ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2018 Vol. 61, Št. 1: 3–12

The effect of thyme essential oil on germination and early growth of wheat

Vpliv timijanovega eteričnega olja na kalitev in zgodnjo rast pšenice

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Abstract: Essential oils (EOs) are becoming an important alternative as seed decontaminating agents opposite to synthetic seed preservatives due to their antimicrobial activities. They also inhibit seed germination which may be problematic for seeds of cultural plants. In the present study, different treatments of wheat (*Triticum aestivum*) grain with thyme (*Thymus serpyllum*) EO were tested. Wheat grains were treated with thyme EO for 6, 12 and 24 hours to determine the optimal time of EO treatment in order to reduce fungal infection of the grain surface and at the same time preserve high germination rate of seeds and good physiological status of seedlings during early stages of their growth. Germination rate, fresh mass and length of shoots and roots, and physiological status of the seedlings were compared according to the duration of the treatment. The 6 h-EO treatment was the most optimal procedure that did not affect seed germination, was not harmful for the seedlings and prevented fungal infections. The thyme EO could potentially be used as a protective agent for wheat grain intended for sowing and food production.

Keywords: essential oil, germination, seedling growth, thyme, wheat

Izvleček: Eterična olja (EO) postajajo pomembna alternativa sintetičnim pripravkom za zaščito semen pred mikrobi. Ker EO zavirajo tudi kalitev semen, je njihova uporaba pri kultiviranih rastlinah lahko problematična. V raziskavi smo preučili različne načine tretiranja semen pšenice (*Triticum aestivum*) z EO timijana (*Thymus serpyllum*). Pšenična semena smo tretirali z EO timijana 6, 12 in 24 ur ter določili optimalni čas tretiranja, ki zavre glivne okužbe na površini semen in obenem ohrani visoko stopnjo kalivosti semen ter dobro fiziološko stanje kalic v začetnem obdobju rasti. Določili smo delež kalivosti, svežo maso, dolžino poganjka in korenin ter fiziološko stanje kalic in jih primerjali glede na čas tretiranja. Šest-urno tretiranje z EO je bilo najbolj optimalno, ker se glivne okužbe niso razvile in hkrati ni vplivalo na kalitev semen in rast kalic. Timijanovo EO bi lahko bilo uporabno kot zaščitni pripravek za pšenična semena, ki se jih uporablja za setev in kot živilo.

Ključne besede: eterično olje, kalitev, rast kalic, timijan, pšenica

Introduction

Wheat (*Triticum aestivum)* is a cultural plant and an important baking cereal. After rice, it is the second most important source of calories in human nutrition. In terms of production worldwide, it is the second most important cereal after maize, but latter is mostly used as animal forage (Awika 2011). With the production of 161 023 tons in 2016, wheat is also the most important cereal in baking industry in Slovenia (Statistical Office of the Republic of Slovenia). On the field or during the storage, wheat grains can be infected with different fungi that cause economic losses and health problems for humans and animals. Especially fungi of the genera *Fusarium*, *Aspergillus* and *Penicillium* pose a big threat for human health due to the production of harmful secondary metabolites, called mycotoxins (D'Mello et al. 1998, Hussein and Brasel 2001). High yield losses are caused by the fungi of the genus *Fusarium* which are transferred from infected grains to the seedlings and affect their growth and development (Knudsen et al. 1995), while fungi of the genera *Alternaria* and *Aspergillus* reduce seed germination (Ruza et al. 2004).

Post harvesting treatment of stored grains with synthetic fungicides affects the quality of cereals and may pose serious hazard for consumers' health (Osman 2011). Essential oils (EOs) are becoming an important alternative as seed decontaminating agents opposite to synthetic seed preservatives. In addition to strong fungicidal effects they are biodegradable and show low toxicity to human and animal health (Sivakumar and Bautista-Baños 2014).

