Trichoderma and sulphoethyl glucan reduce maize root rot infestation and fusaric acid content

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ABSTRACT

Roots of maize seedlings (cv. Pavla) infested by *Fusarium verticillioides* (10^5/ml) were cultivated on Murashige-Skoog medium (MSM, Sigma, USA) containing $CaCl_2$, IAA and kinetin. Simultaneously, a strain of the antagonistic fungus *Trichoderma* sp. and a sulphoethyl glucan (SEG) isolated from the cell walls of *Saccharomyces cerevisiae*, were added. Two evaluations (on 7 and 14 days) were done. Productivity parameters of leaves and roots (fwt, dwt, and length), disease severity index (DSI) and fusaric acid (FA) concentration were evaluated. Both *Trichoderma* sp. and SEG increased productivity parameters of plants in infested variants and maintained it on the level of control plants during 14 days of experiment. *Trichoderma* reduced the DSI, while SEG increased it. DSI correlated with FA concentration. After seven days of cultivation concentration of FA was lower in all infected variants cultivated concomitantly with agents, compared with the one without them. After 14 days of cultivation both agents reduced the concentration of FA up to 50% to the non-measurable concentration in variant with *Trichoderma*. In variant with positive control, where FA was added to SEG, its concentration decreased up to 30%.

Keywords: *Fusarium verticillioides*; biocontrol; *in vitro*; productive parameters; disease severity; sulphoethyl glucan

The fungus *Fusarium verticillioides* (Sacc.) lives in soil and can penetrate stalks and roots directly or spread systemically in the plant after infection that originates from seed-borne inoculums (Soonthornpoct et al. 2000). Besides the capability to synthesize mycotoxins in Slovakian agro-climatic conditions (Šrobárová et al. 2002), *F. verticillioides* also produces the phytotoxin fusaric acid (FA) in naturally infected plants (Nadubinská et al. 2002). *Trichoderma* spp. are common inhabitants of the rhizosphere and are well recognized as biocontrol agents of soil-borne plant pathogens (Chet 1987). Recently defence responses were demonstrated during early stages of root colonization by this fungus (Yedidia et al. 1999). Yeast or yeast cell walls can also be used as adsorbents for mycotoxins. By the use of yeast cell walls only instead of whole cells, the adsorption of mycotoxins can be enhanced (Bata and La'sztity 1999).

The aim of our work was to test a strain of *Trichoderma* sp., a well-known antagonistic fungus, and yeasts polysaccharide derivative – sulphoethylglucans (SEG) for control of root rot caused by *F. verticillioides* in an experiment involving artificial infection of roots.

MATERIAL AND METHODS

Control agents. Fungal glucan was isolated from the cell wall of *Saccharomyces cerevisiae* as previously described (Kogan et al. 1988, Chorvatovičová et al. 1993). Candidate antagonistic *Trichoderma* strains from the collection of ISPA, Bari, Italy were tested against pathogenic *Fusarium* species isolated from maize ears and roots in dual cultures grown on potato-dextrose-agar (Sigma) for 7–21 days. The isolate with the highest activity was selected for this experiment.

Setting up an experiment. Caryopses of maize (susceptible cv. Pavla) were surface-sterilized for 1 min with 2% sodium hypochlorite (commercial bleach), and rinsed three times with sterile distilled water. Afterwards, the seeds were left to germinate for three days on moisturized filter paper in a sterilized box. Seedlings were grown for 14 days on Murashige-Skoog (Murashige and Skoog 1962) medium (MSM, Sigma, USA) containing $CaCl_2$, IAA and kinetin which is the most suitable medium for long and successful plant cultivation *in vitro* (Liu et al. 1997). Along with untreated control the following treatments and their combinations were tested:

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1. growth medium, control
2. *F. verticillioides* 10^5/ml spore concentration
3. *F. verticillioides* 10^5/ml spore concentration with *Trichoderma* sp.10^6/ml
4. *F. verticillioides* 10^5/ml spore concentration with 0.025% SEG
5. *Trichoderma* sp. 10^6/ml
6. 0.025% SEG

After 7 days of cultivation as a toxin positive control 7) variant with 0.025% SEG and fusaric acid (FA) in a concentration 8.40 μg/ml was added according to the average level evaluated *in situ* samples (Nadubinská et al. 2002).

In each variant 15 three-days-old seedlings were inserted, in two repetitions. All variants had volume of 200 ml and after 7 days of cultivation a fresh solution of MSM was added to maintain constant volume. We used the strain of *F. verticillioides* which produced FA *in vitro*. Plants were cultivated hydroponically in a special room under controlled conditions at temperature of 21/15°C day/night and 16/8 h photoperiod provided by white fluorescent lights, irradiance 180 μmol/m^2/s, day/night relative humidity 80/60%.

