2149.
 38. Johnson MA and Croteau R. Ecology and metabolism of plant lipids In: Fuller G and Nes WD (Eds). Washington DC, American Chemical Society, 1987.
 39. Jurc M. Gozdna zoologija. Ljubljana. Biotehniška fakulteta. Oddelek za gozdarstvo in obnovljive gozdne vire. Narodna in univerzitetna knjižnica. 2011.
 40. Kawano T and Muto S. Mechanism of peroxidase actions for salicylic acid induced generation of active oxygen species and an increase in cytosolic calcium in tobacco cell suspension culture. *J. Exp. Bot.* 2000;345(51):685–693.
 41. Klepzig KD, Adams AS, Handelsman J, Raffa KF. Symbioses: A key driver of insect physiological processes, ecological interactions, evolutionary diversification, and impacts on humans. *Environ. Entomol.* 2009;38(1):67–77.
 42. Kozłowski G, Buchala A, Metraux JP. Methyl jasmonate protects Norway spruce (*Picea abies* (L.) Karst.) seedlings against *Pythium ultimum* Trow. *Phys. Mol. Plant Path.* 1999;55(1):53–58.
 43. Kreft I, Škrabanja V, Bonafaccia G. Temelji prehranskih in biotskih vplivov antioksidantov. In: Žlender B, Gašperlin L. (Eds). Antioksidanti v živilstvu. 20. Bitenčevi živilski dnevi 2000. Ljubljana, Biotehniška fakulteta, Oddelek za živilstvo. 2000;33–37.
 44. Krekling T, Franceschi VR, Berryman AA, Christiansen E. The structure and development of polyphenolic parenchyma cells in Norway spruce (*Picea abies*) bark. *Flora* 2000;195:354–369.
 45. Krekling T, Franceschi VR, Krokene P, Solheim H. Differential anatomical responses of Norway spruce stem tissues to sterile and fungus infected inoculations. *Trees* 2004;18:1–9.
 46. Krokene P and Solheim H. Pathogenicity of four blue-stain fungi associated with aggressive and nonaggressive bark beetles. *Phytopathology* 1998;88:39–44.
 47. Krokene P and Solheim H. What do low-density inoculations with fungus tell us about fungal virulence and tree resistance?. In: Lieutier F, Mattson WJ and Wagner MR (Eds). Physiology and genetics of tree-phytophage interactions. INRA Editions Versailles, France, 1999;353–362.
 48. Kugler N. Vpliv temperature in čas ekstrakcije na vsebnost antioksidativnih učinkovin iz iglavcev. Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo. Diplomsko delo 2011.
 49. Larcher, W. Physiological plant ecology. Springer Verlag, Berlin, 2003.
 50. Li S-H, Nagy NE, Hammerbacher A, Krokene P, Niu X-M, Gershenzon J, Schneider B. Localization of phenolics in phloem parenchyma cells of Norway spruce (*Picea abies*). *ChemBio-Chem.* 2012;13:2707–2713.
 51. Lieutier F, Brignolas F, Sauvard D, Yart A, Galet C, Brunet M, Van de Sype H. Intra- and inter-provenance variability in phloem phenols of *Picea abies* and relationship to a bark beetle-associated fungus. *Tree Physiol.* 2003;23(4):247–256.
 52. Linnakoski R, Beer ZW, Niemelä P, in Wingfield MJ. Associations of conifer-infesting bark beetles and fungi in fennoscandia. *Insects* 2012;3:200–227.
 53. Likar M and Regvar M. Praktikum fiziologije rastlin. Ljubljana, Študentska založba. 2003;94.
 54. Malá J, Hrubcová M, Máchová P, Cvrčková H, Martincová O, Cvikrová M. Changes in phenolic acids and stilbenes induced in embryogenic cell cultures of Norway spruce by two fractions of *Sirococcus strobilinus* mycelia. *J. For. Sci.* 2011;57:1–7.
 55. Martin D, Tholl D, Gershenzon J, Bohlmann J. Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiol.* 2002;129:1003–1018.
 56. Martin D, Gershenzon J, Bohlmann J. Induction of volatile terpene biosynthesis and diurnal emission by methyl jasmonate in foliage of Norway spruce. *Plant Physiol.* 2003;132:1586–1599.
 57. Meyer AJ and Hell R. Glutathione homeostasis and redox-regulation by sulfhydryl groups. *Photosynth. Res.* 2005;86:435–457.
 58. Miller B, Madilao LL, Ralph S, Bohlmann J. Insect-

- induced conifer defense: White pine weevil and methyl jasmonate induce traumatic resinosis, de novo formed volatile emissions, and accumulation of terpenoid synthase and octadecanoid pathway transcripts in Sitka spruce. *Plant Physiol.* 2005;137:369–382.
59. Müller M and Job H. Managing natural disturbance in protected areas: Tourists attitude towards the bark beetle in a German national park. *Biol. Conserv.* 2009;142:375–383.
 60. Nagy NE, Fossdal CG, Krokene P, Krokling T, Lönneborg A, Solheim H. Induced responses to pathogen infection in Norway spruce phloem: changes in polyphenolic parenchyma cells, chalcone synthase transcript levels and peroxidase activity. *Tree physiol.* 2004;24:505–15.
 61. Nagy NE, Franceschi VR, Kvaalen H, Solheim H.. Callus cultures and bark from Norway spruce clones show similar cellular features and relative resistance to fungal pathogens. *Trees* 2005;19(6):695–703.
 62. Nebeker T and Hodges J. Host response to bark beetle and pathogen colonization. Schowalter GF (Ed). In: *Beetle - pathogen interactions in conifer forests*, San Diego, Academic, 1993;157–173 pp.
 63. Noctor G, Arisi A-CM, Jouanin L, Kunert KJ, Rennenberg H, Foyer CH. Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. *J. Exp. Bot.* 1998;49:321;623–647.
 64. Noctor G. Metabolic signalling in defence and stress: the central roles of soluble redox couples. *Plant Cell Environ.* 2006;29:409–425.
 65. Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Garcia B, Queval G in Foyer CH. Glutathione in plants: an integrated overview. *Plant Cell Environ.* 2012;35(2):454–484.
 66. Novak M, Urbanek Krajnc A, Lah L, Zupanec N, Kraševc N, Križman M, Bohlmann J, Komel R. Low density *Ceratocystis polonica* inoculation of Norway spruce (*Picea abies*) triggers accumulation of monoterpenes with antifungal properties. *Eur. J. For. Res.* 2014;133:573–583.
 67. Novak M. Cytochromes P450 from blue-stain fungi involved in monoterpene biotransformation. Univerza v Ljubljani. Medicinska fakulteta. Doktorska disertacija 2014.
 68. Paine TD, Raffa KF, Harrington TC. Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Ann. Rev. Entomol.* 1997;42:179–206.
 69. Percival GC. Induction of systemic acquired disease resistance in plants: Potential implications for disease management in urban forestry. *J. Arboric.* 2001;27(4):181–192.
 70. Pont V and Pezet R. Relation between the chemical structure and the biological activity of hydroxystilbenes against *Botrytis cinerea*. *J. Phytopathol.* 2008;130(1):1–8.
 71. Raffa KF, Aukema BH, Bentz BJ, Carroll AL, Hicke JA, Turner MG, Romme WH. Cross-scale drivers of natural disturbances prone to anthropogenic amplification: The dynamics of bark beetle eruptions. *BioScience* 2008;58:501.
 72. Ralph SG, Yueh H, Friedmann M, Aeschliman D, Zeznik JA, Nelson CC, Butterfield YSN, Kirkpatrick R, Liu J, Jones SJM et al. Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant Cell. Environ.* 2006;29:1545–1570.
 73. Riedle-Bauer M. Role of reactive oxygen species and antioxidant enzymes in systemic virus infections of plants. *J. Phytopathol.* 2000;148:297–302.
 74. Rodrigues, KCS, Fett-Neto AG. Oleoresin yield of *Pinus elliottii* in a subtropical climate: Seasonal variation and effect of auxin and salicylic acid-based stimulant paste. *Ind. Crops Prod.* 2009;30:316–320.
 75. Rohde M, Waldmann R, Lunderstädt. Induced defence reaction in the -phloem of spruce (*Picea abies*) and larch (*Larix decidua*) after attack by *Ips typographus* and *Ips cembrae*. *Forest. Ecol. Manag.* 1996;86:51–59.
 76. Sallé A, Monclus R, Yart A, Garcia J, Romary PLF. Fungal flora associated with *Ips typographus*: frequency, virulence, and ability to stimulate the host defence reaction in relation to insect population levels. *Can. J. For. Res.* 2005;35:365–373.
 77. Schmidt A, Zeneli G, Hietala AM, Fossdal CG, Krokene P, Christiansen E, Gershenzon J. Induced chemical defenses in conifers: Biochemical and molecular approaches to studying their function. In: Schmidt A, Zeneli G, Hietala AM, Fossdal CG, Krokene P, Christiansen, Gershenzon J, Romeo JT (Eds). *Chemical ecology and phytochemistry in forest ecosystems*, Vol. 39. Elsevier, Amsterdam, 2005;1–28.
 78. Six DL and Wingfield MJ. The role of phytopathogenicity in bark beetle-fungus symbioses: a challenge to the classic paradigm. *Ann. Rev. Entomol.* 2011;56:255–72.
 79. Sjoström E. *Wood chemistry, fundamentals and applications*. Second Edition. Academic press, Inc., Harcourt Brace Jovanovich, Boston, London, Sydney, Tokyo, Toronto, 1993;293.
 80. Smirnoff N and Wheeler GL. Ascorbic acid in plants: biosynthesis and function. *Crit. Rev. Plant. Sci.* 2000;19:267–290.
 81. Smith GD, Carroll AL, Lindgren BS. Facilitation in bark beetles: endemic mountain pine beetle gets a helping hand. *Agric. For. Entomol.* 2011;13:37–43.
 82. Šuštaršič S. Kako meriti stres pri rastlinah? Univerza v Ljubljani, Biotehniška fakulteta. Diplomski projekt 2012.
 83. Tausz M, Wonisch A, Grill D, Morales D, Jiménez MS. Measuring antioxidants in tree species in the natural environment: from sampling to data evaluation. *J. Exp. Bot.* 2003;54(387):1505–1510.
 84. Tausz M, Šircelj H, Grill D. The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid? *J. Exp. Bot.* 2004;55(404):1955–1962.
 85. Tegischer K, Tausz M, Wieser G, Grill D. Tree-age and needle-age dependent variations of antioxidants and photoprotective pigments in spruce needles at the alpine timberline. *Tree Physiol.* 2002;22:591–596.
 86. Torres P, Avila JG, Romo de Vivar A, García AM, Marín JC, Aranda E, Céspedes CL. Antioxidant and insect growth

- regulatory activities of stilbenes and extracts from *Yucca periculosa*. *Phytochemistry* 2003;64:463–473.
87. Urbanek Krajnc A. Effects of endogenously increased levels of thiol compounds and salicylic acid on *Zucchini yellow mosaic virus* infected Styrian oil pumpkin plants. Thesis. Uni Graz. 2004.
 88. Urbanek Krajnc A. A temporal analysis of antioxidative defense responses in the phloem of *Picea abies* after attack by *Ips typographus*. *Tree Physiol.* 2009;29:1059–1068.
 89. Urbanek Krajnc A, Kristl J, Ivancic A. Application of salicylic acid induces antioxidant defense responses in the phloem of *Picea abies* and inhibits colonization by *Ips typographus*. *ForEcol Manage.* 2011;261:416–426.
 90. Urbanek Krajnc A, Novak M, Felicijan M, Kraševc N, Lešnik M, Zupanec N, Komel R. Antioxidative response patterns of Norway spruce bark to low-density *Ceratocystis polonica* inoculation. *Trees* 2014;28(4):1145–1160.
 91. Veberič R. Bioactive compounds in fruit plants. el. knjiga. Ljubljana. Narodna in univerzitetna knjižnica, Biotehniška fakulteta. 2010. (URL): <http://www.bf.uni-lj.si/agronomija/oodelku/katedre-in-druge-org-enote/za-sadjarstvo-vinogradnistvo-invrtnarstvo/sadjarstvo/>
 92. Viiri H, Annala E, Kitunen V, Niemelä P. Induced responses in stilbenes and terpenes in fertilized Norway spruce after inoculation with blue-stain fungus *Ceratocystis polonica*. *Trees* 2001;15:112–122.
 93. Wallis C, Eyles A, Chorbadian R, McSpadden Gardener B, Hansen R, Cipollini D, Herms DA, Bonello P. Systemic induction of phloem secondary metabolism and its relationship to resistance to a canker pathogen in Austrian pine. *New Phytol.* 2008;177:767–778.
 94. Wermelinger B. Ecology and management of the spruce bark beetle *Ips typographus*—a review of recent research. *Forest Ecol Manag.* 2004; 202(1–3):67–82.
 95. Whitney H. Relationships between bark beetles and symbiotic organisms. Mitton J, Sturgeon K (Eds). In: *Bark beetles in north American conifers*. Austin: Univ. Texas, 1982;183–211pp.
 96. Witzell J, Martin JA. Phenolic metabolites in the resistance of northern forest trees to pathogens – past experiences and future prospects. *Can. J. For. Res.* 2008;38:2711–2727.
 97. Woods JA, Hadfield JA, Pettit GR, Fox BW, McGown AT. The interaction with tubulin of a series of stilbenes based on combretastatin A-4. *Br. J. Cancer* 1995;71:705–711.
 98. Xiang C, Werner BL, Christensen EM and Oliver DJ. The biological functions of glutathione revisited in arabidopsis transgenic plants with altered glutathione levels. *Plant Physiol.* 2001;126:564–574.
 99. Zechmann B, Zellnig G, Urbanek Krajnc A and Muller M. Artificial elevation of glutathione affects symptom development in ZYMV-infected *Cucurbita pepo* L. plants. *Arch. Virol.* 2007;152:747–762.
 100. Zeneli G, Krokene P, Christiansen E, Krekling T, Gershenzon J. Methyl jasmonate treatment of mature Norway spruce (*Picea abies*) trees increases the accumulation of terpenoid resin components and protects against infection by *Ceratocystis polonica*, a bark beetle-associated fungus. *Tree Physiol.* 2006;26:988–997.
 101. Zhao FJ, Tausz M, De Kok LJ. Role of sulfur for plant production in agricultural and natural ecosystems. In: Hell R, Dahl C, Knaff D, Leustek T (Eds). *Advances in photosynthesis and respiration*. Springer, Dordrecht 2008;417–435.

Antioksidativni obrambni odziv lubja navadne smreke ob napadu podlubnikov in z njimi povezanimi glivami modrivkami

IZVLEČEK

Čeprav so podlubniki in z njimi povezane glive sestavni del gozdnih ekosistemov, sta osmerozobi smrekov lubadar (*Ips typographus* LINNAEUS) in z njim povezana patogena gliva modrivka [*Ceratocystis polonica* (SIEM.) C. MOREAU], ena najbolj uničujočih škodljivcev navadne smreke [*Picea abies* (L.) H. KARSTEN]. Lubadarji navadno naseljujejo oslabela in podrta drevesa, v specifičnih pogojih pa lahko napadejo tudi vitalna. S poškodbami floema in kambija ter motnjo transpiracijskega toka v ksilemu, drevesu povzročijo številne fiziološke motnje. Iglavci imajo široko paleto obrambnih mehanizmov, ki temeljijo na anatomiji lubja in fiziološkem stanju drevesa. Njihova osnovna funkcija je zaščita hranilno in energetske bogatega floemskega tkiva, meristematske aktivnosti vaskularnega kambija in transpiracijskega toka v sekundarnem ksilemu debla. Glavno področje obrambnih mehanizmov je namreč sekundarni floem, ki predstavlja fizično in kemično zaščito lubja pred škodljivci, saj vsebuje polifenolne parenhimatske celice, sklerenhim, kristale kalcijevega oksalata in shizogene smolne kanale. Obrambni sistem lubja iglavcev zajema tri vrste obrambnih strategij – konstitutivno in inducirano obrambo ter sistemsko pridobljeno odpornost. Konstitutivna in inducirana obramba zavirata kolonizacijo podlubnikov, rast gliv in zapirata ranitvena mesta. Med uspešnim napadom pa je za drevo ključnega pomena še vzpostavitev sistemsko pridobljene odpornosti (SAR). Le ta se postopoma širi po rastlini ter izzove sistemske spremembe v metabolizmu drevesa, ki se ohranjajo skozi daljše časovno obdobje. Med številnimi obrambnimi odzivi, povezanimi z aktivacijo SAR, sta pomembni sinteza antioksidantov in aktivacija antioksidativnih encimov, ki varujejo rastlino pred reaktivnimi kisikovimi spojinami (ROS). Prispevek zajema pregled dosedanjega znanja o strategijah antioksidativnega odziva lubja smreke pri napadu smrekovega lubadarja (*Ips typographus*) in z njim povezane patogene glive modrivke (*Ceratocystis polonica*).

The accuracy of the germination rate of seeds based on image processing and artificial neural networks

Uroš ŠKRUBEJ^a, Črtomir ROZMAN^b and Denis STAJNKO^{b*}

^aLokovica 8a, SI-3325 Šoštanj, Slovenia

^bUniversity of Maribor, Faculty of Agriculture and Life Sciences, SI-2311 Hoče, Slovenia

ABSTRACT

This paper describes a computer vision system based on image processing and machine learning techniques which was implemented for automatic assessment of the tomato seed germination rate. The entire system was built using open source applications ImageJ, Weka and their public Java classes and linked by our specially developed code. After object detection, we applied artificial neural networks (ANN), which was able to correctly classify 95.44% of germinated seeds of tomato (*Solanum lycopersicum* L.).

