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CAN *Trichoderma asperellum* [T1] STIMULATE GROWTH OF LEMON BALM (*Melissa officinalis* L.) IN DIFFERENT SYSTEMS OF CULTIVATION?

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Abstract. The need to obtaining of healthy plant material and very small range of plant protection products in organic farming forces to searching for alternative means of plant production. Evaluation of *Trichoderma asperellum* as a growth promoter and the control effects of *T. asperellum* to *Septoria melissae* was evaluated. Also the fungi infesting lemon balm were identified. The experiment covered various combinations depending on the farming systems (conventional and organic system) and application of *T. asperellum.* The microorganism was used three times with one month interval as foliar spraying only in organically growing plants. *T. asperellum* was applied as water solution at a concentration of 10 $g \cdot l^{-1}$ by shoulder-carried sprayer. Control plots were treated only with water, no chemical fungicides were used. Plant development and its healthiness were assessed in respect of the effect of *Trichoderma*. *T. asperellum* was capable of stimulating the plant growth and in consequence causing an increase of dried mass of herb. There was no influence on essential oil content and proportion of leaves in the dried herb. The possibility of using of *Trichoderma* could be promising; especially for organic farming. Among pathogens naturally infesting of lemon balm *Septoria melissae* was identified most commonly.

Key words: beneficial microorganism, medicinal plant, organic cultivation, *Septoria melissae*

INTRODUCTION

Intensification of medicinal plants cultivation and high demands of industry are an important cause of research aiming at obtaining high quality raw material. Medicinal

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and aromatic plants used as fresh or dried material for drug production should be free of any pathogens. Therefore, medicinal plant plantations are most often subject to protective chemical treatments, which unfortunately can bring residues. The necessity of limiting the pesticide usage in medicinal plants cultivation gives a good opportunity for biopreparation usage and searching for alternative methods of protection or different systems of cultivation. Lemon balm (*Melissa officinalis* L.) is not often infested by pests, but in some cases the herb yield and its quality can be reduced [Bokor et al. 2008]. Pathogens from *Septoria* genus were found on many plants, including lemon balm [Nagy and Horvath 2010]. *Septoria melissae* infests lemon balm and causes leaf spots which are grey, black. According to data published by Machowicz-Stafaniak et al. [2004], among the fungi occurring on the leaves of lemon balm *Alternaria alternata* was observed most frequently. From the phyllosphere there were also obtained the fungi from the genus *Fusarium* and *Phoma* as well as the species of *Botrytis cinerea*, *Colletotrichum gloeosporioides* and other [Machowicz-Stafaniak et al. 2002, 2004, 2008, Zalewska and Machowicz-Stefaniak 2004, Szczeponek and Mazur 2006, Frużyńska-Jóźwiak and Andrzejak 2007].

Organic cultivation is one of the alternative systems of farming. Organic methods of food production, including medicinal plants, allow obtaining raw material that is completely free of residues. These methods of agricultural production are environmentally friendly and can deliver fresh material as expected by consumers, especially for children and for those suffering from allergies. Obtaining high quality food without any symptoms of disease and pest damage is very important especially for organic growers and processors.

For many years *Trichoderma* isolates have been used in different fields of production and protection in agriculture [Elad 1995, Howell 2003, Harman et al. 2004, Korolev et al. 2008, Jacometti et al. 2010]. *Trichoderma* spp. has no any harmful effects on human or animal health or on the environment. Many companies used these fungi to produce biological plant protection products or plant growth promoters. These microbial products are active by producing toxic compounds with a direct antimicrobial activity against pathogens. They can stimulate a plant to produce its own defense metabolites [Elad and Freeman 2002]. These beneficial microorganisms compete with plant pathogens. They are a nature-friendly and ecological approach to overcome problems caused by standard chemical methods of plant protection and as such they can be introduced into organic system of food production [Harman et al. 2004, Kowalska 2010]. Most microbial plant growth promoters are applied by mixing with soil before planting, but they can also be applied around the plant roots, or are recommended to be used by dipping the roots.

The aim of this study was to evaluate *T. asperellum* as a lemon balm growth promoter applied by foliar spraying during the growing season. The second issue considered was the assessment of the control effects of *T. asperellum* to lemon balm diseases, and identification of the pathogenic fungi that infest balm lemon.