EOs are synthesized as secondary metabolites in aromatic plants. They are very complex natural mixtures that contain volatile terpenes, terpenoids and aromatic and aliphatic compounds. Because of high number of components, EOs seem to have no specific cellular targets. It has been proposed that the antimicrobial activity of EOs may be attributed to their ability to pass through the cytoplasmic membrane, disrupt its structure and permeabilize it. Once inside the cell, they can inhibit cellular functions (Calo et al. 2015). Thyme (*Thymus*) is one of the most widely used plant genera in folk medicine. The EOs of *Thymus vulgaris* are known to have antiseptic, antiviral and antimicrobial activities (Anžlovar et al. 2014, Dorman and Deans 2000, Reichling et al. 2009). EOs from other aromatic plants from genera *Origanum* and *Lavandula* also possess antifungal properties. Lebanese Za'atar (*Origanum syriacum*) EO acts against *Aspergillus niger*, which is a common food spoilage fungus, *Penicillum* spp. and *Fusarium oxysporum* which infect variety of fruits (Daouk et al. 1995). *Lavandula stoechas* ssp. *stoechas* EO also acts against *F. oxysporum* and is highly active against *Rhizoctonia solani,* known for causing various plant diseases such as collar rot, root rot and wire stem (Angioni et al. 2006).

Previously, we isolated five fungal endophytes from wheat seeds: *Alternaria alternata*, *Alternaria infectoria, Aspergillus flavus, Epiccocum nigrum* and *Fusarium poae,* and tested the effectiveness of thyme EO on fungal radial growth *in vitro* (Anžlovar et al. 2017). The thyme EO showed remarkable *in vivo* efficacy in protecting the EO fumigated wheat seeds from fungal infection. Čepin et al. (2016) also studied the effectiveness of thyme EO in order to reduce fungal infection of wheat grain. They found that EO treatment significantly decreased fungal infection but also inhibited seed germination regardless of the duration of the EO treatment. Germination inhibition and high phytotoxic activity of thyme EO has been reported in the past (Angelini et al. 2003, De Almeida et al. 2010).

The aim of this research was to determine the optimal time of seed treatment with thyme EO in order to reduce the infection of seeds with fungi and at the same time preserve high germination rate and good physiological status of wheat seedlings during early stages of their growth.

Material and methods

Materials

We used 100% natural, pure thyme essential oil extracted from *Thymus serpyllum* (Primavera Life GmbH, Germany). Wheat seeds (*Triticum aestivum* L. cv. 'Savinja') were obtained from the Agricultural Institute of Slovenia.

Essential oil treatment

Wheat seeds were soaked in 0.2% thyme essential oil in 10% dimethylsulphoxide (DMSO). 10% DMSO was prepared by diluting 5 ml of concentrated DMSO (Sigma, USA) in 45 ml of distilled water. Approximately 160 wheat seeds (two replicates, each with 80 seeds) were placed in a plastic Petri dish (2r = 5.5 cm) and poured over with 4 ml of 10% DMSO and 8 µl of thyme EO. Petri dishes were sealed with parafilm to prevent EO evaporation and placed on a shaker (Gerhardt, Germany) for 6, 12 and 24 hours at room temperature and 90 rpm. Control treatment was done with 10% DMSO and 6 hours shaking. After the EO treatment, the seeds were placed into automatic sprouters (EasyGreen® MicroFarm System, Easy-Green Factory Inc., Nevada, USA) with programmed water spraying in the intervals of 5-4-4-3-3-5 hours over a 24 h period. The seeds germinated and seedlings grew in the chambers for 7 days at room temperature and ambient light.

Growth of wheat seedlings

Every day, shoot height was measured for 21 randomly assigned seedlings for each replicate and treatment (control and 6, 12, 24 h of EO). At the end (day 7), germination rate, fresh shoot and root mass, shoot height and root length were measured. 100 mg of wheat shoots and roots per each treatment were separated and frozen in liquid nitrogen for further biochemical analysis.