Key words: image processing, artificial neural networks, seeds, tomato

INTRODUCTION

As one of the most important input in agriculture a quality seed is a basis for higher agricultural productivity and a key to economic growth.

A number of methods for seed quality evaluation and sorting have been developed so far, mainly based on the detection of various physical and chemical properties which correlate well with certain vigour and germination parameters (McDonald 1998).

Nowadays, seed testing is performed in accredited laboratories by trained human analysts. The tests are designed to evaluate the quality of the seed lot. Several tests are done. For instance, a germination test determines the maximum germination potential, or viability of the seed. The germination rate of a particular seed lot is a key indicator which shows the seed performances in the field and it is expressed as a percentage (for example a 90% germination rate means 90 out of 100 seeds are likely to germinate under proper growing conditions). This information is important for calculating optimal seeding rates and to determine whether a particular seed lot has the potential to produce a quality crop.

Since the manual counting is time-consuming and labour

intensive process, we are looking at ways we can improve the process efficiency. We have been examining ways of automating a task by means of computer vision systems, based on image processing and machine learning. This can provide an alternative to manual counting and inspection of seed samples.

Image analysis was introduced in the field of seed technology already by Howarth and Stanwood (1994) who have developed a colour image database to characterize the phenotypic variation of genetic resources. Image processing also provided precise results in the field of seed identification or classification (Uchigasaki et al. 2000, Granitto et al. 2002) and germination assessment (McDonald et al. 1998). Dell'Aquila et al. (2000) used image analysis to characterize the imbibition of white cabbage seeds, while Geneve and Kester (2001) evaluated seeding size after germination by computer-aided analysis of digital images from a scanner (Ducournau et al. 2004).

Ureña et al. (2001) proposed a machine vision system which used automated data gathering process and a fuzzy logic-based system for automatic evaluation of germination quality.

Ducournau et al. (2004) presented a machine vision system

*Correspondence to:

E-mail: denis.stajnko@um.si

designed to count the number of emergent radicle tips on seed lots under controlled lighting, temperature and hygrometric conditions. The automated acquisition system employed an algorithm that was able to count the germinated seeds and provided the mean germination time based on the difference between two consecutive pictures.

Modern computer vision mainly based on image processing procedures such as proprietary software MATLAB or other specialized expensive software. In our work a free image processing and analysis program named ImageJ was used, which is readily available, open source and public domain software developed at the National Institutes of Health (NIH), Bethesda, Maryland USA (Rasband 2012).

MATERIALS AND METHODS

Tomato seeds (*Solanum lycopersicum* L.) variety 'Marmande', were obtained from the seed company Semenarna Ljubljana d.d. Slovenia. Before the experiment, the uncovered seeds were stored for a month in an incubator at 4 °C, 50% relative humidity to equilibrate to an identical seed moisture condition. Then we randomly chose 700 seeds from 3 bags as the sample. Next, we placed a dark filter paper inside twenty-eight glass Petri dishes (90x98x18mm) and moistened each with 3ml distilled water. The dark filter paper was used to obtain optimal contrast between seed, radicle and filter paper. Twenty-five seeds were placed on top of the wet filter paper in each dish and spaced them evenly. We put covers on the dishes. The seeds were germinated under a controlled condition and maintained in the dark at 20 to 30 °C (± 1 °C) and 75% relative humidity for seven days in a Jacobsen incubator. The seeds were illuminated for 8 hours in every 24 hour period. Light was provided by a cool white fluorescent source of 750 lux. Images were captured by

a Nikon D80 digital SLR camera with Sigma 18 – 200mm zoom lens. The camera was mounted on a stand with an easy vertical movement, which provided rigid stable support. The camera was set at a distance 450 mm. The images were obtained by 3872x2592 pixels, horizontal resolution 300 dpi, vertical resolution 300 dpi and a bit depth 24. We placed a warm white, 22 W fluorescent tube with a 210 mm diameter circular lamp with a rated voltage of 220 V around the Petri dish with a seeds sample. A light diffuser, a semi-spherical steel bowl of 270 mm diameter, covered the light bulb, prevented external influences and provided diffused light (Figure 1). All images were transferred from the digital camera to a personal computer PC (dual-core microprocessor Intel Pentium B950 2.10 GHz, 4 GB installed memory RAM) via universal serial bus (USB) cable.

Images processing

The ImageJ software was used for image processing and extracting features from original RGB images (Figure 2a). First, we cut off the frame from each RGB image to establish the correct region of interest (ROI) in the centre of each image by using the known radius, so the cropped image was received (Figure 2b). The cropping process reduced the size of the images so all the following manipulations were more efficient. In our study the original matrix of 3872x2592 pixels was reduced to 1854x1836 pixels. In the second step a Gaussian filter with sigma parameter σ set at 2 was used for smoothing the image. This filter used a convolution with a Gaussian function (Eq. 1) described by Rasband (2008):

$$\varphi_{\mu, \sigma^2}(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-\mu)^2}{2\sigma^2}} \quad (1)$$

where x is the intensity of pixel, μ is a mean, σ is the standard deviation, σ^2 is a variance, $\pi=3.14$ and $e=2.718$.

The image was then transformed from RGB color space into an 8-bit grayscale image (Figure 2c) which was finally converted by a threshold operation into a binary image (Figure 2d). For thresholding limits were defined by several pre-testing, so the lower limit was set to 85 and the upper limit to 255 grayscale intensity.

The minimum particle size was set to 1500 pixels and the maximum to 14.000 pixels. With this we additionally avoided the smaller and the bigger areas that could not be accounted as seeds. Finally, presented seeds were separated from the background and automatically labelled with the integer. The external perimeter of the seed was traced in yellow (Figure 2e).

The resulting set of extracted features together with statistics and measurements was saved in a separately table for each Petri dish, where each row represents a single seed and each column a single parameter of particular seed. Feature extraction was automated by using ImageJ's macro language facility, which enables any of ImageJ's GUI features to be invoked programmatically.

Simultaneously to image processing an expert technician

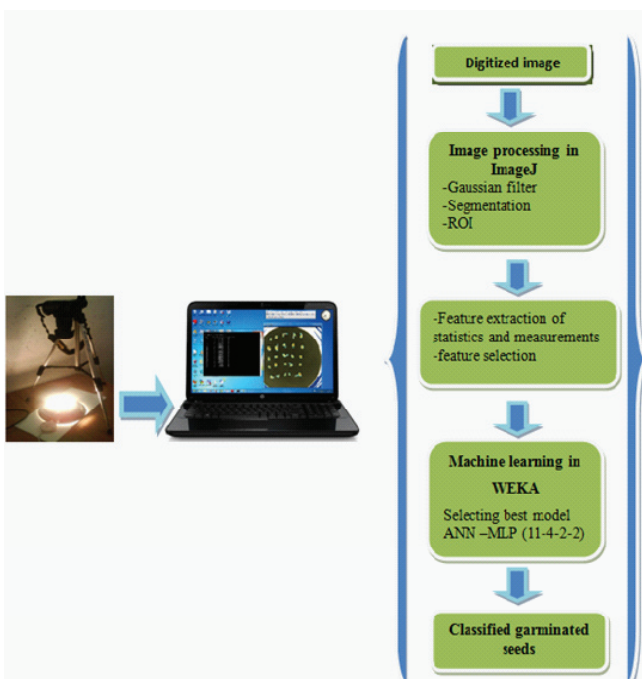


Fig. 1: Proposed computer vision system

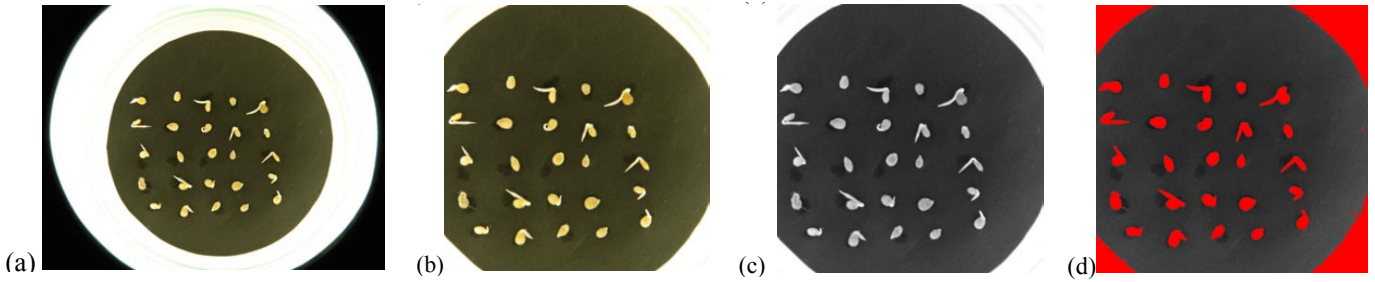


Fig. 2: Image processing. (a) Original image in RGB colour space. (b) Cropped image. (c) 8-bit grayscale image. (d) Binary image after thresholding. (e) Labelled seeds with numbers and yellow line. The next step in the processing was to extract the features from the image containing labelled seeds by Analyze Particles ImageJ command. The description of all measured and used image analysis parameters is reported in Table 1.

examined 28 images of each series of 25 seeds and provided a nominal class (germinated or not germinated) for each of the 700 seeds. Those results were also added to the previous mentioned tables.

Machine learning

Once the feature vectors had been generated and exported as *csv* (comma-separated values) formatted file, we used machine learning software WEKA (Waikato Environment for Knowledge Analysis) to perform further analysis. WEKA is a collection of machine learning algorithms for data mining tasks and contains tools for data pre-processing, classification, regression, clustering, association rules, and visualization. The algorithms can either be applied directly to a dataset or called from Java code. WEKA was developed at the University of Waikato, New Zealand for the purpose of identifying information from raw data obtained from agricultural domains and due to the usability and openness extended also to other fields. It is written in Java and well connected with an ImageJ. The combination of these two tools was first time described by Mayo et al. (2007) for classifying the moths by species after feature vectors were extracted from each of the moth images.

WEKA was first used for future selecting and ranking. For evaluator we used *InfoGainAttributeEvaluator* which estimated the worth of an attribute by measuring the information gain with the respect to the class and *Ranker* which graded attributes by their individual evaluations. The biggest impact on classification had a parameter *Perimeter* which is the length of the outside boundary of the selection and lowest *Mean* which is the sum of the grey values of all the pixels in the selection divided by the number of pixels (Table 1).

For learning classifier models, the data were separated into 10 sets, each consisting of 70 instances. After that, the training was performed by using 9 of these sets, and testing was performed on the one remaining set (630 seeds as training and 70 seeds for testing for each run). This was repeated 10 times for each model.

Classification

For classification artificial neural networks (ANN) multilayer perceptron architecture (MLP) was implemented and compared with manual counting. For training we used a back propagation algorithm. The value of *learning rate* was set up

Table 1: Parameters measured by ImageJ and ranked by WEKA

Parameter	Description
Perim	Perimeter is the length of the outside boundary of the selection.
Kurtosis	Kurtosis is the degree of peakedness of a distribution.
Max	The maximum grey values within the selection.
Skewness	Skewness is a measure of the degree of asymmetry of a distribution.
StdDev	Standard deviation of the grey values used to generate the mean grey value.
Major	Major is the primary axis of the best fitting ellipse.
Area	Area of selection in square pixels
Mode	Modal gray value is a most frequently occurring gray value within the selection. Corresponds to the highest peak in the histogram.
Median	The median value of the pixels in the selection.
Minor	Minor is the secondary axis of the best fitting ellipse.
Mean	Average grey value within the selection. It is the sum of the grey values of all the pixels in the selection divided by the number of pixels.

at 0.3 and *momentum rate* at 0.2. The number of neurons in input and output layers was set to 11 and 2 respectively since the number of features was 11 and the number of possible classes was 2. In the next step several combinations of hidden layers and different number of hidden neurons were tested, so the training time varied from 100 to 2000 epochs.

Evaluation measures for classifier performance

To objectively evaluate the performance of ANN, we used a classification accuracy, precision, recall and the F-measure, which were derived from confusion matrix.

Classification accuracy refers to the percentage of correct predictions made by the ANN model when compared with the manual evaluation of the 25 test data. It is calculated as the number of correctly classified instances divided by the total number of instances:

$$\text{accuracy} = \frac{TP+TN}{TP+TN+FP+FN} * 100\% \quad (2)$$

where TP refers to true positive, TN refers to true negative, FP refers to false positive and FN refers to false negative. Then $TP+TN+FP+FN$ is the total number of instances in the testing set and $TP+TN$ is the number of correctly classified instances (Witten and Frank 2005).

In our case TP represented actually germinated seeds which were also predicted as germinated. TN represented actually not germinated seeds which were also predicted as not germinated. FP were actually not germinated seeds predicted as germinated and FN were germinated seeds predicted as not germinated.

Precision is the proportion of predicted positive instances which are actual positive among all those which were classified as positive. It is calculated as follows:

$$p = \frac{TP}{TP+FP} \quad (3)$$

where TP refer to true positive and FP refer to false positive. A FP occurs when the class is incorrectly predicted as positive

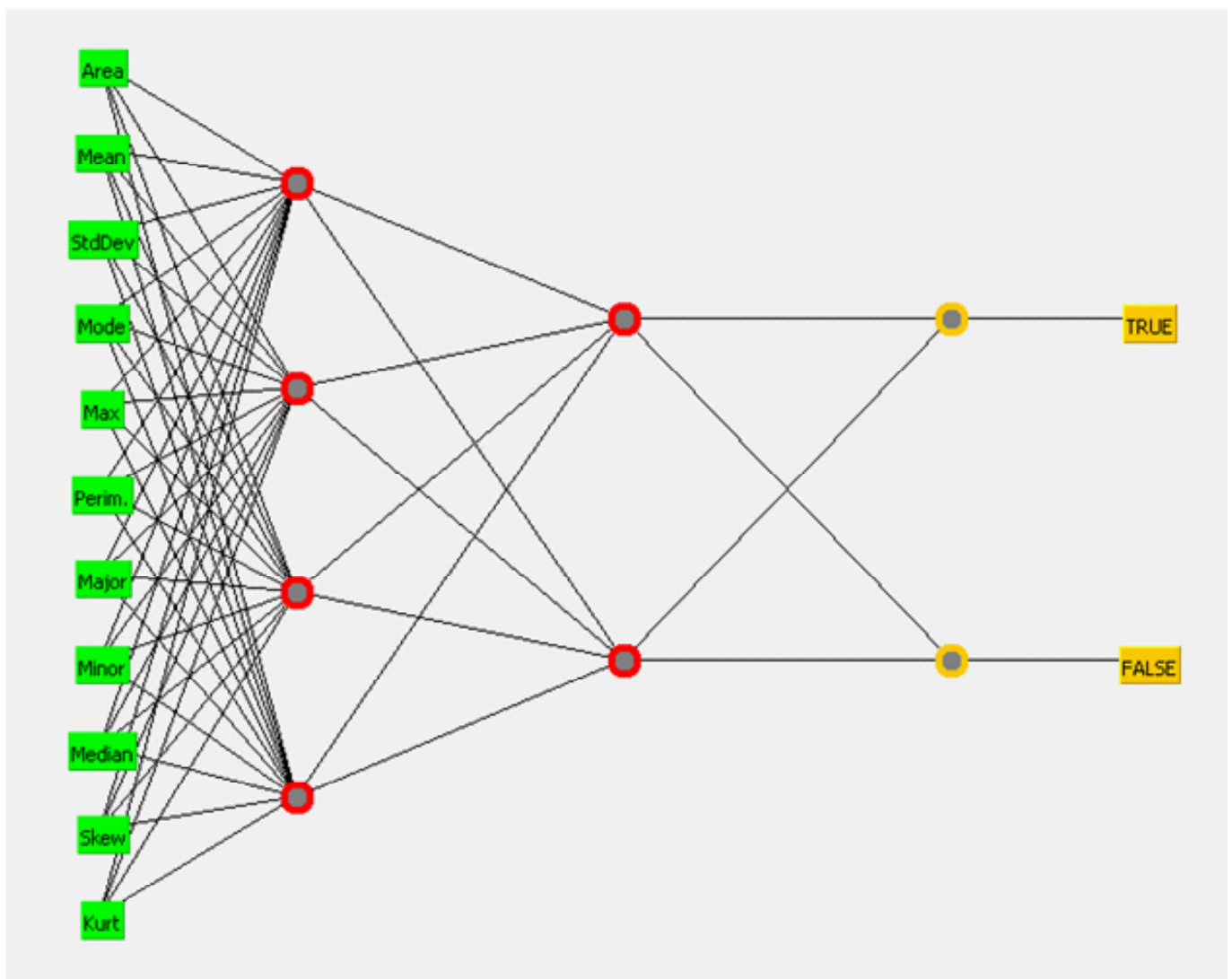


Fig. 3: ANN model with 11-4-2-2 topology

Table 2: Measures for classifier performance

model	Accuracy			Precision			Recall			F-measure		
	Mean	Std. dev	Sig.	Mean	Std. dev.	Sig.	Mean	Std. dev.	Sig.	Mean	Std. dev.	Sig.
ANN	95.44	2.14		0.9722	0.0233		0.9652	0.0218		0.9684	0.0148	

when it is actually negative (Witten and Frank 2005).