MATERIALS AND METHODS

Field trials

The experiment was located in Plewiska, near Poznań (N52º21' E16º48') on a two-year-old plantation of balm lemon owned by the Institute of Natural Fibres and Medicinal Plants. In 2008, the plantation was established on the certified organic field, while the control was set on the conventional field. In March 2008, lemon balm seeds were sown in greenhouse, then plantlets were transplanted into the open plots with the area of 0.45 × 0.45 m. The seeds originated from a breeding project of the Institute. The plantation was established in 2008, before lemon balm on this same site was cultivated rye-vetch mixture. The conventional field had been subjected to mineral fertilization, tillage and crop rotation scheme. In order to maintain the high standards demanded for medicinal plant raw materials, a moderate intensity of management, in line with good agricultural practice, was applied. Thus no herbicide and fungicide inputs were incorporated and manual weeding methods were used in each case before and throughout the medicinal plant cultivation in both systems. In the course of the cultivation on the conventional field, three universal fertilizers (N/CaO/MgO, 60:6:8 kg ha^{-1}, respectively; P_2O_5 – 70 kg ha^{-1}, K_2O – 100 kg ha^{-1}) were applied, at the recommended rates [Kołodziej 2010]. Phosphorus and potassium fertilizers were incorporated in the spring of 2008, whereas nitrogen fertilizer was applied in mid-July 2008–2011. Similarly, one application of universal fertilizer "Azofoska" (300 kg·ha^{-1}), containing N/P_2O_5/K_2O, 1:0.5:1.4, respectively, was given in the spring of 2010 and 2011.

The observations were made in 2010 and 2011 on 2- and 3-old plants, respectively. The experiment was established in the randomized complete block design in three replicates. Each plot had the size of 10 sqm.

The experimental scheme includeded farming systems (conventional and organic system) and application, or not, of *T. asperellum* to organically grown plants. *Trichoderma asperellum* was used three times with one month intervals by foliar spraying. A suspension of Trifender WP® (product of Biovéd Co., Hungary) was used, one gram of the product contained 5 × 10^8 of conidium isolate T1 (NCAIM 68/2006). Control plots were treated only with water, no chemical fungicides were used. The first applications, in each year, were made in May. The product was applied in a water solution at a concentration of 10 g·l^{-1} by shoulder-carried sprayer "Kwazar" (tank volume 10 L.). Seven days after each treatment, the observation of the disease symptoms on the leaves was performed. Plants were also assessed in respect of any effects of the treatment on their development and healthiness. Disease symptoms on lemon balm were assessed on leaves (on the lower and upper parts of the plant) and described as percentage of the leaf area covered with the disease symptoms.

Laboratory experiment

Seven days after the second treatments (in July of 2010 and 2011) 50 leaves were randomly sampled from each plot. The collected leaves were subjected to mycological analysis in laboratory conditions.

Determination of the leaf fungal colonization. Irrespective of any symptoms, the complete leaf blades, without surface sterilization, were placed in Petri dishes (∅ 9cm) filled with the PDA medium, pH 6.5 (Fluka) incubated at 25°C for 3–7 days. After emergence of mycelium, identification was performed by means of a microscope.

Isolation of fungi from the fragments of leaf blades with the disease symptoms. Leaf cuts were harvested from margin of spots and immersed in 70% ethyl alcohol for 10 sec. and in 5% NaOCl (ACE) for 60 sec. Then the cuts were rinsed under tap water and placed on Petri dishes with the PDA medium. Five cuts were placed on each Petri dish. Petri dishes were incubated for 7–14 days at 25°C. All isolates were examined by means of a microscope and, if possible, identified and assigned to the respective species. Identification to species was made according to methods described by: Domsh et al. [1980], Ellis and Ellis [1987], Marcinkowska [2010].

Harvest and analysis of the raw material

In each year of the experiment, at the beginning of the lemon balm flowering period (July), raw material was collected by hand, from the area of 1.0 sqm of each plot. The herbs were dried in room temperature, in a shaded and well ventilated place. Fresh and dried material was weighted.