Lipid peroxidation

Lipid peroxidation was estimated indirectly via malondialdehyde (MDA) content. Previously frozen roots and shoots (100 mg) were homogenized in 2 ml of acidic reagent, consisting of 0.3% (w/v) 2-thiobarbituric acid (Fluka, Spain) and 10% (w/v) trichloroacetic acid (Acros Organics, Germany). After homogenization, samples were incubated at 95 °C for 30 min and then chilled on ice and centrifuged (Eppendorf Centrifuge 5417 R) at 10 000 rpm and 4 °C for 15 min. The absorbance of the supernatant was spectrophotometrically (UV-1800 Shimadzu spectrophotometer, Japan) measured at 532 nm and 600 nm. The concentration of MDA was calculated using the extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$ (Dolenc Koce et al. 2014).

Statistical analysis

The experiment was independently performed twice. Mean values and standard errors were calculated for all treatments. The differences among treated and control plants were tested using the One-way ANOVA. The level of significance was set at $p < 0.05$.

Results

Germination rate, mass and length of shoots and roots, and physiological status of wheat seedlings that were treated with thyme EO were compared according to the duration (6, 12, 24 h) of the treatment.

In control treatment, more than 90% of the seeds germinated on the second day, while in EO treatments a delay in germination was observed. This delay was related to the duration of the treatment, i.e. greater delay in longer EO treatments (12 and 24 h). Germination of wheat seeds was delayed and decreased for up to 93.3% and 97.4% on the second day in 12 and 24 h treatments, respectively. Shorter (6 h) treatment inhibited germination on the first three days but then germination rate increased and after 7 days it reached 85.7%. After 7 days, when the experiment ended, a difference of 11.9% in germination rate between control and the 6 h-treatment was noticed, while in the 12 h- and 24 h-treatments the percentages of germinated seeds were 52.4% and 45.2%, respectively (Tab. 1).

The delayed germination reflected on the growth dynamics during the first 7 days after EO treatment. Wheat seedlings were least affected after the 6 h-treatment with thyme EO while 12 h- and 24 h-treatments inhibited shoot growth more severely (Fig. 1).

The germination of EO-treated seeds was delayed and further growth of seedlings was reduced as indicated by the values in trend line equations (Fig. 1). The 6 h-treatment with thyme EO affected germination and growth moderately (coefficient of the second order higher than value 2 as in control treatment) while 12 h- and 24 h-treatments inhibited shoot growth more severely (coefficient of the second order had value 1 or lower, negative coefficients of the first order).

Table 1: Germination rate of wheat seeds during 7 days of growth after the treatment with thyme EO for 6, 12 and 24 h (n=160).

Tabela 1: Delež kalivosti pšeničnih semen v 7 dneh po 6, 12 in 24-urnem tretiranju s timijanovim EO (n=160).

	Germination rate (%)			
	Control	6 h	12 h	24 h
Day 1	69.0	0 .0	0.0	0.0
Day 2	92.9	50.0	7.1	2.4
Day 3	95.2	61.9	28.6	9.5
Day 4	97.6	78.6	35.7	28.6
Day 5	97.6	83.3	42.9	31.0
Day 6	97.6	85.7	50.0	40.5
Day 7	97.6	85.7	52.4	45.2

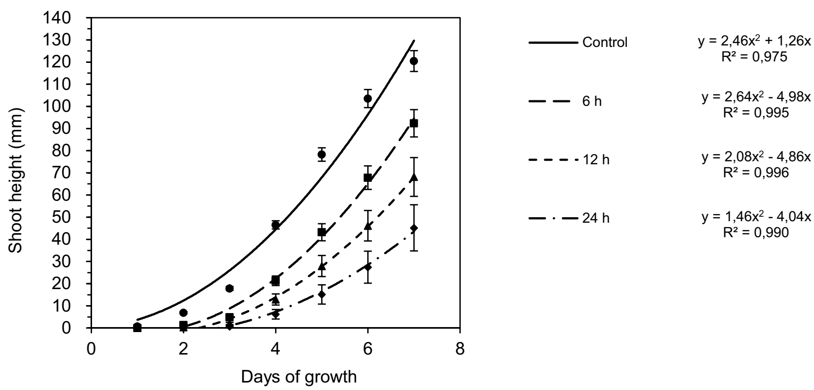

Figure 1: Shoot growth of wheat seedlings during 7 days of growth after the treatment with thyme EO for 6, 12 and 24 h. Mean values ± standard errors are shown (n=42). Trendline equation describes the polynome of the 2nd order. R^2 – coefficient of determination.