Recall is defined as the ratio of the true positive to the sum of the TP and FN. Recall is also known as sensitivity in some fields. It is calculated as follows:

$$\text{recall} = \text{TP} / (\text{TP} + \text{FN}) \quad (4)$$

FN occurs when the class is predicted as negative when is actually positive (Baeza and Riberio 1999, Witten and Frank 2005).

F-measure is defined as the harmonic mean of precision and recall. It is calculated as follows:

$$\text{F-measure} = (2 \times \text{Precision} \times \text{Recall}) / (\text{Precision} + \text{Recall}) \quad (5)$$

It has a high value when both precision and recall have high values, and it is seen as way of finding the best compromise between these two measures (Baeza and Riberio 1999).

RESULTS AND DISCUSSION

The best accuracy of 95.44% (Table 2) was obtained with 2 hidden layers that contained 4 and 2 hidden neurons, respectively, and a training time set at 500. The algorithm realized a 97% TP rate, with a 3% FN rate and a 93% TN rate with a 7% FP rate. ANN achieved the precision (0.9722), which is also very high. The ANN reached a recall (0.9652) and F-measure (0.9684), respectively.

The main advantage of our procedure over the study of (Dell'Aquila et al. 2000), who detected the germinated seeds by comparing the difference of seed XY position or area size of seeds on two consecutive images (before and after germination), was that we used eleven features derived from a single picture instead, to assess the germination rate. The single acquisition is also favourable, because it decreased a possibility of fungal contamination caused by several opening of the Jacobsen incubator.

Additional significant difference between our study and the one of Jossen et al. (2010) is that in our case the image processing, feature extraction and classification were entirely automatic, without any manual interventions for transferring data between different software applications.

CONCLUSIONS

In the present study, a computer vision system based on

image processing and machine learning techniques was developed which was implemented for automatic assessment of the seed's germination rate. The entire system was built by using open source applications ImageJ, Weka and their public Java classes and linked together by our specially developed code, which made it non-expensive and acceptable for many laboratories. The results show that ANN accuracy was very high (95.44%). The prototype system has classified one sample of germinated seeds in 4s and has out performed trained analysts, thus it shows a great opportunity for exchanging the time consuming manual counting in the laboratories for estimating the seed quality.

REFERENCES

1. Baeza R, Ribeiro B. Modern Information Retrieval. Addison-Wesley Longman Publishing Co., Boston, 1999: 544 p.
2. Dell'Aquila A, van Eck JW, van der Heidjen GWAM. The application of image analysis in monitoring the imbibition process of white cabbage (*Brassica oleracea* L.) seeds. Seed Sci. Res. 2000;10:163–169.
3. Ducournau S, Feutry A, Plainchault P, Revillon P, Vigouroux B, Wagner MH. An image acquisition system for automated monitoring of the germination rate of sunflower seeds. Comp. Electron. Agricult. 2004;44:189–202.
4. Geneve RL, Kester ST. Evaluation of seedling size following germination using computer-aided analysis of digital images from flat-bed scanner. Hortscience 2001;36(6):1117–1120.
5. Granitto PM, Navone HD, VerdesPF, Ceccato HA. Weed seeds identification by machine vision. Comp. Electron. Agricult. 2002;33:91–103.
6. Howarth MS, Stanwood PC. Extracting 3-D information using 2-D images of seeds. Comp. Electron. Agricult. 1994;10:175–188.
7. Jossen R, Kodde V, Willems L, Ligterink W, Van der Plas L, Hilhorst H. Germinator: a software package for high-throughput scoring and curve fitting of Arabidopsis seed germination. Plant J. 2010; 62(1):148–159.
8. Mayo M, Watson AT. Automatic species identification of live moths. Knowledge-Based Systems, 2007;20:195–202.
9. McDonald MB. Seed quality assessment. Seed Sci. Res. 1998;8:265–275.
10. Rasband WS. Image. US National Institutes of Health, Bethesda, Maryland, USA, 2008.
11. <<http://rsb.info.nih.gov/ij/index.html>> Accessed August, 2012.

12. Uchigasaki M, Serata K, Miyamoto S. An automated machine vision system for classification of seeds using color features. *J. Agricult. Struct.* 2000;30(4):325–332.
13. Ureña R, Rodriguez F in Berenguel M. A machine vision system for seeds germination quality evaluation using fuzzy logic. *Comp. Electron. Agricult.*, 2001,32:1–20.
14. Witten, I., and Frank, E. (2005). *Data Mining: Practical Machine Learning Tools and Techniques* (2nd Ed.). Morgan Kaufmann,

Natančnost določanja kalečih semen s pomočjo obdelave slik in nevronske mreže

IZVLEČEK

Članek opisuje sistem računalniškega vida, ki temelji na tehnikah obdelave slik in strojnega učenja, ki je bil izdelan za avtomatsko oceno stopnje kaljenja semen paradižnika. Celoten sistem je bil zgrajen s pomočjo odprtokodnih aplikacij ImageJ, Weka in njihovih javno dostopnih javanskih kod, ki smo jih povezali v lastno originalno razvito kodo. Po odkrivanju predmetov na RGB slikah, smo uporabili umetne nevronske mreže (ANN), ki so bile sposobne pravilno razvrstiti 95,44% nakaljenih semen paradižnika (*Solanum lycopersicum* L.).

Impact of various types of anti-hail nets on light exposure in orchards and quality parameters of apples– a review

Marinka BRGLEZ SEVER, Stanislav TOJNKO, Tatjana UNUK*

University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 11, Hoče

ABSTRACT

The new Common Agricultural Policy (CAP) has been designed to shrink funds that Hungarian agricultural reform will ineNowadays, anti-hail nets are a part of basic equipment in a modern apple orchards. They decrease the risks of apple production and thus allow regular and quality apple harvest. Colours of nets differently obstruct the passing of light through the net, which directly affects some quality parameters of the yield, especially the fruit skin colour. The article includes a brief overview of studies associated with the impact of light exposure under different types of anti-hail nets on quality of apple fruits, as well as investigations which deal with adaptation of various technological measures, such as the use of reflective ground foil. A part of data from the existing literature explains the effect of anti-hail net usage on forming a microclimate under the net, and consequently, its effect on the development and dynamics of diseases and pests.

Key words: Anti-hail net, light, microclimate, apples, yield quality

INTRODUCTION

The benefits of anti-hail net usage are reducing the alternate bearing incidence due to protection of fruit trees, reduction of sunburn in hot years, protection against birds and insects, lesser impact of strong wind and continuous market supply of high quality fruits. Anti-hail nets point to the importance of consistently carrying out the technical support measures with the intention of balancing the growing conditions, mostly the light exposure of the fruit in the tree crown. (Germšek 2010, Germšek and Unuk 2014). As the distinct advantages of anti-hail net usage, Klein (2000) states a smaller day and night temperature variations under the net, a stronger and more uniform growth, and a partial protection against frost and of heavy rain.

Another benefit of using the anti-hail nets, as seen in practice, is also the possibility of combining it with the plant support systems. Due to the elimination of production risks, which in some regions poses a distinct problem because of the hail, the placement of anti-hail nets allows for a quicker return of the invested assets in orchard establishment, earlier

and more regular yields of high quality, and thus a higher economy of apple production (Germšek in Unuk 2014).

Anti-hail net light transmittance

Light has a crucial role when it comes to the intensity of the photosynthesis, differentiation of floral buds, and consequently also affects the quality of fruit. It is also a key factor affecting colour, taste and resilience of fruits (Jazbec and Hanzak 1982, Holzwarth 2008).

Photosynthetically active radiation (PAR), which is needed for photosynthesis, has the wavelength between 400 and 700 nm. Approximately from 85 to 95% PAR - radiation is absorbed by a leaf, the rest is either reflected or penetrates the leaf (Štampar et al. 2002). The level of light is decreased for approximately 7% (PAR) – 20% (UV) with a white net (which allows for the maximum light exposure under a net): 11% (PAR) –28% (UV) with a red-white net, 12% (PAR) – 23% (UV) with a green-white net, 13% (PAR) with a light grey net, 15% (PAR) – 26% (UV) with a black and green, 16%

*Correspondence to:
E-mail: tatjana.unuk@um.si

(PAR) – 23% (UV) with a black net and 18% (PAR) – 29% (UV) with a red net, measured 50 centimetres below the net. The level of UV light is decreased by 29% with a black net (Blanke 2007). With a grey net the level is decreased by 13% (PAR), whereas with a black net by 18% (PAR) (Blanke 2007, cited.).

Larcher (2003) states that the leaves exposed to the sunlight, compared to those in the shade, have a higher level of net photosynthesis. The leaves on the trees protected by anti-hail net could behave similarly to the leaves in the shade, due to somehow decreased exposure to light. Widmer (2001) claims that swings in day temperature show that weather (cloudiness, fog) can affect the light exposure to a much higher degree than the anti-hail net; he states that the assimilation of leaves experiences a small decrease only on cloudy days, while it remains unchanged on sunny days.

A part of UV rays is transmitted through the nets as a visible light, therefore they do not represent additional protection against UV rays with the wavelength above 300 nm. Trees and fruits under such nets are, during their growth, exposed to the same level of UV – A and partially UV – B radiation as well as the level of visible light. The effect of UV radiation on plants depends on strength of the visible light and is even stronger when the light is weaker, for example in dapple shade, in cloudy weather and under the net. For fruit farming, nets with higher level of light transmittance in regard to durability of materials and price are recommended. It's not yet entirely clear which part of the radiation they reflect (Nobel 1991, cit. in Germšek 2010).

Different types of anti-hail nets obstruct the passing of the light differently and have different life expectancies. The thinner the net and the bigger the meshes, the higher are the swings in transmittance levels (Blanke 2007). Grey nets represent the biggest share of the standard production. They consist of crystal fibres with added black fibres for better durability. Black anti-hail nets, in comparison to white ones, have longer life expectancy, lasting from 15 to 20 years, and blend into the colour composition of the landscape more easily, but decrease the light transmittance by 20% (Zadravec 2002, Germšek and Unuk 2014). White nets transmit more light (86%), but at the same price come with less than a half of the life expectancy of a black net (Dobaja 2005); white net's life expectancy is between 7 and 9 years. Its weakness is also in its noticeability in nature, compared to the black net, which led to a compromise in net colours – grey and green nets. Due to dirt accumulation over the years, the difference in light transmittance between the types of nets decreases with time (Dobaja 2005). Light transmittance in sunny weather is lower with white and black net compared to the uncovered part. With the black net it is decreased by 25% and with the white one by 12%. With red-white nets the transmittance is decreased by 14%, with red-black ones by 18% and with green-black ones by 23% (Blanke and Salomaklin 2008). Under a grey net, shading reaches up to 12% (Zadravec 1998, as cited in Kuzma 2011). Iglesias and Alegre (2006) proved that the differences in light transmittance due to the colour of the net were smaller in cloudy weather.

Blanke (2007) states that in the ecological conditions of Southern Europe, for single-colour and two-colour varieties of apples, which achieve appropriate colouring, or

for varieties which are prone to sunburn, black nets are the most appropriate. White or crystal and grey nets are more appropriate for the apple orchards in NW Europe, where the lack of sunlight is noted and where there is no risk of sunburn. Red nets seem inappropriate because of the higher level of shading and also because they cannot blend with the landscape.

Anti-hail nets are mostly made out of highly compressed polyethylene fibres, 228–356 µm wide. One of special characteristics of black and black-green nets is that they have two black longitudinal threads, while transparent and red nets have two transparent or two red longitudinal threads. Transverse single threads in black, red and green nets are black. Nets also differ in thread density. The anti-hail net with double knitting lets through only the smallest possible grain of hail. It has a double longitudinal and a single transverse knitting (Blanke 2007).

Light exposure under the anti-hail net and substituting the lack of light with a reflective ground foil

The fruit skin (fruit exterior) colour represents one of the basic parameters which attract the consumers; in the eyes of a buyer, it represents direct connection to the internal quality of the fruits (Sadar et al 2013). This is the main reason why growers use numerous measures to improve the colouring of fruits. Because of the decrease in light transmittance in orchards under the anti-hail net, the skin colour is even a bigger challenge for the growers.

Blanke (2004) states that a white reflective foil laid out between the rows of trees, improves the colouring of fruits of the 'Jonagold Wilmuta' variety. This impact is even bigger when the foil is laid out early, meaning in July instead of August. The improved colouring of the fruits with the use of reflective foil is confirmed in combination with the white and black net. It was established that the improved colouring of the fruits is achieved under white and black nets, if the reflect foil is laid on the ground at least six weeks prior to the harvest. While studying the impact of the reflective foil, Jakopič et al. (2007) confirmed that the foil under the net could compensate the decrease of light transmittance even in the last month before harvest, and that covering the ground of the orchard with the reflective foil had a positive effect on the fruits' (variety 'Fuji') exposure to light.

In the ecological conditions of the Fruit Growing Centre in Maribor (NE Slovenia), the impact of the anti-hail net and the ground reflective foil on the size and quality of yield (variety 'Jonagold' and 'Elstar') was not documented. However, the positive impact of the reflective foil on the fruit colouring was confirmed. The impact was the highest in combination with the crystal net (91.7% on the variety 'Elstar'). The highest impact on fruit colour of the variety 'Elstar' was recorded with the use of reflective foil (91.6%), followed by the crystal net (91.7%), the black net (84.9%), control – without nets and reflective foil (84.1%), reflective foil in combination with the black net (80.8%) and reflective foil in combination with the crystal net (79.7%). In 2002, the highest impact on fruit

colour of the variety 'Elstar' was recorded with the use of crystal net (64.4%), followed by reflective foil in combination with the crystal net (58.3%), control (54.5%), reflective foil in combination with the black net (51%) and reflective foil (47.7%). Regarding the variety 'Jonagold', the highest impact on fruit colour in 2001 was recorded when using the reflective foil in combination with the crystal net (61.5%), followed by the crystal net (58.8%), the black net (58.5%) and reflective foil in combination with the black net (47.8%). In 2002, the highest impact on fruit colour of the variety 'Jonagold' was recorded when using the reflective foil in combination with the black net (78.1%), followed by reflective foil (59%), the crystal net (58.6%), the black net (41%), reflective foil in combination with the crystal net (34.1%) and control (21.7%) (Dobaja 2005).

The impact of anti-hail net on some quantity and quality parameters of fruits

The optimal light conditions in the tree crown improve the health status and colour of the fruits (anthocyanin synthesis) (Awad et al. 2001). Light is very important for the increase in the anthocyanin level (Macheix et al. 1990). The less light reaches the treetops, the smaller is the intensity of photosynthesis, which in the end results in the lesser fruit colouring (Ubi et al. 2006). Different authors (Vercammen 1999, Widmer 2001, Zadravec 2002, Germšek and Unuk 2014) prove that the quantity of yield under the protective net does not decrease; many of them even state the opposite. Zadravec et al. (2009) and Germšek (2008) proved that the net does not impair the bearing fruit – on the contrary, they noted a better differentiation of flower buds on the trees under the net (which might also be due to the uncovered trees being damaged by hail). Anti-hail net does not affect the trunk width, growth of the annual shoots, the number of nodes, or any other aspect of the exuberance of growth (Germšek 2008).

Widmer (2001), Blanke (2007) and Zadravec et al. (2009) established that the external quality of the fruit (diameter) under the net does not worsen. Germšek and Unuk (2014) state that the average weight of fruits is bigger under the black net, while Zadravec (2002), Štampar et al. (2002) and Iglesias and Alegre (2006) did not notice any affect on the average weight of the fruits.

Internal quality is also becoming more and more important for the consumer. Many authors have established that the internal quality of fruits somewhat changes when grown under an anti-hail net; fruit firmness and acid content increase in certain years, but the content of dry matter typically decreases (Widmer 2001, Steinbauer 2008, cit. in Zadravec et al. 2009). Zadravec (2002) establishes that there is more soluble dry matter in fruit grown outside the net (fruit with better colour contains more dry matter). Likewise, Germšek and Unuk (2014) establish that apple fruit (variety 'Gala Brookfiel' and 'Fuji Kiku 8') grown under the black anti-hail net has a lower content of dry matter (from 0.6 to 1 °Brix). Štampar et al. (2002), Iglesias and Alegre (2006), Germšek and Unuk (2014) claim that hardness of the fruit is not linked

to the anti-hail nets. Štampar et al. (2002) also establish that cultivation under anti-hail nets does not influence the content of malic and fumaric acid, although the fruit grown under a black net contains more citric acid.

Zadravec (2002) and Germšek and Unuk (2014) noted that the starch decomposition in the fruit is slower under a net, which is a sign of slower ripening. More dry matter was measured in the fruit grown without a net (fruit with better colour contains more dry matter) and a lower starch index was established in the fruit grown under a net (Štampar et al. 2002, Zadravec 2002, Germšek and Unuk 2014). An anti-hail net causes minimal differences of individual sugars (2x more glucose and fructose than sucrose) (Zadravec 2002). Štampar et al. (2002) prove the differences in glucose content (higher under black) and sucrose (higher under white), with no differences in fructose and sorbitol.