Content of the essential oil was evaluated by hydro-distillation from a herb without stems, with Dering's apparatus following the methods recommended by the European Pharmacopoeia [2008]. For distillation process, 20.0 g of the herbal drugs were placed in a 1000 ml round-bottomed flask, next 500 ml of water as distillation liquid was added. Distillation was carried out for 2 hours.

Weather conditions (2010–2011)

Data on the weather conditions come from the meteorological observation station nearby the experimental fields. In 2010, the monthly temperature ranged from 12°C to 21 (May–July). In the growing season, the relative humidity (RH) in the region was from 63% (July) to 82% (May). In 2011, the monthly temperatures were noted in the range from 14°C (April) through 20°C (June) to 19°C (July). In the same time, the relative humidity (RH) ranged from 64% to 78%.

Statistical analysis

The normality of distribution of the trait – occurrence of *Septoria melissae* was tested using the Shapiro-Wilk's normality test [Shapiro and Wilk 1965]. The trait was non-normal and was therefore transformed using the square root transformation. The three-way fixed model analysis of variance (ANOVA) was carried out to determine the effect of 1) year, 2) crop system, 3) time of observation as well as year × crop system, year × time of observation, crop system × time of observation and year × crop system × time of observation interactions on the variability of the studied trait. The least significant differences (LSDs) of trait were calculated, and on this basis homogeneous group (not significantly different from each other) were determined. Data analyses were performed using the statistical package GenStat v. 7.1 [Payne et al. 2003].

RESULTS AND DISCUSSION

Field trials

The significant differences ($P < 0.001$) in the incidence of *Septoria melissae* were noted for years of the experiments and for the interaction "year × time of observation" and for the interaction effect of "crop system × time of observation". It means that these factors could influence the results of the experiments (tab. 1).

Table 1. Mean squares from the analysis of variance for the incidence of *Septoria melissae* (data transformed)

Source of variation	d.f.	Occurrence of *Septoria melissae*
Year	1	23.433***
Crop system	2	2.394*
Time of observation	2	0.961
Year × Crop system	2	0.197
Year × time of observation	2	13.875***
Crop system × time of observation	4	3.062***
Year × Crop system × time of observation	4	1.405*
Residual	657	0.563

$P < 0.05$, * $P < 0.01$, ** $P < 0.001$***

In both years of observation, the necrotic spots, black or dark brown, sometimes grayish inside, round or shapeless were noted on the lemon balm leaves. These spots were 3–5 mm in diameter. During the disease development, blotches could merge together. Inside the spots were sometimes visible as black dots up to 0.5 mm with picnidia. Incidences of spots were described as symptoms of *S. melissae*.

The evaluation of the health status of lemon balm was differentiated yearly (tab. 2). In 2011, the pressure of *S. mellisae* was lower in all the tested systems. The disease incidence in July 2010 in organic cultivation and organic plus *Trichoderma* was lower compared to 2011 in the same systems and to the conventional system in both years. In July 2011, the organic lemon balm was the most infested (18.7%), while the lowest occurrence of the disease was noted on the plants treated with *Trichoderma* (8.5%). Similar results, (non-significantly different) were observed in the combination with conventional plants (10.8%).

The strain of *Trichoderma asperellum* tested in the presented research is a biocontrol agent that is highly effective against many pathogens in different crops, it was developed by some companies, which commercialize this fungus as a microbial pesticide under patent and registration [Nagayama et al. 2007]. The antagonism of *Trichoderma* spp. to *Fusarium solani* and others the soil-borne diseases on Chinese herbal plants was confirmed in paper by Liu-yan Qin et al. [2011]. There *T. harzianum* and *T. viride*

showed prospects in the control of soil-borne disease on Chinese herbal medicines. In other experiment under field conditions, seeds of faba bean were coated with essential oils of selected herbal plants, then were sown in soil treated with *T. harzianum* [Mokhtar Mohamed Abdel-Kader et al. 2011]. Reduction in disease incidence was reflected in a yield increase. These results show that application of essential oils integrated with *T. harzianum* may be considered as an applicable, safe and cost-effective method for controlling soilborne diseases. Our investigations on foliar application of *Trichoderma* could be prospective for leaf diseases controlling and may lead to an increase of yield. In our earlier trials, in the second season of research with strawberry, significantly higher yield of the strawberry was obtained compared to the untreated plants [Kowalska 2011]. Significant positive correlation between yielding and duration of the experiment was observed also by Porras et al. [2009] in strawberry plantation.