Slika 1: Rast poganjka pšeničnih kalic v 7 dneh po 6, 12 in 24-urnem tretiranju s timijanovim EO. Prikazane so povprečne vrednosti ± standardne napake (n=42). Enačba trendne črte opisuje polinom 2. stopnje. R^2 – koeficient determinacije.

After 7 days, the length of shoots was reduced for 23.3% ($p < 0.001$) in 6 h-treatment when compared to control. The reduction of root length was smaller and non-significant ($p = 0.610$). Other two EO treatments, 12 and 24 h, significantly inhibited growth of both shoots and roots. Shoot length was reduced for 43.4% in 12 h-treatment and for 64.7% in 24 h-treatment, while root length was not affected as much: it was reduced for 19.6% in 12 h and 52.8% in 24 h-treatment (Fig. 2).

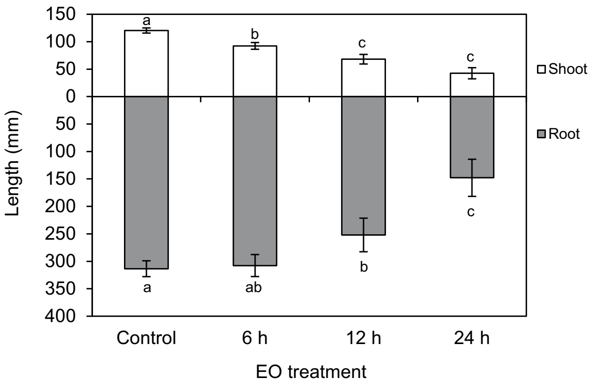

Figure 2: Shoot and root length of wheat seedlings after 7 days of growth and treatment with thyme EO for 6, 12 and 24 h. Mean value ± standard error is shown (n=42). Different letters indicate statistically significant differences (ANOVA, $p < 0.05$).

Slika 2: Dolžina poganjka in korenine pšeničnih kalic po 7 dneh rasti po 6, 12 in 24-urnem tretiranju s timijanovim EO. Prikazane so povprečne vrednosti ± standardne napake (n=42). Različne črke označujejo statistično značilne razlike (ANOVA, $p < 0,05$).

The same reduction was observed in fresh mass of shoots and roots of wheat seedlings (Fig. 3). After 7 days, the inhibitory effect of thyme EO was most evident in 24 h-treatment which significantly ($p < 0.001$) reduced shoot mass for 55.5% and root mass for 50.3% ($p = 0.067$). The 12 h-treatment showed similar effects: shoot mass was significantly smaller for 44.1% ($p < 0.001$), while root mass non-significantly decreased for 14.2% ($p = 0.497$). The 6 h-treatment significantly decreased shoot mass for 23.5% ($p < 0.001$) in comparison to the control treatment while root mass was at the control level.

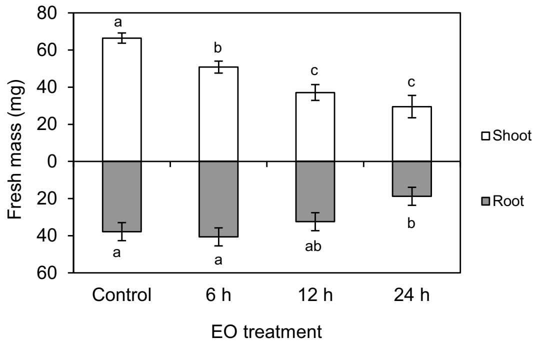

Figure 3: Fresh mass of wheat seedlings after 7 days of growth and treatment with thyme EO for 6, 12 and 24 h. Mean value ± standard error is shown (n=42). Different letters indicate statistically significant differences (ANOVA, $p < 0.05$).