Fruit grown under the net is less exposed to sunburn, despite unchanged maximal leaf temperatures on trees (Zadravec 1998, cit. in Kuzma 2011). A lower risk of sunburn under anti-hail nets, especially under black nets, and consequently higher quality of the fruit's skin is also established by Štampar et al. (2002), Dussi et al. (2005), Iglesias and Alegre (2006). Zadravec (2002) establishes that 12% of fruit was sunburnt on trees without a net, 6% on trees under a white net and 5.5% on trees under a black net. Therefore, a reduction of light exposure is very welcome in extreme circumstances. Concerning the presence of sunburn, Steiermark recommends the use of black nets, woven with two fibres. Their experience shows that there was no sunburn on trees planted under a black net, whereas 6.5% of fruit was sunburnt on trees grown without a net; when a reflective foil was used for better fruit colour 9% of the fruit was sunburnt, on organic trees, where lime sulphur was used to protect from diseases, 16% of fruit was sunburnt (Caf 2009, cit. in Germšek 2010).

Casierra-Posada and Lüdders (2001), when studying the effect of summer pruning and mineral content of apple trees under anti-hail nets, have determined that anti-hail nets effect the content of minerals in sprouts and leaves. When pruned in June, the content of calcium and magnesium was higher in leaves. The fruit grown outside of a net had a higher content of calcium and potassium in comparison to the fruit grown under an anti-hail net. The fruit grown outside of a net has a higher calcium – potassium ratio in comparison to the fruit grown under a net. Growing fruit under a net decreases the ration between $(K+Mg) / Ca$.

Fruit skin colour under an anti-hail net

The basic colour of apples comes from chlorophyll pigments, most of which is chlorophyll a and b in a ratio 3 : 1. During ripening, the green colour turns to yellow, because of the forming of carotenoids (Hribar 1989). The ripening of red fruits leads to red colour in the fruit's skin. The decrease of green colour is connected to the decomposition of chlorophyll, while the red colour stems from the accumulation of anthocyanin (Macheix et al. 1990). The visual perception of colour is not only effected by the absolute concentrations of individual pigments in the fruit's skin but also by the proportions of the vacuoles and the distribution and size of

the cells in the apple's skin. The final percept of the colour is a consequence of a combination of all previously listed factors (Curry 1997).

It has been proven that a net can influence the chlorophyll synthesis in leaves (depending on variety, growth period and planting distance), as the average levels of photosynthesis pigments in leaves under a net are lower than outside of it. According to Horvat (2011), anti-hail nets also have an influence on the smaller decomposition of chlorophyll and formation of red pigments in fruits' epidermis, which has been confirmed by Germšek and Unuk (2014). Because of the small variability in the fruit grown under the net, compared to that grown without a net, Germšek and Unuk (2014) claim, that at the time of the technological ripeness the an anti-hail net (black) does not have a negative impact on fruit skin colour in cases where the study was conducted on the better coloured clones (like the variety 'Gala Brookfield').

Also according to Preiffer and Jankovič (2004), the net has a positive effect on the fruit's colour, because the colouration of the fruits is more similar. The fruit skin colour is mostly genetically determined and depends on weather conditions, special Low night temperatures during ripening have a positive effect on the accumulation of anthocyanins in apples. Ubi et al. (2006) claim that low temperatures cause a lower consumption of carbohydrates for breathing and increase photosynthesis, which ultimately affects the biosynthesis of anthocyanin. Night time temperatures between 3 and 11 °C are supposedly most suitable for coloration (Iglesias and Alegre 2006). Optimal daytime temperatures for anthocyanin accumulation are between 15 and 25 °C, whilst optimal night time temperatures are between 10 and 20 °C. Vidrih and Hribar (2002) claim that the fruit's colour is more intense when the differences between day and night time are bigger, and that coloration is optimal if cold nights (10–15 °C) are followed by warm (20–25 °C) and sunny days.

Horvat (2011) believes that the research of anthocyanin content shows that anti-hail nets effect and slow down the decomposition of chlorophyll and formation of red pigments in the fruit's epidermis. Germšek and Unuk (2014) point to an average 7 day (variety 'Gala') and an average 10 day (variety 'Fuji') delay in the decomposition of chlorophyll in the fruit's epidermis.

This effect was reduced at the time of optimal technological ripeness or was not perceived in favourable weather conditions at the time of ripening.

Concerning fruit colour, Jakopič et al. (2007) states that the variety 'Fuji' shows no differences in colour under a net or outside of it. Zadavec (2002) claims that the use of anti-hail nets causes an inferior fruit colour, which is most obvious when the black net is used. In another experiment, Zadavec et al. (2009) establishes no negative impact of anti-hail nets on fruit colour, just a slight reduction of the quantity of fruits with the most desired colour. The red skin colour is slightly worse in two-coloured varieties, while fully red varieties show no negative impact on the colour of the epidermis (Blanke 2007). Germšek and Unuk (2014) have made an effort to show that the position of the fruit (variety 'Gala Brookfield' and 'Fuji Kiku') has a stronger effect on the dynamics of colour development than the anti-hail net itself.

The impact of anti-hail nets on the microclimate of the orchard

In an experiment that lasted several years in the Fruit Growing Centre Maribor - Gačnik, the measurements of daily temperatures have shown that nets somehow reduce momentary temperature fluctuations caused by sun exposure changes. Similar results were found when measuring relative air humidity. A smoother curve in daily temperatures and relative air humidity can mean somehow better or less stressful conditions for a tree, which could explain why the reduction of light does not result in lower production capabilities of the orchard under an anti-hail net (Zadavec 2002).

The use of the net in an experiment in Spain has shown differences in temperature under the net and outside of it. The maximum temperatures under the net were 3 °C lower, but only on sunny days. On cloudy days, the differences were negligible. The decrease of maximum temperatures are also confirmed by Torggler (2002) (cit. in Zadavec et al. 2009) by, from 0.3 to 0.4 °C, Kührt et al. (2006) by 0,8 °C, and Solomakhin and Blanke (2008) by 0,2 °C, but only on sunny days. Zadavec (1998, cit. in Kuzma 2011) states that the somewhat smaller differences between maximum and minimum air temperatures could partly be the reason for delayed and lower fruit colouration.

Iglesias and Alegre (2006) also found a slight increase in relative air humidity under a net. They found a 2–6% increase of relative air humidity and, at the same time, a 11% decrease in evapotranspiration, which was associated with lower wind velocity. In comparison to measurements outside the net, an increase in relative air humidity from 0 to 8.9% was also determined under an anti-hail net by Torggler (2002, cit. in Zadavec et al. 2009).

A positive effect of anti-hail nets on fruit temperature has also been shown. Reduced fruit and leaf temperatures consequently mean less sunburn damage. A black net reduced the temperature of fruit by 4 °C, a white net by 2.5 °C. On cloudy days with less light, the differences were smaller. The differences between air and fruit temperatures were also smaller than on sunny days (Iglesias and Alegre 2006).

The anti-hail net also affects the quantity of rain that gets to the ground. The differences in the measurements of rain fall (less under an anti-hail net) are probably the consequence of the measuring instrument accuracy, and only with very small quantities of rain would it be possible that a part of the rain is "intercepted" by the net and evaporates from it – so the rain would not actually fall on the plants and ground in the orchard under the net (Zadavec et al. 2009).

The duration of the leaf dampness after rain, which is (c. 1 hour) longer under an anti-hail net, could only change the course of the incubation period for the apple scab in very specific infection circumstances. It is believed that black anti-hail nets do not prolong leaf dampness significantly and therefore do not increase the danger of certain diseases (Zadavec et al. 2009).

The impact of anti-hail nets on disease and pest development and mobility

Reigne (1997) (cit. in Iglesias and Alegre 2006) argues that anti-hail nets do not have an effect on the occurrence of the codling moth. Demaria et al. (2006, cit. in Iglesias and Alegre 2006) and Zadravec et al. (2009) observed that there was a lower number of this pest under an anti-hail net and the damage to fruit was lower when compared to the part which was outside the net. Ferjanc (2009) believes that the net hinders the movement of codling moths, but claims that despite this there is no distinctive impact on the degree of fruit damage. Graf et al. (1999) have established that the net is an obstacle when the codling moths move in, but mostly does not hinder these moths moving out. In investigations associated with the effects of nets on mating of codling moths, there were no differences between the part of the orchard under the net and the part outside it, when the lure was placed on the height of 1.5 m, while the difference was visible when the lure was placed on the height of 3 m, as there were more fertilised females outside of the net (Tasin et al. 2008). Kührt et al. (2006) claim that individual stages of the codling moth development occur a few days later under the net because of lower temperatures. In accordance with thermoregulation in the phenology model, the stages appear four days later under the net, except for the first generation, where the occurrence is three days late. The development of the whole generation was one day shorter than outside the net. The delay of the occurrence of individual stages under the net, where thermoregulation was not integrated, was four days for all stages.

Reigne (1997, cit. in Iglesias and Alegre 2006) establishes that anti-hail nets do not influence the occurrence of plant lice, while Racskó et al. (2005) states the importance of temperature in the development of this pest. Because of lower wind speeds under the net, the migration conditions of plant lice are better, while the possibility of other pests is decreased. They also believe that delayed ripeness does not influence the development of larvae in the fruit. Ferjanc (2009) states that anti-hail nets with regular mesh size cannot physically prevent the plant lice from entering the orchard. He believes that the impact on the size of the plant lice population varies according to the type and manner of migration between hosts.

Graf et al. (1999) and Tasin et al. (2008) have established that more fruit was damaged from caterpillars in orchards outside of nets. Zadravec et al. (2009) claim that the plant lice population develops faster and better under an anti-hail net. Ferjanc (2009) believes that the red plant lice develops better outside the anti-hail net, but states that the impact on the occurrence of this pest differs depending on fruit varieties ('Braeburn', 'Gala' and 'Fuji').

Reigne (1997) (cit. in Iglesias and Alegre 2006), Zadravec et al. (2009), Ferjanc (2009) note that anti-hail nets do not have an effect on diseases, such as, for example, apple scab and powdery mildew. Ruegg (1997) (cit. in Widmer 2001) also emphasizes that the leaves under the net, compared to those outside the net, do not stay damp long enough, which is why the possibility of apple scab infections is lower. Zadravec et al. (2009) state that the duration of the leaf dampness after

rain, which is (app. 1 hour) longer under an anti-hail net, could only change the course of the incubation period for apple scab in very specific infection circumstances.

CONCLUSION

Numerous researchers have demonstrated different experiences with anti-hail nets. It maybe reasonable to set experiments for each specific situation, where a wider selection of different types of anti-hail nets (with different colours and structures) would be integrated. In order to obtain reliable data, more experiments would have to be carried out in individual topics of great interest (the impact of anti-hail nets on the development and movement of pests, development of diseases, fruit quality, vegetative reactions of cultivated plants to conditions under anti-hail nets...), since the current research shows that some subjects and factors are poorly explored and that information on the effects of anti-hail nets is incomplete. In the future, it would be useful that experiments associated with anti-hail nets and their impact would include less explored topics (irrigation needs, tendencies of natural undeveloped fruit pruning under anti-hail nets...). More coherent findings and a more comprehensive understanding of these impacts in different researches would lead to more uniform conclusions which are urgently needed in practice.

REFERENCES

1. Awad MA, Wagenmakers PS, Jager A. Effects of light on flavonoid and chlorogenic acid levels in the skin of 'Jonaglod' apples. *Sci. Hortic.* 2001;88:289-298.
2. Blanke M. Can reflective ground cover compensate for light losses under hail nets? *Acta Hortic.* 2004;732:669-673.
3. Blanke M. Farbige Hagelnetze: Ihre Netzstruktur sowie Licht- und UV - Durchlässigkeit bestimmen die Ausfärbung der Früchte. *Erwerbs-Obstbau.* 2007;49(4):127-139.
4. Blanke M, Solomakhin A. Coloured hail nets alter light transmission, light spectra, phytochrome as well as vegetative growth, leaf chlorophyll and photosynthesis and reduce flower induction in apple. *Plant Growth Reg. J.* 2008;11:211-218.
5. Casierra – Posada F, Lüdders P. Influence of summer pruning and nitrogen nutrition on mineral content in apple trees under hail protection net. *Erwerbobstbau* 2001;43:106-113.
6. Curry EA. Temperatures for optimum anthocyanin accumulation in apple tissue. *J. Hortic. Sci.* 1997;72:723-729.
7. Dobaja K. Vpliv talne reflektivne folije, protitočne kristalne in črne mreže na notranjokakovost plodov pri jablani (*Malus domestica* Borkh.) sorte 'Jonagold' in 'Elstar' [diplomsko delo]. Maribor: Fakulteta za kmetijstvo in biosistemske vede, Univerza v Mariboru, 2005.
8. Dussi MC, Giardina G, Reeb P. Shade nets effect on

- canopy light distribution and quality of fruit and spur leaf apple cv. 'Fuji'. Span. J. Agric. Res. 2005;3(2):253-260.
9. Ferjanc B. Vpliv protitočne mreže na razvoj škodljivcev in boleznih jablane (*Malus domestica* B.) [diplomsko delo]. Maribor: Fakulteta za kmetijstvo in biosistemske vede, Univerza v Mariboru, 2009,
 10. Germšek B. Vpliv protitočne mreže na rast, rodnost in kakovost pridelka jabolk (*Malus domestica* B.) sorte 'Gala' [diplomsko delo]. Maribor: Fakulteta za kmetijstvo in biosistemske vede, Univerza v Mariboru, 2008,
 11. Germšek B. Razvoj parametrov kakovosti jabolk sort 'Gala' in 'Fuji' kot posledica vpliva protitočne mreže in položaja plodov v krošnji [magistrsko delo]. Maribor: Fakulteta za kmetijstvo in biosistemske vede, Univerza v Mariboru, 2010,
 12. Germšek B, Unuk T. Kakovost jabolk sort 'Gala Brookfiel' in 'Fuji Kiku' pod in izven protitočne mreže. Acta agricult. Slov. 2014;103(1):137-144.
 13. Graf B, Höpli H, Rauscher S, Höhn H. Hagelnetze beeinflussen das Migrationsverhalten von Apfel – und Schalenwickler. Obst – Weinbau 1999;12:289-292.
 14. Holzwarth R. Hagelschutznetze: Moderne Technik schützt das Obst. Besseres Obst 2008;6:25.
 15. Horvat S. Vpliv protitočne mreže na obarvanost plodov jablane (*Malus domestica* Borkh.) [diplomsko delo]. Ljubljana: Biotehnična fakulteta, Oddelek za agronomijo, Univerza v Ljubljani, 2011,
 16. Hribar J. Spremembe kemičnih in mehaničnih lastnosti jabolk sorte 'Jonagold' pri različnih pogojih skladiščenja [doktorska disertacija]. Ljubljana, BF, VTOZD za živilsko tehnologijo, 1989,
 17. Iglesias I, Alegre S. The effect of anti-hail nets on fruit protection, radiation, temperature, quality and profitability of 'Mondial Gala' apples. J. App. Horticult. 2006; 8(2):91-100.
 18. Jakopič J, Veberič R, Štampar F. The effect of reflective foil and hail nets on the lighting, color and anthocyanins of 'Fuji' apple. Sci. Horticult. 2007;115(1):40-46
 19. Jazbec M, Honzak J. V sadnem vrtu, moj vrt moje veselje. Ljubljana, Kmečki glas, 1982:38.
 20. Klein K. Vpliv protitočne mreže na kakovost plodov jablan [diplomsko delo]. Maribor: Fakulteta za kmetijstvo in biosistemske vede, Univerza v Mariboru, 2000,
 21. Kuzma S. Vpliv protitočnih mrež na kakovost plodov žlahtne jablane (*Malus domestica* Borkh.) [diplomsko delo]. Ljubljana: Biotehnična fakulteta, Oddelek za agronomijo, Univerza v Ljubljani, 2011,
 22. Kührt U, Samietz J, Höhn H, Dorn S. Modelling the phenology of codling moth: Influence of habitat and thermoregulation. Agric. Ecosyst Environ. 2006;117:29-38.
 23. Larcher W. Physiological plant ecology: Ecophysiology and stress physiology of functional groups, 4th edn., Berlin: Springer, 2003,
 24. Macheix J.-J, Fleuriet A, Billot J. Fruit phenolics. Boca Raton, CSR Press, 1990,
 25. Preiffer M, Jankovič P. Obvladovanje tveganja pri pridelavi sadja s pomočjo protitočnih mrež sistema Wiesel. V: Zbornik referatov 1. Slovenskega sadjarskega kongresa z mednarodno udeležbo. Krško, 24.- 26. marec. Ljubljana, 2004:249-253.
 26. Sadar N, Urbanek Krajnc A, Unuk T. 2013. Spectrophotometrically determined pigment contents of intact apple fruits and their relations with quality: a review. Zemdirbyste-Agriculture vol. 2013;100:105-111., No. 1 (2013)
 27. Steinbauer L. Treffen der Arbeitsgruppe »Obstbau unter Hagelnetzen. Haidegger Perspektiven 2008;3:6-7.
 28. Štampar F, Veberic R, Zadavec P, Hudina M, Usenik V, Solar A and Osterc G. Yield and fruit quality of apples cv. 'Jonagold' under hail protection nets. Gartenbauwissenschaft 2002;67(5):205-210.
 29. Tasin M, Demaria D, Ryne C, Cesano A, Galliano A, Anfora G, Loriatti C, Alma A. Effect of anti-hail nets on *Cydia pomonella* behaviour in apple orchards. Entomologia experimentalis et applicata 2008;129(1):32-36.
 30. Ubi BE, Honda C, Bessho H, Kondo S, Wada M, Kobayashi S, Moriguchi T. Expression analysis of anthocyanin biosynthetic genes in apple skin: Effect of UV – B and temperature. Plant Sci. 2006;170(3):571-578.
 31. Vercammen J. Eerste ervaringen met hagelnetten - ten in België. Fruitteelt – nieuws 1999;12:6-8.
 32. Vidrih R, Hribar J. Optimalni rok obiranja sadja. Brstika. Priloga tednika kmečki glas za sadjarje in vinogradnike. 2002;1,3:4-5.
 33. Zadavec P. Vpliv protitočne mreže na količino in kakovost pridelka pri jablani. Sodobno kmetijstvo. Kmečki glas 2002;35(3):108-111.
 34. Zadavec P, Donik B, Beber M, Unuk T, Tojnko S, Lešnik M, Germšek B, Ferjanc B. Odziv jablane in škodljivih organizmov na spremenjene razmere pod protitočnimi mrežami. Monografija, Sadjarski posvet, 2009:38-44.
 35. Widmer A. Beschattung unter weissen und grauen Hagelnetzen. Obst und Weinbau 2001a;133:581-583.
 36. Widmer A. Light intensity and fruit quality under hail protection nets. Acta Hort. 2001b;557:421-426.