Table 2. Mean values (%) and standard deviations (SD) for the studied trait – the incidence of *S. mellisae* in years 2010–2011 (non transformed data)

Time of observation		2010				2011			
		organic system	organic + *Trichoderma* system	conventional system	$LSD_{0.05}$	organic system	orgnic + *Trichoderm* system	conventional system	$LSD_{0.05}$
May	mean	20.37a	20.07a	13.00b	6.86	4.87a	8.63a	4.50a	4.52
	s.d.	13.59	14.47	11.93		7.69	11.13	7.06	
June	mean	14.44a	10.24ab	7.47b	4.68	15.42a	10.07ab	7.62b	5.49
	s.d.	9.06	16.17	5.89		15.34	12.74	11.03	
July	mean	6.47b	7.22b	13.44a	3.89	18.73a	8.5b	10.87b	5.66
	s.d.	5.74	6.37	13.71		13.36	10.73	8.48	
Incidence mean		13.76	12.51	11.3		13.0	9.06	7.66	

Means marked with different letters are significantly different at $P < 0.05$

Trichoderma spp. are fungi present in nearly all agricultural soils [Harman 2004]. They are not normally regarded as resident organisms on foliar surfaces. However, *Trichoderma* spp. was found also on the phylloplane [Mangiarotti et al. 1987]. It was demonstrated that *Trichoderma* strains can survive in the aerial part of the plants for long periods [Perello et al. 2003] and can act as antagonists of foliar fungal pathogens in a wide range of crops [Elad 2000]. Some publications demonstrated that biological control is a promising strategy for managing septoria and alternaria spot [Perello et al. 2009]. However, little information is available on the utility of *Trichoderma* for controlling herbal plants under field condition**.**

According to the requirements for medicinal raw materials, lemon balm has to be free of pathogens and pesticide residues. Szczeponek et al. [2006] reported that natural

product like chitosol shows protection activity against lemon balm pathogens and it is dependent on the used concentration. They indicated the protective and plant growth promoter action and induced some plant resistant mechanisms. The results obtained after the *Trichoderma* treatment are ambiguous, but it is possible that *Trichoderma* spp. can stimulate the immune-response in plants. The greenhouse experimental data showed that it is possible to reduce the incidence or severity of the septoria and alternaria disease on tomato by using other beneficial leaf colonizing organisms like *Cryptococcus laurentii*, *Candida tenuis, C. oleophila* and *Pseudomonas putida* [Blum 2000]. In field experiment this fungus applied by spraying at the beginning of growth season was highly effective against *Botrytis cinerea* in strawberry plantations [Kowalska 2011].

The highest weight of the fresh herb in both years was obtained from the conventional combination (2.7 $kg \cdot m^{-2}$ and 1.6 $kg \cdot m^{-2}$ in 2010 and 2011, respectively). Comparing the results from combinations in 2010 and 2011, an increase of weight of the dried herb collected in 2010 from organic plants treated by *Trichoderma* (0.57 $kg \cdot m^{-2}$) and from organic plants in 2011 (0.53 $kg \cdot m^{-2}$) were noted (tabs 3 and 4). The obtained results indicate that foliar treatment with this microorganism can stimulate the growth of plants, despite the fact that our earlier knowledge on it was focused on soil application. Increase of dried mass of herbal material after foliar treatments was performed.