Slika 3: Sveža masa pšeničnih kalic po 7 dneh rasti po 6, 12 in 24-urnem tretiranju s timijanovim EO. Prikazane so povprečne vrednosti ± standardne napake (n=42). Različne črke označujejo statistično značilne razlike (ANOVA, $p < 0,05$).

The physiological status of wheat seedlings was estimated by lipid peroxidation which is a marker of oxidative stress. None of the treatments with thyme EO had any effect on lipid peroxidation in shoots of wheat seedlings (Fig. 4A). There was also no effect on the roots except in seedlings, treated with thyme EO for 24 hours, where higher MDA content was measured (Fig. 4B).

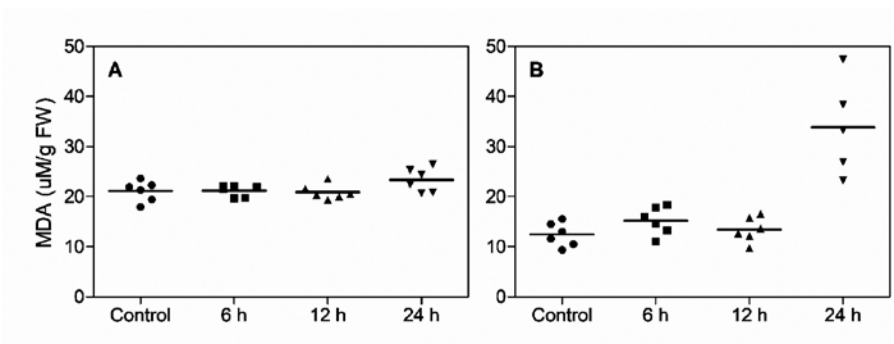

Figure 4: Malondialdehyde (MDA) content in shoots (A) and roots (B) of wheat seedlings after 7 days of growth and treatment with thyme EO for 6, 12 and 24 h. Mean value is indicated with the line (n=6).

Slika 4: Vsebnost malondialdehida (MDA) v poganjkih (A) in koreninah (B) pšeničnih kalic po 7 dneh rasti po 6, 12 in 24-urnem tretiranju s timijanovim EO. Povprečna vrednost je označena s črto (n=6).

Discussion

Wheat is susceptible to fungal infection, which can affect crop yield and have negative effect on human health (Anžlovar et al. 2017). The negative effects of synthetic chemicals on human and animal health and on the environment have caused an increasing demand for natural additive options. Essential oils exhibit antimicrobial properties which can make them suitable alternatives to synthetic chemicals (Calo et al. 2015). In the present study wheat seeds were exposed to thyme essential oil for 6, 12 and 24 h. During the 7-day experiment the seeds and later, the seedlings were monitored and a few growth related (fresh mass and length of shoots and roots) and physiological parameters (lipid peroxidation) were measured. In comparison to the study of Čepin et al. (2016), this experiment was carried out in automatic sprouters instead of Petri dishes. Germination tests in Petri dishes allow the germination rate to be monitored only for the first three days when seedlings are very small, while sprouters are larger and allow for longer growth. Also, humidity conditions can be better controlled in sprouters and field conditions can be more adequately imitated. In sealed Petri dishes, the effect of essential oil can be enhanced due to limited aeration.

Kotan et al. (2013) found the direct application of the essential oil suspension of *Satureja hortensis* to have a toxic effect on the tomato seeds, but the concentrations of essential oil were 4 and 8 times higher than in our experiment. In our case, even with lower concentration of EO, the 24 h-treatment showed toxic effect on the wheat seedlings. Most favourable thyme EO treatment that reduced fungal infection and at the same time preserved germination rate and normal physiological parameters of wheat seedlings was the 6 h-treatment. The growth of wheat seedlings was very similar to the control and the germination rate reached 85.7%. The 6 h-treatment was also selected as the most suitable by Čepin et al. (2016). In their experiment more seeds germinated in 6 h-treatment than in 12 h- and 24 h- while fungal infection decreased by 48.0% in comparison to the control.