Vpliv različnih tipov protitočnih mrež na prepustnost svetlobe in parametre kakovosti jabolk – pregledni članek

IZVLEČEK

Danes protitočne mreže predstavljajo osnovno opremo v nasadih jablan. Zmanjšujejo rizik pridelave in hkrati omogočajo redne pridelke jabolk visoke kakovosti. Različne barve mreže različno močno ovirajo prehod svetlobe, kar ima neposredni vpliv na parametre kakovosti pridelka, posebej obarvanost. Članek vključuje kratki pregled objav, povezanih s prepustnostjo svetlobe svozi različne tipe protitočnih mrež ter posledično vpliv na kakovost plodov. Vključene so tudi vsebine raziskav, ki tečejo na temo prilagoditve posameznih tehnoloških ukrepov v nasadu, kot je to npr. uporaba reflektivne folije. Nekaj zbranih informacij, ki jih lahko najdemo v literaturi, pa pojasnjuje vpliv protitočnih mrež na vzpostavitev mikroklimne v nasadu ter posledično vpliv na razvoj in dinamiko pojava posameznih boleznin in škodljivcev.

Sweet maize growth and yield response to organic and mineral fertilizers, N rates and soil water regimes

Franc BAVEC^{1*}, Martina BAVEC¹, Silva GROBELNIK MLAKAR¹, Milojka FEKONJA²

¹University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 10, 2311 Hoče, Slovenia

²Development Agency Slovenske gorice, Trg osvoboditve 9, 2230 Lenart

ABSTRACT

Sweet maize is an underutilized vegetable in European temperate areas, and its consumption is increasing. For better understanding of cultivation practices, this pot experiment aimed to determine the effects of different water regimes and nitrogen (N) rates calculated from N target values. N rates of 0 (control), 0.6 and 2 g N pot⁻¹ were applied as organic by-products pumpkin cake and pig manure digestate, and mineral fertilizers CAN 27 and ENTEC[®]26. Treatments of water supply were based on measured soil matric potentials of 2.8 pF (drought stress), 2.6 pF (optimal water) and 2.4 pF (overwatered). In comparison to mineral fertilizers, pumpkin cake proved to be equal in effectiveness in plant height (155.8 cm), cob (85.8 g), green (124.9 g) and leaf mass per plant (44.2 g), or even better in root (72.3 g) and broom mass per plant (3.0 g). Yield parameters, cob mass (70.1 g), its length (6.3 cm) and diameter (2.0 cm), as well as the residual mineral N (59 mg N kg⁻¹) significantly increased at the highest N rate. Significantly lower values of the evaluated morphological parameters and photosynthetic rates (at brooming and harvesting) were associated with drought stress. The matric tension of 2.6 pF was established as an appropriate water regime for sweet maize growth.

Key words: *Zea mays* L. *saccharata* Sturt., nitrogen, fertilizers, soil water potential, growth conditions

INTRODUCTION

In the tropics, from where sweet maize (*Zea mays* L. *saccharata* Sturt.) originates, the most important limiting factors for its growth are water and nitrogen (N) supply (Moser et al. 2006). The crop is grown at latitudes between 50°N and 40°S, and at altitudes 0–3,000 m above sea level (Ghorpade et al. 1998), and to establish sweet maize production in a temperate climate requires research into cultivation practices. Moreover, climate change (i.e. increased temperatures) suggests the possibility of its wider production, which will require further research in areas currently considered atypical for maize (Bavec and Bavec 2002).

Limiting factors for sweet maize production are high temperatures and water stress. High air temperatures (> 38°C) and concurrent water stress decrease yields (Ramadoss et al. 2004). In semi-arid regions, where temperatures are high

and problems with water and irrigation are common, Sari et al. (2000) reported that April is the preferred sowing time for cob and yield quality, and that later sowing in May reduces yield. Öktem et al. (2003) recommended a drip irrigation system with two-day irrigation frequency (100 % of evapotranspiration) to achieve optimum growth of sweet maize in semi-arid regions. Stone et al. (2001) analyzed the drought responses of sweet maize water use, biomass, yield, and yield components and found that yield was strongly related to biomass accumulation (especially to biomass accumulated after brooming). Biomass reduction occurred mainly due to the effects of drought stress on radiation use efficiency, particularly at early growth stages.

When considering the growth of sweet maize, special attention needs to be devoted to N nutrition and possible environmental pollution by N residues after harvesting the crop (Silgram and Shepherd 1999). N residues include the residual soil mineral N (N_{min}) and N in crop residues. Using

*Correspondence to:
E-mail: franci.bavec@um.si

the recommended N rates for vegetables, however, may leave large amounts of residual soil N_{min}, especially if crops such as sweet maize are harvested before maturity (Neeteson et al. 1999). Olaniyan et al. (2004) suggested that the use of organo-mineral fertilizers reduces nitrate losses due to leaching and improves soil structure; and that the highest yield and total dry matter were attained at the highest N rate of 120 kg N ha⁻¹. Juntharapthep et al. (2007) found that fermented chicken manure was an efficient fertilizer and produced the highest yields of unhusked, husked and standard unhusked cob for sweet maize (14.25, 10.56 and 13.62 t ha⁻¹, respectively).

Many aspects of plant growth, such as plant photosynthetic activity, N concentration and protein content are affected by drought stress, which also influences nitrate reductase activity in species such as maize (Foyer et al. 1998) and winter wheat (Xu and Yu 2006). Prolonged periods of dehydration stress inhibit photosynthesis of most active mesophyll cells, suppressing metabolism and lowering water use efficiency. Effects on stomatal conductivity are as important as those on photosynthesis (Taiz and Zeiger 2002). Research on maize (Jacob and Lawlor 1992), wheat (Xu et al. 2009) and sweet maize (Xu et al. 2004; Fletcher et al. 2008) has shown that photosynthetic functions depend on many growth factors. Xu et al. (2004) noted that an increase in photosynthetic activity of sweet maize and the quantity of dry matter might be associated with stomatal opening and biochemical activities.

The lack of information on sweet maize cultivation practices limits its introduction into temperate climate zones. For this reason, data obtained from pot experiments provides valuable knowledge regarding the (i) influences of different N rates, (ii) the applied form of fertilizers (i.e. organic or mineral), and (iii) of various water regimes on morphological and yield characteristics, and photosynthetic parameters of sweet maize.

MATERIAL AND METHODS

The effects of different N target values, fertilizer types and water regimes were studied on sweet maize morphological, photosynthesis and yield parameters in a greenhouse pot experiment (University Research Centre Maribor, Slovenia: 46°39' N, 15°41' E and 282 m.a.s.l.) under natural light and temperature conditions. N rates of 0 (control), 0.6 and 2.0 g N pot⁻¹ were calculated similarly to Rodrigues (personal communication), as equivalent approximations of N target values: 70 and 170 kg N ha⁻¹, and control (presowing N_{min}). The examined N rates (41 and 141 kg N ha⁻¹) were calculated according to the following formula: N rate (kg N ha⁻¹) = N target value – kg N_{min} in the soil (i.e. 29 kg N_{min} ha⁻¹). Different organic by-products (pumpkin cake from oil processing with 9 % N, and digestate from biogas production of pig manure with 1.6 % N) and mineral fertilizers (CAN – 27% ammonium nitrate NH₄NO₃, and ENTEC26 – 26% ammonium nitrate and ammonium sulphate (NH₄)₂SO₄ with nitrification inhibitor (DMPP)) were applied to pots maintained at three water regimes.

A three-factor randomized block design was used. The basic block consisted of four pots (0.14 m in diameter and

0.90 m in height) and was carried out in three replications using sweet maize hybrid 'Gold cup F1' with normal sugar content 'su'.

Top soil of sandy loam texture was collected near the greenhouse, homogenized and used to fill the pots. The chemical and physical properties of used soil were: organic matter 11.2 mg g⁻¹, P₂O₅ 52.2 mg kg⁻¹, and available K₂O 233.2 mg kg⁻¹. After filling the experimental pots soil (15.8 kg per pot), with a bench mat at the bottom, were placed to the constructed skeleton of building net. The iron net supported the pots and maintained a constant distance of 30 cm between the plants.

Before sowing, N_{min} was analyzed (N_{min} = nitrate-N + ammonium-N = 8 mg kg⁻¹) in the soil (Scharph and Wehrmann 1975; ISO/DIS 14255 1998), and on this basis the N rate was calculated. Fertilizers were applied before sowing, mixed in the upper 0.1 m of soil in the pot. Soil N_{min} was also analyzed at BBCH 75–79 growth stage (milk stage) (Zadoks et al. 1974) and samples were taken from all pots. In total, 300 pots were used. Due to technical limitations (i.e. greenhouse area) the control treatment without added N was analyzed only at the optimal water regime (2.6 pF).

Three days after filling pots, sweet maize was sown into pots (after emergence, thinning reduced the numbers to one plant per pot) and tensiometer tubes (30 cm long) were placed randomly into pots with one tensiometer per treatment (i.e. only in the first replication). After sowing, pots were subjected to the three different soil water regimes. Water regimes were controlled as by Van der Vecken et al. (2003) with tensiometers at soil matric potentials of 2.40 pF (considered as overwatered), 2.60 pF (optimal watered) and 2.80 pF (drought stress) as measured by SMS model 2500 (SDEC, France), where pF units are logarithms of hPa. At BBCH 13–14 stage, all treatments were sustained at optimal water regime (2.6 pF). The tensiometers were placed in pots for daily measurements of the pressure generated in the water column and to calculate the amount of water needed for a given water regime. Tensiometer measurements were performed daily before irrigation. The relationship between matric potential and gravimetric soil moisture content (from wilting point to field capacity) was read from the calculated desorption curve (ISO 11274 1998).

Morphological and yield parameters for all plants were determined at harvest. Measurements included (masses are all in fresh weights): cob mass, plant mass (whole plant), plant green mass (aboveground biomass), root mass, leaf mass, stem mass, broom mass, root length, cob length, cob diameter and plant height.

During the growing season, photosynthetic parameters (photosynthetic rate – A, and stomatal conductance – g_s) were monitored by gas-exchange equipment (LCpro+, ACD BioScientific Ltd, UK). Environmental conditions in the leaf chamber were set by LCpro+ equipment. Measurements were made during 10:00–14:00 h on the upper fully expanded leaf at stages BBCH 15–17 (A1 and g_s1), 65–69 (A2 and g_s2) and 75–79 (A3 and g_s3) using the method of Hirasava and Hsiao (1999). They were repeated five times at min⁻¹ intervals and the mean of the five readings was taken as the measured value.

An analysis of variance (ANOVA; P < 0.001, P < 0.01 and P

< 0.05) for a factorial experiment (fertilizer × N rate × water regime) was performed using the Statgraphic® Centurion (2005) statistical package. Significant differences among treatments were determined using least significant difference (LSD) test at $P < 0.05$. Pearson's correlation coefficients between morphological and yield parameters, photosynthetic parameters and Nmin were calculated using SPSS 15.0 for Windows statistical package (2005). A quadratic regression was calculated only for some significant properties.

RESULTS AND DISCUSSION

Morphological characteristics

In the case of N rates, there were no significant differences in root, leaf and broom mass (Table 1). In contrast, plant mass, green mass, stem mass and plant height significantly increased as N rates were increased. There was a significant effect of N on root length, with significantly longer roots at the lower N rate of 0.6 g pot⁻¹. This indicated the possibility that plants experiencing less N in the soil develop deeper root systems. Similar results were also reported for common maize by Rhoads and Bennett (1990), who found out that crop roots took up nutrients and water from upper levels of the soil under low-water stress or non-stress conditions. Furthermore, based on the measured morphological parameters, pumpkin cake fertilizer appeared to be an acceptable substitute for the N mineral fertilizers. When ENTEC[®]26 fertilizer, which has

a longer fertilization effect, was compared with either CAN 27 or pumpkin cake, there were no significant differences. Similarly, Guertal (2000) reported results with bell pepper where slow-release fertilizers had no consistent improvement over a soluble N source. The use of digestate in the present study resulted in significantly lower plant mass and plant height, and also in less N residues at harvest compared to the other three fertilizers. These results can be explained by the use of fresh digestate, which can lead to phytotoxic effects or to N loss by ammonia volatilization (Abdullahi et al. 2008; Fuchs et al. 2008).

Water regimes also had a significant effect on all investigated morphological parameters of sweet maize. Except for root length, there was no significant difference between overwatered and optimal water regimes in any morphological parameter. However, all parameters were significantly lower under drought stress conditions. Only root length significantly differed in all water regimes; in comparison to the optimal water regime, there were significantly longer roots in the overwatered condition, and significantly shorter roots for drought stress. In contrast, Kirtok (1998) found in common maize that frequently watered plants produced a shallow root system, whereas occasionally watered plants produced a deep root system. However, in the present study the root mass of overwatered compared to optimally watered plants was not different (Table 1).

Table 1: Effects of water supply and N fertilization on morphological parameters (cob mass–CM; plant mass–PM; plant green mass–GM; root mass–RM; leaf mass–LM; stem mass–SM; broom mass–BM; root length–RL; cob length–CL; cob diameter–CD; plant height–PH) of sweet maize (mean values per plant)

Treatments	CM (g)	PM (g)	GM (g)	RM (g)	LM (g)	SM (g)	BM (g)	RL (cm)	CL (cm)	CD (cm)	PH (cm)
Fertilizer (F)	***	**	NS	**	NS	*	***	NS	***	***	***
N rate (N)	*	**	**	NS	NS	**	NS	***	***	***	*
Water regime (W)	**	***	***	***	***	***	***	***	***	***	***
Interaction											
F×N	NS	**	***	***	**	**	*	NS	***	***	***
F×W	NS	NS	***	***	NS	***	***	***	***	***	**
N×W	*	**	**	NS	*	*	NS	NS	**	**	NS
F×N×W	NS	NS	NS	**	NS	*	*	***	*	NS	NS
Fertilizer											
Oil pumpkin cake	85.8 a	210.7 a	124.9	72.3 a	44.2	77.6 a	3.0 a	84.4	5.7 a	1.7 b	155.8 a
Digestate	21.5 b	138.9 c	117.4	58.2 b	39.3	75.3 a	2.8 ab	76.7	1.5 b	0.7 c	131.3 b
CAN 27	61.9 a	181.5 ab	119.7	51.1 b	42.9	74.1 a	2.6 b	84.2	6.6 a	2.1 ab	156.8 a
ENTEC [®] 26	66.2 a	175.4 b	109.2	52.8 b	43.0	64.3 b	1.8 c	85.0	7.0 a	2.3 a	157.9 a
N rate (g pot ⁻¹)											
0.60	47.6 b	158.6 b	110.9 b	54.6	40.2	68.1 b	2.6	88.0 a	4.1 b	1.3 b	147.0 b
2.00	70.1 a	194.6 a	124.6 a	62.6	44.5	77.5 a	2.5	77.2 b	6.3 a	2.0 a	153.9 a
Water regime											
Drought stress	30.4 b	115.3 b	84.9 b	20.2 b	27.9 b	55.1 b	1.9 b	70.6 c	1.2 b	0.4 b	114.0 b
Optimal water	74.5 a	210.0 a	135.5 a	79.8 a	49.8 a	82.7 a	2.9 a	82.6 b	7.6 a	2.4 a	170.7 a
Overwatered	71.6 a	204.6 a	132.9 a	75.8 a	49.5 a	80.6 a	2.9 a	94.6 a	6.8 a	2.3 a	166.7 a

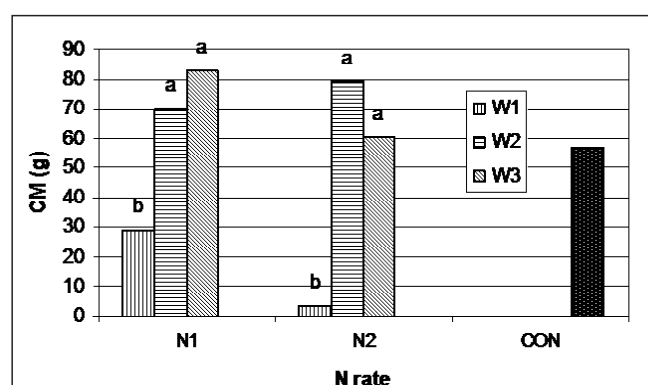
*, **, ***, NS: significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, and not significant, respectively
a-c values within column followed by the same letter are not significantly different at $P < 0.05$ (LSD test).