Table 3. Mean values of selected yield parameters of balm lemon in 2010

Cultivation system	Fresh mass of raw material ($kg \cdot m^{-2}$)	Dried mass of material ($kg \cdot m^{-2}$)	Part of leaves in dried material (%)	Content of essential oil in dried material (%)
Organic	1.4	0.28	60	0.19
Organic farming with *Trichoderma* application	2.5	0.57	66	0.14
Conventional	2.7	0.41	60	0.19

Table 4. Mean values of selected yield parameters of balm lemon in 2011

Cultivation system	Fresh mass of raw material ($kg \cdot m^{-2}$)	Dried mass of material ($kg \cdot m^{-2}$)	Part of leaves in dried material (%)	Content of essential oil in dried material (%)
Organic	1.3	0.53	59	0.12
Organic farming with *Trichoderma* application	1.2	0.51	60	0.12
Conventional	1.6	0.44	67.3	0.12

An enhancement of crop productivity as well as beneficial effects on plant morphology and physiology were emphasized [Harman et al. 2004, Di Marco and Osti 2008]. Treatment with formulated *T. asperellum* promoted the growth and the development of

potato plants and finally significantly increased the yield [Kowalska 2010]. Liopa-Tsakalidi et al. [2010], who studied the effect of chitin on the growth of lemon balm in organic cultivation, reported that the chitin presence in the peat substrate did not affect the weight of plants, but in the peat-sand substrate it increased plant weight compared to the respective substrates without chitin.

It is possible that in the organic system of production the dried mass of herb is similar or higher comparing to the conventional system. No differences between the combinations and system of production in respect of the essential oil content in dried herb and the share of leaves in dried material were noted. Patora et al. [2003], who evaluated different populations of lemon balm grown in Poland, reported that the content of essential oil ranged between 0.08% and 0.25% (leaves) and between 0.06% and 0.17% (herb). Saglam et al. [2004], who examined the effect of plant density and propagation method, obtained the results of 0.20–0.28% of essential oil content in dry leaves and there was no significant effect of any treatment observed. The data presented by Cosge et al. [2009] showed that investigated plant material of lemon balm grown in Turkey contained between 0.04% and 0.10% of essential oil.

It is possible that treated plants can produce natural substances that induce a defense reaction against the pathogens infection and help to take more nutrients from the soil by plants. A positive correlation was observed between the application of *Trichoderma* and plant healthiness. Experiments with microbial treatments on medicinal crops are a novelty and the authors have not found more data on the subject.

Laboratory experiments

Alternaria alternata and *Septoria melissae* overgrow leaves in the conventional and organic experiments with or without *Trichoderma* application (tab. 5). However, the lowest incidence of *S. melissae* was observed in the organic experiment where the plants were treated with *Trichoderma*, while in case of *A. alternata* the lowest percentage of 7.7% was noted only in the organically grown plants. *Mycelia sterilia* was found only in the organic samples (tab. 6).

Table 5. Occurrence of filamentous fungi colonizing the leaf blades of lemon balm in different cultivation systems (identification of fungi from complete leaves, without surface disinfection)

Cultivation system	Replicate	Occurrence of filamentous fungi	Identification of fungi colonizing leaf surface
Conventional cultivation	1	yes	*Alternaria alternata, Septoria melissae*
	2	yes	*Alternaria alternata*
	3	yes	*Alternaria alternata, Penicillium* spp.
Organic cultivation with *Trichoderma* application	1	yes	*Alternaria alternata, Penicillium* spp.
	2	yes	*Septoria melissae*
	3	yes	*Alternaria alternata*
Organic cultivation	1	yes	*Alternaria alternata*
	2	yes	*Alternaria alternata, Penicillium* spp.
	3	yes	*Alternaria alternata*

Table 6. Fungi obtained from the leaf cuts with symptoms described as septoria leaf spot (%)

Cultivation system	*Septoria melissae*	*Alternaria alternata*	*Mycelia sterilia*
Conventional cultivation	85.7	14.3	0
Organic cultivation with *Trichoderma* application	80	20	0
Organic cultivation	84.6	7.7	7.7

Fungi of the *Septoria* genus are known as the most frequently occurring pathogens of plants. *Septoria melissae* Desm. was the most frequently isolated fungus from the leaves with spots described as septoria leaf spot collected from the evaluated plants. It could be concluded that the described symptoms were indeed caused by S. *melissae* as the primary infection. No differences were found between the cultivation systems and the incidence frequencies of septoria – in the conventional system it was 85.7%, and in the organic system it was 84.6%. In some cases *A. alternata* was obtained from the infected leaves. *A. alternata* was also isolated in Serbia from the older sage leaves (*Salvia officinalis* L.) with symptoms of round or shapeless black spots [Bokor et al. 2008]. The spots often merged together and caused necrosis. *A. alternata* was also obtained with the 80% frequency from infected leaves of lemon balm with black spots of 3–7 mm in diameter by Machowicz-Stefaniak et al. [2004]. However, it should be noticed that *A. alternata* is described often as the secondary colonizer of the dead plant material [Ogórek et al. 2011] and it frequently colonizes herbal plants [Szczeponek and Mazur 2006].