The germination of seeds treated with thyme EO for 12 and 24 hours was delayed and further growth of these seedlings was reduced as well. However, 6 h-treatment caused only a delay of seed germination while seedlings growth was not affected. Poonpaiboonpipat et al. (2013) also noticed a delay in germination when barnyardgrass seeds were exposed to lemongrass essential oil. Our experiment did not focus on fungal infection of the seeds, but we noticed more fungi on the seeds in control treatments and after 6 h- and 12 h-treatment compared to 24 h-treatment. Based on the results of lipid peroxidation, which is a marker of oxidative stress, these fungal infections had no effect on the physiological status of wheat seedlings. On the contrary, increased levels of MDA were measured in older rotten tissues of *Phaseolus vulgaris* infected with *Botrytis cinerea* (Muckenschnabel et al. 2001).

The fact that after 7 days there were no differences in MDA content in the shoots for any of the EO treatments when compared to the control, proves that thyme EO did not induce stress in shoots of wheat seedlings. MDA content was at the control level also in roots, except in the case of 24 h-treatment when significantly higher MDA content was measured (high coefficient of variation of 28.18%). This finding indicates that this EO treatment represented stress for the roots of wheat seedlings. Longer monitoring of seedlings growth would reveal if this change reflects on the structure and function of treated plants.

Based on our results we can conclude that the optimal protective treatment with thyme EO that reduces fungal infection, but still does not affect seed germination and is not toxic for the seedlings, is the 6 h-treatment. This treatment has a potential for use as a protection agent for grain intended for sowing and food production, but further analysis to determine the cost and safety for the cultural plants should be performed.

Povzetek

Eterična olja (EO) postajajo pomembna alternativa sintetičnim pripravkom za zaščito semen pred mikrobi. Ker EO zavirajo tudi kalitev semen, je njihova uporaba pri kultiviranih rastlinah lahko problematična. Za timijanovo EO je dokazano, da ima dobro protimikrobno aktivnost, tako proti bakterijam kot proti glivam. Poleg tega je timijan znano zelišče in zdravilna rastlina z blagodejnimi učinki za človeka.

V raziskavi smo želeli določili optimalni čas tretiranja semen pšenice (*Triticum aestivum*) z EO timijana (*Thymus serpyllum*), ki zmanjša glivne okužbe semen ter hkrati ohrani visoko raven njihove kalivosti in dobro fiziološko stanje kalic v zgodnjih fazah rasti. Pšenična semena smo namočili v 0,2 % EO timijana v 10 % DMSO za 6, 12 in 24 ur. Po tretmaju smo jih prenesli v kalilnike s programiranim načinom pršenja, kjer so kalila 7 dni pri sobni temperaturi in osvetlitvi. Vsak dan smo pri 21 naključno izbarnih kalicah izmerili dolžino poganjka. Po 7 dneh smo za vsak tretma določili delež kalivosti ter dolžino in svežo maso poganjkov in korenin. Po 100 mg poganjkov in korenin smo zamrznili za meritve lipidne peroskidacije, ki je pokazatelj oksidativnega stresa.

Ugotovili smo, da je 6-urno tretiranje pšeničnih semen s timijanovim EO najbolj optimalno, saj je bilo glivnih okužb malo, kalitev semen in rast kalic pa sta ostali na ravni kontrolnega tretmaja. Daljše tretiranje z EO je tudi zmanjšalo glivne okužbe, vendar je upočasnilo kalitev semen in rast kalic. Pri 24-urnem tretmaju z EO se je tudi povišala lipidna peroksidacija v koreninah kalic. Rezultati raziskave kažejo, da bi bil kratkotrajen (6-urni) tretma s timijanovim EO uporaben kot zaščitni protiglivni postopek za pšenična semena, ki se jih uporablja za setev in kot živilo.

Acknowledgments

This study was financially supported by the Slovenian Research Agency, grant no. P1-0212. The authors are thankful to dr. Matevž Likar for statistical assistance.

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