Parameters of yield formation

There were significant differences in cob mass for the different fertilizers, N rates and water regimes (Table 1), but the only significant interaction was N rate \times water regime (Figure 1). The pumpkin-cake organic fertilizer produced cobs of similar mass than those fertilized by CAN 27 and ENTEC[®]26. Rodrigues et al. (2010) found similar results for tall cabbage where ENTEC[®]26 and urea were not statistically different in dry matter yield and nitrogen use efficiency, in field and pot experiments. In the present study, the digestate resulted in significantly lower values of cob mass, length and diameter compared to the other fertilizers. A similar pattern to cob mass was also found for cob length. Treatment with ENTEC[®]26 resulted in significantly greater cob diameter compared to pumpkin cake and digestate.

The cob mass, length and diameter were significantly affected by different N rates and water regimes (Table 1). There was higher cob mass, length and diameter at the higher N rate (2 g pot⁻¹). Drought stress resulted in significantly lower cob mass, length and diameter in comparison to optimal and overwatered regimes; furthermore, these parameters were similar for the overwatered and optimal water conditions. Zhang and Davies (1989) reported a similar reduction in yield when crops were exposed to water stress for prolonged periods.

There was a strong positive correlation $r = 0.778$ ($P < 0.001$) between cob mass and plant mass (Table 2). The significant quadratic regression ($y = -0.014x^2 + 0.0954x - 9.95461$; $R^2 = 0.88$, $P > 0.001$) can explain the high influence of plant mass (x) on cob mass (y). These results were similar to those of Stone et al. (2001) who reported that yield of sweet maize is strongly related to biomass accumulation, especially to biomass accumulation after silking. There were moderate correlations between cob mass and root mass ($r = 0.529$, $P < 0.001$), and strong correlations between cob mass and plant height ($r = 0.775$, $P < 0.001$). Plant mass was also correlated with root mass ($r = 0.509$, $P < 0.001$) and with plant height ($r = 0.634$, $P < 0.001$).



Different letters indicate significant differences between treatments ($P < 0.05$).

Fig. 1: Interactions between examined treatments, water supply (drought stress-W1, optimal water-W2 and overwatered-W3) and N rates (N1-0.6 g pot⁻¹; N2-2 g pot⁻¹) treatments for the cob mass (CM) at sweet maize growth stage BBCH 75-79; parallel is shown control plot at optimal water regime (CON)

Table 2: Correlations (Pearson) between morphological characteristics (n=72) of sweet maize (plant mass-PM; plant green mass-GM; cob mass-CM; root mass-RM; plant height-PH; root length-RL)

Analyzed parameters	GM	CM	RM	PH	RL
PM	0.777***	0.778***	0.509***	0.634***	0.213
GM		0.617***	0.642***	0.615***	0.233*
CM			0.529***	0.775***	0.196
RM				0.562***	0.421***
PH					0.389***

* significant at 0.05, *** significant at 0.001.

Photosynthetic parameters

Photosynthetic rate (Table 3) was significantly affected by N rate (at BBCH 15-17), by water regime (at BBCH 65-67), and by fertilizer and water regime at harvest (BBCH 75-79). At BBCH 15-17, the measured photosynthetic rate was significantly higher at lower N rate (0.6 g pot⁻¹), but not at brooming and harvest. Zhao et al. (2005) reported that decreased photosynthetic rate of sorghum plants was associated with N deficiency. In comparison to the optimal water regime, there was a lower photosynthetic rate (BBCH 65-69 and 75-79) under drought stress conditions. Photosynthetic rate at BBCH 75-79 was lowest for digestate (4.0 $\mu\text{mol m}^{-2}\text{s}^{-1}$) and was higher for pumpkin cake (5.2 $\mu\text{mol m}^{-2}\text{s}^{-1}$), CAN 27 (6.5 $\mu\text{mol m}^{-2}\text{s}^{-1}$) and ENTEC[®]26 (8.5 $\mu\text{mol m}^{-2}\text{s}^{-1}$). Investigations of Efthimiadou et al. (2009) under field conditions, showed that photosynthetic rate (66 d after sowing) was significantly higher with application of cow manure at 240 kg N ha⁻¹ compared to barley mulch and poultry manure (both at 35, 70 and 140 kg N ha⁻¹) and mineral fertilizer (240 kg N ha⁻¹). In the present study, of all investigated factors only water regime influenced the stomatal conductance at BBCH 65-67: it was significantly lower in drought stress compared to optimal conditions and also lower in the overwatered regime. Stomatal conductance was not significantly different between N rates and between fertilizer treatments. Similar results were achieved also by Efthimiadou et al. (2009) who investigated organic manures and conventional fertilizer in field conditions.

There were moderate correlations (data not shown) between stomatal conductance and photosynthetic rate at BBCH 15-17 ($r = 0.553$, $P < 0.001$) and BBCH 65-67 ($r = 0.699$, $P < 0.001$), but a weak correlation at BBCH 75-79 ($r = 0.332$, $P < 0.001$). Xu et al. (2004) also concluded that leaf stomatal opening for sweet maize was positively related to increases in photosynthetic functions and quantity of plant dry matter. The correlations between photosynthetic parameters (A1-A3 and g_s1-g_s3) and morphological characteristics were calculated (Table 4).

Correlations between photosynthetic rates and plant height (Table 4) were not strong, but increased during successive growth stages ($r = 0.166$, $r = 0.406$, $P < 0.001$, and $r = 0.495$, $P < 0.001$, respectively). Similarly, correlation strength between photosynthetic rate and plant mass, cob mass, cob diameter and cob length with growth increased.

Table 3: Effects of water supply and N fertilization on Nmin residuals in the soil at harvest, photosynthetic rate (A1–A3 at BBCH 15–17, 65–67 and 75–79, respectively) and stomatal conductance (g_s 1–3g1 at BBCH 15–17, 65–67 and 75–79, respectively) of sweet maize (mean values per plant)

Treatments	Nmin	A1	A2	A3	g_s 1	g_s 2	g_s 3
	mg kg ⁻¹		$\mu\text{mol m}^{-2}\text{s}^{-1}$			$\text{mol m}^{-2}\text{s}^{-1}$	
Fertilizer (F)	***	NS	NS	***	NS	NS	NS
N rate (N)	***	**	NS	NS	NS	NS	NS
Water regime (W)	NS	NS	*	***	NS	*	NS
Interaction							
F×N	***	NS	*	**	NS	NS	NS
F×W	***	NS	NS	***	NS	NS	NS
N×W	***	NS	NS	NS	NS	*	NS
F×N×W	***	NS	NS	*	NS	NS	NS
Fertilizer							
Oil pumpkin cake	56 a	18.0	13.0	5.2 bc	0.12	0.10	0.07
Digestate	6 c	18.8	12.7	4.0 c	0.14	0.10	0.06
CAN 27	49 ab	19.2	15.6	6.5 b	0.13	0.10	0.08
ENTEC*26	43 b	19.4	11.4	8.5 a	0.14	0.09	0.11
N rate (g pot ⁻¹)							
0.60	17 b	20.2 a	13.8	5.8	0.14	0.10	0.07
2.00	59 a	17.5 b	12.5	6.2	0.13	0.09	0.08
Water regime							
Drought stress	38	17.6	10.1 b	3.8 b	0.12	0.08 b	0.07
Optimal water	37	19.1	15.7 a	7.6 a	0.13	0.12 a	0.09
Overwatered	41	19.8	13.7 ab	6.6 a	0.15	0.10 ab	0.08

*, **, ***, NS: significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, and not significant, respectively
a-c values within column followed by the same letter are not significantly different at $P < 0.05$ (LSD test).

Table 4: Correlations (Pearson) between photosynthetic parameters (A1–A3 and g_s 1–3 at BBCH 15–17, 65–67 and 75–79, respectively) and morphological characteristics, n=72

Analyzed parameters	PH	PM	GM	CM	CD	CL
g_s 1	0.270*	-0.006	0.083	0.038	0.072	0.064
A1	0.166	0.087	0.258*	0.090	0.133	0.105
g_s 2	0.356**	0.163	0.209	0.271*	0.263*	0.266*
A2	0.406***	0.256*	0.304**	0.404***	0.362**	0.429**
g_s 3	0.171	0.124	0.060	0.198	0.238*	0.238*
A3	0.495***	0.387**	0.202	0.589***	0.630***	0.649***

*, **, *** significant at the 0.05, 0.01, 0.001, respectively.

Only correlations between green mass (plant mass without cob) and photosynthetic rate at harvest were lower than at brooming stage. Correlations between stomatal conductance and morphological characteristics were weak; but the values increased to brooming growth stage and then declined by harvest.

Nmin at harvest

Residual Nmin in soil varied significantly with fertilizers and N rate; as well as with interactions of fertilizer × N rate, fertilizer × water regime, N rate × water regime, and fertilizer

× N rate × water regime (Table 3). Residual Nmin values were significantly higher at higher N rates.

The digestate resulted in the lowest Nmin residuals (6 mg N kg⁻¹) at harvest (BBCH 75–79), and can be explained by N immobilization risk (Fuchs et al. 2008). Schievano et al. (2009) explained that immobilization of N in the soil may occur with the use of not-fully matured digestates.

There was significantly higher residual soil Nmin following fertilization using pumpkin cake, CAN 27 and ENTEC*26 (56, 49 and 43 mg N kg⁻¹, respectively); the corresponding converted values were approximately (using 1200 kg m⁻³ specific mass of the soil) 205, 179 and 154 kg N ha⁻¹. High residual values imply potential for groundwater pollution. Additionally, it is important to account for higher temperatures in the greenhouse compared to field conditions, which can increase mineralization processes in the soil. A laboratory leaching study (Huett and Gogel 2000) showed that controlled-release fertilizers at higher temperatures (30–40°C) had an N-release increased and period was shorter. But, this results lead to more clearly picture in case of interaction fertilizer × N rate (Figure 2). The mineral fertilizers at a higher N rate (2 g pot⁻¹) resulted in higher Nmin residuals compared to the lower N rate (0.6 g pot⁻¹). Especially high Nmin residuals were measured after CAN 27 application in comparison to pumpkin cake and digestate. In general, in the case of mineral fertilizers the lower N rate (0.6 g pot⁻¹) resulted in environmentally acceptable Nmin residuals (in accordance with legislation); and also the use of digestate at both N rates resulted in low Nmin residuals (5 and 6 mg N

kg⁻¹). Based on a pot and field study with tall cabbage and controlled-release fertilizers, Rodrigues et al. (2010) found that ENTEC²⁶ was not a fertilizer that prevented N from leaching during winter conditions. Furthermore, in our case no significant differences were observed when water regimes were analyzed.

CONCLUSIONS

The by-product of pumpkin cake as an organic fertilizer had comparable effects on growth (i.e. morphological parameters) and yield parameters (cob mass, length and diameter) of sweet maize as the mineral fertilizers. ENTEC²⁶ as a slow-release N fertilizer showed no advantage for sweet maize in any observed parameter (morphological, yield and photosynthetic parameters) in comparison to pumpkin cake or CAN 27. Fertilizing with a lower N rate (0.6 g pot⁻¹) resulted in significantly lower values of plant mass, cob mass, green mass, stem mass, cob length, cob diameter and plant height, compared to the higher N rate (2 g pot⁻¹); however, there were high soil N_{min} residuals at the highest N rate (59 mg N kg⁻¹). There were similarly high N_{min} residuals for all investigated fertilizers (56, 49 and 43 mg N kg⁻¹), except for pig manure digestate (6 mg N kg⁻¹). In addition, it is important to account for the higher temperatures in the greenhouse than in field conditions, since they can increase mineralization processes in soil. Furthermore, drought stress was associated with significantly lower values for all measured morphological characteristics in comparison to the optimal and overwatered regimes; however, soil N_{min} residuals were not significantly different. The optimal watered regime of 2.6 pF matric potential was confirmed as an appropriate water regime, since the same or significantly higher morphological, photosynthetic or yield parameters were achieved compared to overwatered (2.4 pF) and drought stress (2.8 pF) conditions. Photosynthetic rate was significantly lower at brooming and at harvest under drought stress conditions, in comparison to the optimal and overwatered regimes. Moreover, correlations between photosynthetic rate and yield parameters were weak to moderate, but increased during successive growth stages. This study contributes results and offers opportunities for wider investigations in the field, especially for temperate climates, considering the lack of available sweet maize research results.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of national projects CRP V4-0472 (Sweet maize – development of new, economic and environmentally acceptable products) and V4-0104 (Organic Vegetable Production) conducted by the Government of the Republic of Slovenia, Ministry of Agriculture, Forestry and Food.

REFERENCES

1. Abdullahi YA, Akunna AC, White NA, Hallett PD, Wheatley R. Investigating the effects of anaerobic and aerobic post-treatment on quality and stability of organic fraction of municipal solid waste as soil amendment. *Bioresour. Technol.* 2008;99: 8631-8636.
2. Bavec F, Bavec M. Effects of plant population on leaf area index, cob characteristics and grain yield of early maturing maize cultivars (FAO 100-400). *Eur. J. Agron.* 2002;16:151-159.
3. Efthimiadou A, Bilalis D, Karkanis A, Froud-Williams B, Eleftherochorinos I. Effects of cultural system (organic and conventional) on growth, photosynthesis and yield components of sweet corn (*Zea mays* L.) under semi-arid environment. *Notulae Botulae Botanicae Horti Agrobotanici Cluj-Napoca.* 2009;37:104-111.
4. Fletcher AL, Moot DJ, Stone PJ. Radiation use efficiency and leaf photosynthesis of sweet corn in response to phosphorus in a cool temperate environment. *Eur. J. Agron.* 2008;29:88-93.
5. Foyer CH, Valadier MH, Migge A, Becker TW. Drought-induced effects on nitrate reductase activity and mRNA and on the coordination of nitrogen and carbon metabolism in maize leaves. *Plant Physiol.* 1998;117:283-292.
6. Fuchs JG, Baier U, Berner A, Mayer J, Schleis K. Effects of digestate on the environment and on plant production – results of a research project. ECN/ORBIT e.V. Workshop 2008. "The future of Anaerobic digestion of Organic Waste in Europe". Nürnberg, 2008; Pres. nr. 19.
7. Ghorpade VM, Hanna MA, Jadhav SJ. Sweet corn. In: Salunkhe DK, Kadam SS. *Handbook of Vegetable Science and Technology; Production, Composition, Storage and Processing.* Marcel Dekker, Inc., New York, USA, 1998: 609 - 647.
8. Guertel EA. Preplant slow-release nitrogen fertilizers produce similar bell pepper yields as split applications of soluble fertilizer. *Agron. J.* 2000;92: 388–393.
9. Hirasawa T, Hsiao TC. Some characteristics of reduced leaf photosynthesis at midday in maize growing in the field. *Field Crops Res.* 1990;62:53-62.
10. Huett DO, Gogel BJ. Longevities and nitrogen, phosphorus, and potassium release patterns of polymer-coated controlled-release fertilizers at 30°C and 40°C. *Commun. Soil Sci. Plan.* 2000;31:959-973.
11. ISO/DIS 14255. International Organisation for Standardisation (ISO), ISO/DIS 14225, Soil quality – determination of soluble nitrogen fractions, Geneva, 1998.
12. ISO 11274. International Organisation for Standardisation 11274, Soil quality determination of the water retention characteristic – Laboratory methods 1998 (E), Geneva, 1998.
13. Jacob J, Lawlor D. Dependence of photosynthesis of sunflower and maize leaves on phosphate supply, ribulose-1,5-bisphosphate carboxylase/oxygenase activity, and ribulose-1,5-bisphosphate pool size. *Plant Physiol.* 1992;98:801-807.