In the presented study, *A. alternata* was obtained from both symptomless and spotted leaves. In the isolation from spots, mainly *S. melissae* were isolated. Therefore, it could be concluded in the macroscopic assessment that the described symptoms were caused by the S. *melissae* infection. This suggestion was made after a high number of isolates of *S. melissae* was obtained from spotted leaves (80–85.7% of isolates). This observation needs to be proven by the Koch's postulates. Wiaderny [2006] reported that infestation of lemon balm by a mitosporic fungus *S. melissae* is the greatest threat to the plant. Our results confirmed this opinion. *S. melissae* was obtained most frequently from affected leaves of lemon balm in the region of Poznan and Malopolska [Frużyńska-Jóźwiak and Andrzejak 2007]. In the same experiments other pathogens were also identified such as: *Fusarium oxysporium* (roots and stem base), *Botrytis cinerea* (leaves and stems).

CONCLUSIONS

1. *Trichoderma asperellum*, as applied by foliar spraying, was capable of stimulating, the plant growth, and in consequence causing intensive development and an increase of the dried herb weight, it did not influence the content of the essential oil and share of leaves in the material. These last two properties are most important for herbal stocks, but unfortunately *Trichoderma* not did influence them.

2. The possibility of using *Trichoderma* can be promising; especially for organic cultivation, as some fungicides based on copper compounds will be removed from application in the future.

3. Among the pathogens that naturally infest lemon balm, *Septoria melissae* caused the primary infection, while *Alternaria alternata* was the secondary colonizer.

4. Experiments with *T. asperellum* in lemon balm cultivation in different weather conditions should be continued.

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CZY *Trichoderma asperellum* [T1] MOŻE STYMULOWAĆ WZROST MELISY LEKARSKIEJ (*Melissa officinalis* L.) UPRAWIANEJ W RÓŻNYCH SYSTEMACH ROLNICZYCH?

Streszczenie. Konieczność uzyskania zdrowego materiału roślinnego i bardzo niewielki zakres środków ochrony roślin w systemie ekologicznym zmusza do poszukiwania alternatywnych środków produkcji roślinnej. Oceniono *Trichoderma asperellum* jako stymulator wzrostu, który stosowano poprzez nalistne opryskiwanie w trakcie sezonu wegetacyjnego oraz jego wpływ na występowanie *Septoria melissae*. Zidentyfikowano także naturalnie występujące grzyby chorobotwórcze zasiedlające melisę lekarską. Doświadczenia poletkowe obejmowały kombinacje dwóch systemów rolniczych (konwencjonalny i ekologiczny) oraz aplikowanie *T. asperellum*. Mikroorganizm stosowano trzy razy w odstępie jednego miesiąca. Poletka kontrolne były traktowane wodą, nie stosowano chemicznych środków grzybobójczych. Rośliny zielarskie (2- i 3-letnie) oceniano pod kątem ich rozwoju i zdrowotności. W efekcie *T. asperellum* stosowany jako roztwór wodny o stężeniu 10 $g \cdot L^{-1}$ stymulował wzrost roślin, powodując intensywniejszy ich rozwój i w konsekwencji wzrost suchej masy ziela, nie wpływał jednak na zawartość olejku i zwiększenie udziału liści w zielu. Możliwość wykorzystania *T. asperellum* może być obiecująca, szczególnie dla producentów upraw ekologicznych. Spośród patogenów naturalnie zasiedlających melisę najczęściej identyfikowano *Septoria melissae*.

Słowa kluczowe: ekologiczny system uprawy, mikroorganizm pożyteczny, roślina zielarska, *Septoria melissae*

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