14. Juntharathep P, Suanphairoch S, Klaocheed S, Wongvarodom V. Effects of fertilizer types on sweet corn in organic production systems. In: Proceedings of the 45th Kasetsart University Annual Conference, Bangkok, Thailand, 2007:647-654.
15. Kirtok Y. Corn production and use. Kocaoluk, Istanbul, TR, 1998.
16. Moser SB, Feil B, Jampatong S, Stamp P. Effects of pre-anthesis drought, nitrogen fertilizer rate, and variety on grain yield, yield components, and harvest index of tropical maize. *Agr. Water Manage.* 2006;81:41-58.
17. Neeteson JJ, Booij R, Withmore AP. A Review on Sustainable Nitrogen Management in Intensive Vegetable Production Systems. *ISHS Acta Horticulturae*, 506: International Workshop on Ecological Aspects of Vegetable Fertilization in Integrated Crop Production. *Acta Hort.* 1999;1:17-26.
18. Olaniyan AB, Akintoye HA, Balogun MA. Effect of different sources and rates of nitrogen fertilizer on growth and yield of sweet corn. National Horticultural Research Institute, Vegetable Production, Nigeria [online], 2004. date: 16/June/2009 (website:<http://tropentag.de/2004/abstracts/full/146.pdf>)
19. Öktem A, Simsek M, Öktem AG. Deficit irrigation effects on sweet corn (*Zea mays saccharata* Sturt) with drip irrigation system in a semi-arid region I. water-yield relationship. *Agric. Water Manage.* 2003;61:63-74.
20. Ramadoss M, Birch CJ, Carberry PS, Robertson M. Water and high temperature stress effects on maize production. In: Proceedings of the 4th International Crop Science Congress, Brisbane, Australia. [online], 2004. date: 10/December/2008 (website: <http://cropscience.org.au>.)
21. Rhoads FM, Bennett JM. Corn. In: Stewart BA, Nielsen DR (eds.), *Irrigation of agricultural crops*. ASAE Agronomy Monograph 30, Madison, WI, 1990:569-596.
22. Rodrigues MA, Santos H, Ruivo S, Arrobas M. Slow-release N fertilizers are not an alternative to urea for fertilization of autumn-grown tall cabbage. *Eur. J. Agron.* 2010;32:137-143.
23. Sari N, Yıldız D, Abak K. Effects of sowing times on yield and some agronomic characteristics of sweet corn in the GAP area Turkey. *Acta Hort.* 2000;533:307-313.
24. Scharph HC, Whermann J. Die Bedeutung des Mineralstickstoffvorrates des Bodens zu Vegetationsbeginn für die N-Düngung zu Winterweizen. *Landwirt. Forsch.* 1975;32:100-114.
25. Schievano A, Adani F, Tambone F, D'Imporzano G, Scaglia B, Genevini PL. What is the digestate? Ricola Group, University of Milano, Faculty of Agricultural Sciences, [online], 2009. date: 10/November/2009 (website: <http://users.unimi.it/ricicla/>)
26. Silgram M, Shepherd MA. The effects of cultivation on soil nitrogen mineralization. *Adv. Agron.* 1999;65:267-311.
27. SPSS for Windows. Standard Version (Release 15.0) SPSS, Inc., 2005.
28. Statgraphics® (Release Centurion XV) StatPoint, Inc., 2005.
29. Stone PJ, Wilson DR, Reid JB, Gillespie RN. Water deficit effects on sweet corn. I. Water use, radiation use efficiency, growth, and yield. *Aust. J. Agric. Res.* 2001;52:103-113.
30. Taiz L, Zeiger E. *Plant Physiology*. Sunderland, Sinauer Associates Inc., Sunderland, USA, 2002.
31. Van der Vecken L, Michels P, Feyen J, Benoit F. Optimization of the water application in greenhouse tomatoes by introducing a tensiometer-controlled drip irrigation system. *Sci. Hortic.* 2003;18:9-23.
32. Xu ZZ, Yu ZW. Nitrogen metabolism in flag leaf and grain of wheat in response to irrigation regimes. *J. Plant Nutr. Soil Sci.* 2006;169:118-126.
33. Xu HL, Quin F, Du F, Xu R, Xu Q, Tian C, Li F, Wang F. Photosynthesis in Different parts of a wheat plant. *J. Food Agric. and Environ.* 2009;7: 399-404.
34. Xu HL, Wang JH, Xu RY, Zhao AH. Leaf turgor potential, plant growth and photosynthesis in organically fertilized sweet corn. *Pedosphere* 2004;14:165-170.
35. Zadoks JC, Chang TT, Konzak CF. A decimal code for the growth stages of cereals. *Weed Res.* 1974;14:415-421.
36. Zhao D, Reddy K R, Kakani VG, Reddy VR. Nitrogen deficiency effects on plant growth, leaf photosynthesis, and hyperspectral reflectance properties of sorghum. *Eur. J. Agron.* 2005;22:391-403.
37. Zhang J, Davies WJ. Changes in the concentration of ABA in xylem as a function of changing soil water status will account for changes in leaf conductance. *Plant Cell Environ.* 1989;13:277-285.

Rast in pridelek sladke koruze v odvisnosti od organskih in mineralnih dušikovih gnojil, odmerkov dušika in oskrbe z vodo

IZVLEČEK

Sladka koruza je v evropskih območjih z zmernim podnebjem premalo pridelovana zelenjadnica, njena poraba pa narašča. Za boljše razumevanje načinov oskrbe sladke koruze, je namen lončnega poskusa ovrednotiti vpliv različnih vodnih režimov in odmerkov dušika (N) izračunanih iz ciljnih vrednosti. N v odmerkih 0 (kontrola), 0,6 in 2 g N lonca⁻¹ je bil dodan v obliki organskih ostankov – bučne pogače in digestat iz prašičje gnojevke, ter mineralnih gnojil – CAN 27 in ENTEC*26. Obravnavanja oskrbe z vodo so temeljila na izmerjenih talnih matričnih potencialih: 2,8 pF (sušni stres), 2,6 pF (optimalna preskrbljenost z vodo) in 2,4 pF (prekomerna oskrbljenost tal z vodo). V primerjavi z mineralnimi gnojili, so se bučne pogače izkazale za enako učinkovite glede na izmerjeno višino rastlin (155,8 cm), maso storža (85,8 g), zeleno maso (124,9 g) in maso listov na rastlino (44,2 g), oziroma celo kot boljše gnojilo glede na izmerjeno maso korenin (72,3 g) in metlic na rastlino (3,0 g). Parametri pridelka, kot masa (70,1 g), dolžina (6,3 cm) in premer storža (2,0 cm), kakor tudi ostanki mineralnega dušika (59 mg N kg⁻¹) so bili značilno višji pri gnojenju z najvišjim odmerkom N. Statistično nižje vrednosti vrednotenih morfoloških in parametrov fotosintetske aktivnosti (merjeno ob metličenju in spravilu) so bile povezane s sušnim stresom. Matrični potencial 2,6 pF se je izkazal kot najustreznejši za rast sladke koruze.

Variations in leaf total protein, phenolic and thiol contents amongst old varieties of mulberry from the Gorizia region

Tina UGULIN^{1,*}, Tamas BAKONYI², Rebeka BERČIČ², Andreja URBANEK KRAJNC¹

¹Faculty of Agriculture and Life Sciences, University of Maribor, Pivola 10, 2311 Hoče, Slovenia, e-mail: andreja.urbaneck@um.si

²Faculty of Veterinary Science, Szent István University, Hungária krt. 23-25, H-1143 Budapest, Hungary

ABSTRACT

Preserving the plant genetic resources of genus *Morus* is insufficient but undoubtedly vital for conservation of the world's germplasm for our successors. This research was focused on old mulberry varieties from the Gorizia region in Slovenia which were assessed for their contents on crucial metabolites (proteins, phenolics and thiols) in leaves regarding their antioxidant and nutraceutical potentials. Total proteins were measured spectrophotometrically by following the procedure of Bradford, the total phenolic contents were determined using the Folin-Ciocalteu method and thiols were established with monobromobimane fluorescent dye. The presented metabolite screening showed that some of the evaluated genotypes had higher concentrations of glutathione and were superior in contents of proteins and phenolics when compared to the results of other authors and could be propagated as highly recommendable feed for silkworms, and other animals.

Key words: feeds, glutathione, *Morus*, phenols, proteins

INTRODUCTION

Preserving the plant genetic resources of *Morus* genus is still insufficient but undoubtedly vital for the conservation of the world's germplasm for our successors. Plant material can be preserved in herbariums and museums, while the main strategies of conservation can be divided into *in situ* conservation, *ex situ* conservation, *in vitro* conservation, and DNA banking (Vijayan et al. 2011a). Old varieties are a chance for sustainable leaves production for the growing silk industry. The main method of mulberry propagation is through stem cuttings or grafting of dormant buds (Vijayan et al. 2011b).

Mulberry trees originate from Asia and are currently cultivated in subtropical, tropical, and moderate environments. *Morus* L. (Moraceae) is a genus that is comprised of 10 to 15 species (Nepal, 2008). After expansion from Venice, sericulture and mulberry cultivation within the Gorizia region of Slovenia started to flourish in the middle of the 16th century (Ipavec 2008). Today sericulture in Slovenia has perished, and with plantations gone, only individual trees can be found, mostly near houses. The number of these types of trees in Slovenia is at the present time unknown. Still,

sericulture is today existent in 70 other countries amongst which the leaders are China, India, Vietnam, Uzbekistan, Brazil, and Thailand.

Old genotypes are interesting for research because of their adaptations to environmental conditions. As a result of naturalisation the classification of cultivars based on morpho-phenological attributes is problematic. Leaf shapes are often diverse, even on the same tree, and are influenced by several factors such as plant maturity, growth, leaf position on the branch, and period of growth. Mulberry trees can be monoecious or dioecious which is a challenge for phylogeny.

Throughout history when in the cases of drought or when typical fodder wasn't available, mulberry leaves were given to livestock. Fresh leaves were provided to goats, cattle, horses and pigs, although in small quantities. Mulberry leaves are a well-known fodder in sericulture, where it is the sole source because of its suitable nutritional composition which includes proteins, carbohydrates, chlorophyll and carotenoids. These nutritional parameters of mulberry leaves and silk production are directly proportional to each other (Kumar et al. 2010). Leaves have a high protein content (18 to 25 % DM) and high digestibility (75 to 85 %) (Ba et al. 2005).

*Correspondence to:
E-mail: tina.ugulin@um.si

The type of leaves preferred by silkworms are characterized by lower levels of minerals and crude fibers (Krishnaswami et al. 1970). They can also be used in human diet as non-toxic effects on internal organs were found (Srivastava et al. 2003). Regarding the high contents of proteins in mulberry leaves, they have potential as a forage supplement for animals such as sheep (Liu et al. 2001), goats (Bakshi and Wadhava 2007), and rabbits (Martinez et al. 2005).

Different parts of mulberry preparations have been used in traditional phytomedicine mainly due to high concentrations of antioxidants such as phenolics (Nakamura et al. 2009). Among them phenolic acids and flavonoids possess the highest antioxidant potential. Data also shows that mulberry leaves are abundant in caffeoylquinic acids (6.8 – 8.5 mg/g DM) and flavonols (3.7 – 9.8 mg/g DM) as recorded by Sánchez-Salcedo et al. (2015). Because of the high antioxidant properties of *Morus* species, these leaves have the potential of being used as dietary supplements and food products for human consumption also. On the grounds of the high phenolic content of mulberry extracts there is a potential exploitation of a phytochemical nature.

Morus alba is widely valued in traditional medicine, where it is known by the name *Mori folium*, for diabetes, protecting the liver, and lower blood pressure (Kim et al. 2014). Confections containing *Morus alba* extracts contribute to the prevention and qualities of life for pre-diabetic and diabetic patients (Nakamura et al. 2009; Ma et al. 2014). Mulberry leaf polyphenols possess antiatherogenesis effects that could be studied further (Yang et al. 2011). Yang et al. (2011) came to the conclusion that a single administration of a water extract from leaves of *M. alba* lowers systolic blood pressure in a dose-dependent way. Another study showed that a long-term administration of *M. alba* leaves resulted in decrease in body weight and hepatic lipid accumulation (Oh et al. 2009).

Part of the main objective of the study was to screen the biochemical contents of important primary (proteins) and secondary metabolites (phenolics, cysteine, glutathione) in leaves of local mulberry varieties obtained from the Gorizia region, in order to test their relevance as animal feed.

MATERIALS AND METHODS

Plant material

The samples of mulberry leaves were collected from 22 selected old mulberry genotypes from the Gorizia region in Slovenia. Five fully developed sun-exposed leaves (7th leaf from apex) were gathered randomly from each tree on August 20th 2015 and used as one sample. The mulberry trees were of old genotype origin with perimeters ranging from 80 to 370 cm. The meteorological information is given in the Table 1.

Meteorological information was obtained from the nearest weather station BILJE on August 20th 2015 from 07:00 to 21:00 hours: Temperature: 16.8 – 26.9 °C; Relative humidity: 48 – 99 %; Wind direction: NE, W, WNW; Wind speed: 0.6 – 2.1 m/s.

Table 1: Coordinates of mulberry habitat

Sample number	Coordinates	Additional information
1 - 6	45.89846038818359, 13.61314487457275	Miren 91
7	45.88313293457031, 13.61183166503906	Vrtoče
8 - 9	45.88530731201172, 13.60030746459961	Miren 219
10	45.88692092895508, 13.60146427154541	Miren 192
11	45.90574645996094, 13.61686134338378	Njiva pri Vrtojbi
12	45.90725708007812, 13.61715316772461	Njiva pri Vrtojbi B
13	45.90815353393555, 13.62518024444580	Njiva pri Vrtojbi C
14	45.91115126082022, 13.62616417914690	Njiva pri Vrtojbi D
15	45.91320419311523, 13.62628936767578	Njiva – Krožna cesta
16	45.91765213012695, 13.63195037841796	Vrtojba
17	45.89608383178711, 13.64458942413330	Bilje 149
18 - 20	45.89556884765625, 13.62782192230224	Pot Bilje - Miren
21 - 22	45.89221954345703, 13.63083553314209	Bilje – pri vodnjaku

Sample preparation for biochemical analysis

Immediately after collection, samples were stored in liquid nitrogen. Afterwards they were transferred into the freezer at -80 °C. The tissue samples were subsequently lyophilized and ground. The prepared samples were then stored in air tight vials at -20 °C prior to biochemical analysis.

Extraction and determination of total protein content (tPr)

Total proteins were determined by using the Bradford reagent following the procedure of Bradford (Bradford 1976). The standards were prepared with Bovine Serum Albumine (BSA). TRIS/HCl (1 ml, 0,1 M) (Doucheffa) was added to 25 mg of each sample. After being vortexed for 1 min, the samples were centrifuged for 10 min at 14000 RPM. 80 µl of water was added to the 20 µl of mixture. Bradford reagent (900 µl) (Sigma-Aldrich) was added to the samples, standards, and the reference blank. The mixture was then vortexed for 1 min and proteins were spectrophotometrically determined at 595 nm.

Extraction and determination of total phenolic content (tPh)

The total amount of phenolic compounds was determined using the Folin-Ciocalteu method following the procedure of Ainsworth and Gillespie (2007). Briefly, 1.5 ml of 95 % methanol was added to the 25 mg of each lyophilized sample. After 5 min of homogenisation at 30 Hz in an ultrasonic bath, the samples were centrifuged at 4 °C and 14000 RPM. Subsequently 100 µl of supernatant was pipetted and 200 µl of F-C reagent (10 %) (Merck) was added and vortexed. After exactly 3 minutes 800 µl of 700mM Na₂CO₃ (Sigma-Aldrich) was applied and the mixture was maintained at room temperature in the dark for 120 min. The absorbance was measured at 765 nm against a reagent blank using the Varian Cary UV/VIS spectrophotometer. Gallic acid (Sigma-Aldrich) was used as the reference standard. The total phenolic content was expressed as mg of gallic acid equivalents per gram of each extract on dry basis (mg GAE/g DM).

Extraction and determination of thiols (CYS, GSH)

For the glutathione and cysteine analysis a method with the thiol-specific fluorescence dye monobromobimane was used as described by Tausz et al. (2003). HCl (2 ml of 0.1 M, 32 %) (Sigma-Aldrich) was added to 60 mg PVP (Sigma-Aldrich) and after being allowed to rest overnight the mixture expanded. PVP was used here as a method for excluding any disruptive phenols. 40 mg of lyophilized sample was added to the mixture and homogenized with Ultra-Turrax for 20 sec and afterwards centrifuged for 15 min at 3000 RPM at 4 °C. For the extraction of reduced thiol forms (GSH and CYS) 280 µl of each extract was incubated along with 420 µl of CHES puffer [5,2g CHES (Sigma-Aldrich) + 100 ml bi-distilled water + 40 mg EDTA (Sigma-Aldrich) with the pH 9.3] and 70 µl of 5mM DDT [19,2 mg DTT (Fluka) + 25 ml bi-distilled water] solution. After one hour of incubation the reaction started with the application of 50 µl of 8mM monobromobimane [25 mg monobromobimane (Invitrogen) + 12 ml acetonitrile (Sigma-Aldrich)], followed by 15 min incubation in the dark at room temperature. The derivatisation was stopped with the addition of 600 µl of methansulphonic acid (0.25 %, v/v) (Merck). The suspension was centrifuged for 45 min at 14000 RPM at 4 °C and ultimately 1 ml of each extract was pipetted into vials.

The separation and determination of thiols was carried out using the gradient method, namely, liquid chromatography on the HPLC system Waters 2695, Waters 2475 Multi Fluorescence detector (excitation: 380 nm; emission: 480 nm), column Spherisorb S5 ODS2 25 x 4.6 mm, column temperature 23 ± 1 °C. Solvent A: acetic acid (0.25 %, v/v) in water with the addition of methanol (5 %, pH 3.9) (Sigma-Aldrich). Solvent B: methanol (90 %, v/v) in water with the addition of acetic acid (0.2 %, v/v) gradient: 5 % solvent B to 15 % solvent B in 20 minutes, 100 % solvent B for 6 minutes, and 5 % solvent B for another 8 minutes. Flow rate was 1 mL min⁻¹.

Statistical analysis

The results of biochemical analyses represented the means and standard deviations (S.D.) of three replicate measurements of each sample. The results were evaluated by one-way ANOVA using the program IBM SPSS Statistics 22. Additionally, correlation between secondary metabolites was analyzed. The differences between the samples were compared with the Duncan test at $p \leq 0.05$. Significant differences were indicated by different lowercase letters (a-k).

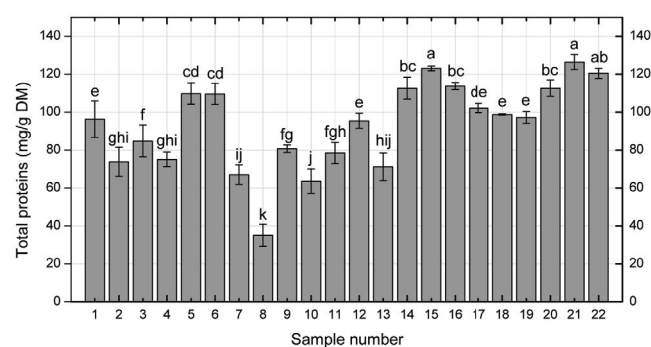


Fig. 1: Total proteins' concentrations

RESULTS AND DISCUSSION

Total protein content

Amino acids represent a primary class of nutrients. The highest levels of total protein content were analyzed in samples 21, 15 and 22, and the lowest in sample 8, as shown in Figure 1. The protein contents were determined within a range from 35 to 126 mg/g DM.

Kandyli et al. (2008) performed a study of the crude protein content in mulberry leaves with concentrations reaching 200 g/kg DM. The major protein fractions are represented by prolamins (44.1 %) and albumins (11.1 %) followed by globulins (9.7 %) and glutelins (8.5 %). It is known that the total protein contents change with mulberry leaf aging (Matei et al. 2006). As the leaves' content of protein decreases with maturity, it is possible this is the source of differences in concentrations between our study and the one conducted by Kandyli. In another study, it was found that amino acids' content increase during leaf maturity though the differences were insignificant. The most represented amino acids were reported to be glutamine, asparanin and leucine (Yao et al. 2000).

Total phenolic content

Phenolics are a group among three main categories of secondary plant compounds. Mulberries possess antioxidant properties, mainly because of the content of phenolic compounds (Thirumalaisamy 2009, Memon et al. 2010, Radojković et al. 2012, Flaczyk et al. 2013). The DPPH radical-scavenging potential was determined to have a positive correlation ($R = 0,803$) with the amount of total phenolics (Memon et al. 2010). In leaves of *M. alba* the main phenolic

components were identified as chlorogenic acid, followed by caffeic, vanillic, sinapic, ferulic and gallic acid. The flavonols fraction contains rutin, quercetine, and kaempferol. Meanwhile, Radojković et al. (2012) determined ferulic acid as the predominant phenolic compound in *Morus alba* leaf extract, followed by rutin, sinapic, chlorogenic and gallic acid. Quercetine was not detected. However, in another study conducted by Katsube et al (2005) the predominant flavonol glycoside was indeed quercetine. Mulberry leaves are free of tannins, as reported by Singh and Makkar (2002).

In the presented study, the maximum amount of total phenolic content was documented among samples 17, 11, 19, 18 and 7, where the concentration reached 15.7 g GAE/100 g extract. The lowest levels were analyzed in samples 14, 13, 5, 4, 10, 2, 6, 3, 22 and 21, as shown in Figure 2.

Our results are similar to those reported by Flaczyk et al. (2013), who analyzed total concentration of phenolic compounds equal to 14.4 g GAE/100 g in *M. alba* leaf extracts. In another study a much lower total phenolic concentration was determined in *M. alba* leaves reaching a maximum value of 4.56 % (Thirumalaisamy 2009). Some of our genotypes expressing higher tPh contents could be valuable sources of genes in future breedings. Additional research is required focused on single phenolic compounds in order to determine the predominant phenols and their concentration within each genotype.

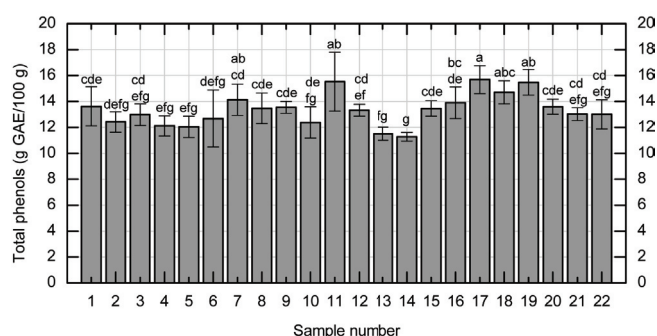


Fig. 2: Total phenols content

Reduced glutathione and cysteine content

Glutathione is the primary low-molecular-weight thiol in most cells. Ordinarily, glutathione is mostly present in its reduced form (GSH), which was measured in this study, with only a small part present in its oxidized state (GSSG) (Dixon et al. 1998). In the absence of stress, plant tissues, including leaves, usually maintain a ratio between GSH:GSSG of a minimum of 20:1 (Noctor et al., 2011). GSH is crucial for plant development by influencing critical functions in embryo and meristem development (Frottn et al. 2009).

Statistically significant differences in the reduced glutathione and cysteine content were determined ($p \leq 0.05$). The data was then grouped into homogeneous subsets, 10 groups for cysteine and 11 for glutathione, as shown in Figure 3. The highest rate of cysteine was measured in sample 15 reaching almost 73 nmol/g DM and the lowest consisted in the samples 16, 17, 14, 7 and 8, with concentrations of 24nmol/g. The highest reduced glutathione concentrations were found in samples 5 and 3, with levels of 2000nmol/g,

while the lowest levels were found among samples 8, 16, 17, 14, 10 and 19, with concentrations above 700 nmol/g.

In comparison with beech trees (*Fagus sylvatica* L.) mulberries have manifold higher concentrations of glutathione (Herbinger et al. 2005).

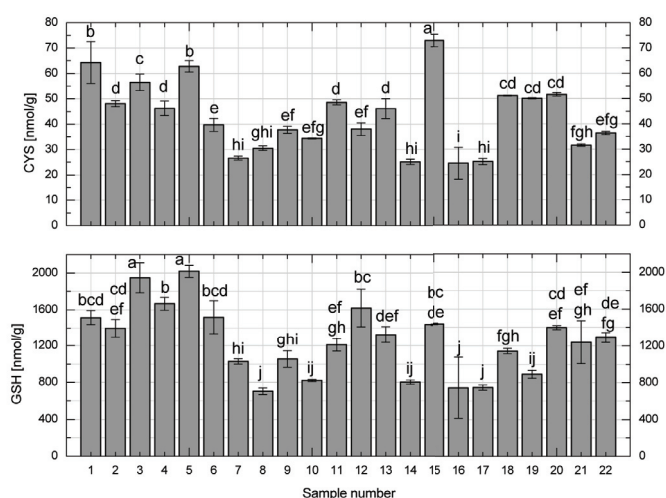


Fig. 3: Cysteine and reduced glutathione concentrations

Correlation between studied biochemical components

The correlation was calculated with the Pearson correlation coefficient, as shown in Table 2. Glutathione is made up of the amino acids glutamine, cysteine and glycine so a high correlation factor between GSH and cysteine was expected. Statistically significant correlation was documented between glutathione and cysteine, with the coefficient reaching 0.707. A lower but statistically significant link was noted between glutathione and proteins, with the coefficient of 0.293. The correlation between cysteine and proteins was 0.241, which was not statistically significant.

Phenolics are known as important antioxidants. As a result of scavenging activities phenolics become oxidized to phenoxyl radicals. The regeneration of phenoxyl radicals can be carried out via ascorbate-glutathione cycle (Szöllösi 2014). However, the expected correlation between phenols, glutathione and cysteine was not statistically important, as the coefficient between the phenols and thiols was - 0.204 and -0.010. respectively. Correlation between proteins and phenols was not expected.

CONCLUSION

The presented metabolite screening in leaves of old local mulberry varieties (*M. alba*) from Gorizia region showed that some evaluated genotypes expressed high contents of metabolites. Samples 21, 15 and 22 contained the highest protein concentrations, samples 17, 11, 19, 18 and 7 contained the most phenols, sample 15 contained the highest rate of cysteine, and samples 5 and 3 contained the highest reduced glutathione concentrations. The nutritively richer varieties could be propagated as highly recommendable feed

Table 2: Correlation between studied biochemical components

		GSH [nmol/g]	Proteins	CYS [nmol/g]	Phenols
GSH [nmol/g]	Pearson Correlation	1	,293*	,707**	-,204
	Sig. (2-tailed)		,037	,000	,151
Proteins (mg/g)	Pearson Correlation	,293*	1	,241	-,032
	Sig. (2-tailed)	,037		,089	,772
CYS [nmol/g]	Pearson Correlation	,707**	,241	1	-,010
	Sig. (2-tailed)	,000	,089		,942
Phenols (mg/g)	Pearson Correlation	-,204	-,032	-,010	1
	Sig. (2-tailed)	,151	,772	,942	

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

for silkworm and other animals. These conclusions may be a stepping stone for selecting cultivars with desired nutrient profiles for breeding.

ACKNOWLEDGMENT

This research was funded by the Slovenian Research Agency (ARRS, N1-0041). The authors thank Mateja Felicijan and Anja Ivanuš for laboratory assistance. The authors also thank the reviewers for valuable comments and suggestions.

REFERENCES

- Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. *Nat. Protoc.* 2007;2:875-877.
- Ba NX, Giang VD, Ngoan LD. Ensiling of mulberry foliage (*Morus alba*) and the nutritive value of mulberry foliage silage for goats in central Vietnam. *Livest. Res. Rural Develop.* 2005;17(15).
- Bakshi MPS, Wadhawa M. Tree leaves as complete feed for goat bucks. *Small Rum. Res.* 2006;69:74-78.
- Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976;72:248-254.
- Dixon DP, Cummins I, Cole DJ, Edwards R. Glutathione-mediated detoxification systems in plants. *Curr. Opin. Plant Biology.* 1998;1:258-266.
- Flaczyk E, Kobus-Cisowska J, Przeor M, Korzak J, Remiszewski M, Korbas E, Buchowski M. Chemical characterization and antioxidative properties of Polish variety of *Morus alba* L. leaf aqueous extract from the laboratory and pilot-scale processes. *Agricul. Sci.* 2013;4:141-147.
- Frottin F, Espagne C, Traverso JA, Mauve C, Valot B, Lelarge-Trouverie C, Zivy M, Noctor G, Meinnel T, Giglione C. Cotranslational proteolysis dominates glutathione homeostasis to support proper growth and development. *Plant Cell* 2009;21(10):3296-314.
- Herbinger K, Then C, Löw M, Haberer K, Alexous M, Koch N, Remele K, Heerdt C, Grill D, Rennenberg H, Häberle K-H, Matyssek R, Tausz M, Weiser G. Tree age dependence and within-canopy variation of leaf gas exchange and antioxidative defence in *Fagus sylvatica* under experimental free-air ozone exposure. *Environ. Pollut.* 2015;137:476-482.
- Ipavec VM. Murve in kavalirji. *Svilogojstvo na Goriškem. Inštitut za slovensko narodopisje, Ljubljana*, 2008.
- Kandylis K, Hadjigeorgiu I, Harizanis P. The nutritive value of mulberry leaves (*Morus alba*) as a feed supplement for sheep. *Trop. Anim. Health Pro.* 2008;41:17-24.
- Katsube T, Imawaka N, Kawano Y, Yamazaki Y, Shiwaku K, Yamane Y. Antioxidant flavonol glycosides in mulberry (*Morus alba* L.) leaves isolated based on LDL antioxidant activity. *Food Chem.* 2006;97:25-31.
- Kim DS, Kang YM, Jin WM, Sung YY, Choi G, Kim HK. Antioxidant activities and polyphenol content of *Morus alba* leaf extracts collected from varying regions. *Biomed. rep.* 2014;2:675-680.
- Kumar RV, Cahuhan S, Kumar D, More N. Nutritional composition in leaves of some mulberry varieties: A comparative study. *Bioinformatics and biomedical technology, 2010 International conference.* 2010;438-442.
- Krishnaswami S, Noamani KR, Asan M. Studies on quality of mulberry leaves and silkworm cocoon crop production, Part I, Quality difference due to varieties. *Indian J. Sericult.* 1970;9(1):1-10.
- Liu JX, Yao J, Yan B, Yu JQ, Shi ZQ. Effects of mulberry leaves to replace rapeseed meal on performance of sheep feeding on ammoniated rice straw diet. *Small Rum. Res.* 2001;39:131-136.
- Ma ST, Liu DL, Deng JJ, Peng YJ. Protective effect of mulberry flavonoids on sciatic nerve in alloxan-induced diabetic rats. *Braz. J Pharm. Sci.* 2014;50(4):765-771.
- Martinez M, Motta W, Cervera C, Pla M. Feeding mulberry leaves to fattening rabbits: effects of growth, carcass characteristics and meat quality. *Animal Sci.* 2005;80(3):275-280.
- Matei A, Tanase DB, Diaconescu C, Constatinescu M, Dolis M. Contributions to the study of the leaf protein value in different mulberry varieties. *Arch. Zootech.* 2006;9.
- Memon AA, Memon N, Luthria DL, Bhangar MI, Pitafi AA. Phenolic acids profiling and antioxidant potential of

- mulberry (*Morus laevigata* W., *Morus nigra* L., *Morus alba* L.) leaves and fruits grown in Pakistan. *Pol. J. Food Nutr. Sci.* 2010;60:25-32.
20. Nakamura M, Nakamura S, Oku T. Suppressive response of confections containing the extractive from leaves of *Morus alba* on postprandial blood glucose and insulin in healthy human subjects. *Nutr. Metab.* 2009;29.
 21. Nepal M.P. Systematics and reproductive biology of the genus *Morus* L. (Moraceae) [dissertation]. Retrieved from ProQuest Dissertations and Theses, 2008 (Accession Order No.3325640.).
 22. Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Garcia B, Queval G, Foyer CH. Glutathione in plants: an integrated overview. *Plant Cell Environ.* 2011;35:454-484.
 23. Oh KS, Ryu SY, Lee S, Seo HW, Oh BK, Kim YS, Lee BH. Melanin-concentrating hormone-1 receptor antagonism and anti-obesity effects of ethanolic extract from *Morus alba* leaves in diet-induced obese mice. *J. Ethnopharmacol.* 2009;122:216-220.
 24. Radojković MM, Zeković ZP, Vidović SS, Kočar DD, Mašković PZ. Free radical scavenging activity and total phenolic and flavonoid contents of mulberry (*Morus* spp. L., Moraceae) extracts. *Hem. Ind.* 2012;66:547-552.
 25. Sánchez-Salcedo EM, Mena P, García-Viguera C, Hernández F, Martínez JJ. (Poly)phenolic compounds and antioxidant activity of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves: Their potential for new products rich in phytochemicals. *J. Funct. Foods.* 2015;18:1039-1046.
 26. Singh B, Makkar HPS. The potential of mulberry foliage as feed supplement in India. In: *Mulberry for animal production.* Anim. Health Prod. Paper. 2002;147:139-153.
 27. Srivastava S, Kapoor R, Thathola A, Srivastava RP. Mulberry (*Morus alba*) leaves as human food: a new dimension of sericulture. *Int. J. Food Sci. Nutr.* 2003;54(6):411-416.
 28. Szöllősi R. Superoxide dismutase (SOD) and abiotic stress tolerance in plants: an overview. Ed.: Ahmad P. In: *Oxidative damage to plants: Antioxidant networks and signaling.* Academic press, 2014, 672 p.
 29. Tausz M, Wonisch A, Grill D, Morales D, Jiménez MS. Measuring antioxidants in tree species in the natural environment: from sampling to data evaluation. *J. Exp. Bot.* 2003;54(387):1505-1510.
 30. Thirumalaisamy R, Gowrishankar J, Sunganthapriya S, Prakash B, Ashok Kumar L, Arunachalam G. Genetic variability in *Morus alba* L. by biochemical and bioassay methods for increased silk productivity. *J. Biomed. Sci.* 2009;1:11-18.
 31. Vijayan K, Saratchandra B, Teixeira da Silva JA. Germplasm conservation in mulberry (*Morus* spp.). *Sci. Hortic.* 2011a;128(4):371-379.
 32. Vijayan K, Tikader A, da Silva JA. Application of tissue culture techniques for propagation and crop improvement in mulberry (*Morus* spp.). *Tree Forest. Sci. Biotechnol.* 2011b;5(1):1-13.
 33. Yang NC, Jhou KY, Tseng CY. Antihypertensive effect of mulberry leaf aqueous extract containing γ -aminobutyric acid in spontaneously hypertensive rats. *Food Chem.* 2011;132:1796-1801.
 34. Yao J, Yan B, Wang XQ, Liu JX. Nutritional evaluation of mulberry leaves as feed for ruminants. *L. R. R. D.* 2000.12(2).

Spremenljivost vsebnosti skupnih proteinov, fenolov in tiolov, v listih starih genotipov murv v Goriški regiji

IZVLEČEK

Ohranjanje genskih virov rodu *Morus* je nezadostno, čeprav je nedvomno bistvenega pomena za ohranjanje svetovne zapuščine za naše naslednike. Ta raziskava temelji na določitvi ključnih metabolitov (proteinov, fenolov in tiolov) v listih starih genotipov murv iz Goriške regije v Sloveniji z namenom proučitve antioksidativne aktivnosti in hranilne vrednosti. Skupni proteini in fenoli so bili določeni spektrofotometrično s pomočjo Bradfordove in Folin-Ciocalteu metode, tioli so bili določeni s pomočjo HPLC po predhodnem markiranju s fluorescenčnim barvilom monobromobimane. Rezultati določitve metabolitov kažejo, da imajo nekateri genotipi visoko vsebnost glutationa, v primerjavi z rezultati drugih avtorjev so superiorni v vsebnosti proteinov in fenolov in bodov nadaljnjih raziskavah razmnoženi z namenom uporabe listov za krmo sviloprejk in ostalih živali.