Evaluation of experimental results. After 7 and 14 days, the roots were separated from the leaves and evaluated for the degree of infestation, the content of FA, and productivity parameters: fresh weight (fwt), dry weight (dwt), and length. The longest roots and leaves were measured in each plant. To determine the dry weight, leaves and roots were dried separately at 105°C to constant weight. The data were subjected to standard statistical analysis, using SAS program 6.08 (1990). The means where applicable were tested for significant differences, using the *LSD* ($P < 0.05$).

Disease severity. The disease severity index (DSI) was rated on the 14th day using a scale according to Kroon and Elgersma (1993) in plants of each treatment, using a 0 – 5 disease index, where 0 indicated a healthy plant; 1 implied penalty of leaves; 2 – wilting of leaves; 3 – yellowing and necrosis of some leaves, wilting of all leaves; 4 – yellowing and necrosis of most of leaves, some leaves fallen; 5 – plant death. Plants only with dark brown spots on roots were checked. Scores were computed to determine DS index where DSI = € (seedlings/class × class score)/total seedlings. Data were subjected to the analysis of variance.

Fusaric acid analysis. Samples (dried roots and leaves) were ground and extracted with methylene chloride and analyzed by a modified thin layer chromatography method, as described by Bacon et al. (1996). After evaporating methylene chloride to dryness, the residue was shortly redissolved in 3 ml ethanol (UV-grade) and applied on Silufol plates (Silufol UV 254) together with FA standard. The plates were developed in n-butanol: acetic acid: ethyl acetate: water (3:2:2:2, v/v). Subsequently, plates were dried at 80°C and the putative FA was detected under UV lamp (CAMAG) at the wavelength 254 nm and localized comparing with a reference Rf value of the synthetic FA run on the plates. All detected places with FA were cut out to small pieces and eluted at occasional whip pining with 3 ml of 80% ethanol for UV range. After elution for one hour the content of FA in each sample was quantified spectrophotometrically on a Specord M 40 spectrophotometer (Carl Zeiss, Jena, Germany) at λ = 270 nm, basing on a calibration curve of FA standard.

RESULTS AND DISCUSSION

The highest DSI in plants on the 14th day was observed in the variants involving inoculation of *F. verticillioides* and that one with simultaneously added SEG. The treatment with *Trichoderma* kept the DS as low as in control. Natural occurrence of *Fusarium* in caryopsis is conceivable, and may account for the DSI observed in non-inoculated variants. Variant 7 with FA added fwt decreased and the degree of DSI probably revealed FA phytotoxicity. On the 7th day, of experiment *Trichoderma* and SEG reduced the content of FA in the variant with roots of inoculated plants (3, 4) compared to the one without them (2). Statistical significant differences were only between infected (2) and infected with SEG added (4). From the 1st to the 2nd evaluation, the content of FA in the roots decreased of about 30%, because of a partial breakdown of FA by the plant, fungus or hampered by SEG. The used polysaccharide (glucan) exhibited numerous and easily accessible adsorption centres for FA including different adsorption mechanisms, e.g. hydrogen bonding, ionic, or hydrophobic interaction (Karlovsky 1999) in an experimental variants with SEG.

After 14 days, FA was completely broken down in the roots of the variant involving co-inoculation of *Trichoderma.* At the time of the 1st evaluation, FA was found only in the leaves of *Fusarium* variant (2), while at the 2nd evaluation it was found only in the leaves of plants treated with it (variant 7). FA has been reported to play an important role in disease, but no correlation between pathogenicity and the amount of FA produced was found (Davis 1969). The relationship between FA concentration and DSI values observed in the present study proved it. FA concentration in roots decreased upon addition of either one of the potential control agents.

The productivity parameters of maize leaves and roots are given in Figures 1–4. After 7 days of cultivation, the productivity parameters of leaves and

roots (fwt and length) were statistically important only in control when compared to the infested and simultaneously added *Trichoderma* or SEG. Dry weight of roots and leaves is significantly different only in infested variant and those without infestation (variant 6). After 14 days (Table 1) all the productivity parameters, are in the same ratio as in first evaluation with the exception of control and fwt which increased rapidly only in control. All parameters are without statistic importance with exception of those (dwt) in infested variant (2) and SEG (6). Recently, using an *in vitro* test, the nature and efficacy of mycotoxin adsorption on a natural organic adsorbent made of isolated *Saccharomyces cerevisiae* cell wall fraction a ligand-toxin interaction model was developed and proposed (Dawson et al. 2001). Therefore, as it was possible to bind zearalenone using cell walls of yeasts (Karlovsky 1999), there is a possibility of SEG to bind fusaric acid, too. It may be the cause why FA content is lower in variants with SEG (4). DSI in that variant was the highest among all variants and in both evaluations. It means that beside the mechanism mentioned above by which FA may be decreased, another mechanism exists by which SEG stimulated the growth of fungi. Probably hydrolytic enzymes of *F. verticillioides* digest SEG and then some free molecules of glucans act as a signal molecules to stimulate further growth not only of the plant (Tamás et al. 2002), but of the pathogen, too. It was demonstrated by Kogan et al. (2004), that chitin and β-glucans are the major components of fungal wall and it means such free glucans from SEG may be incorporated into fungal cell wall of

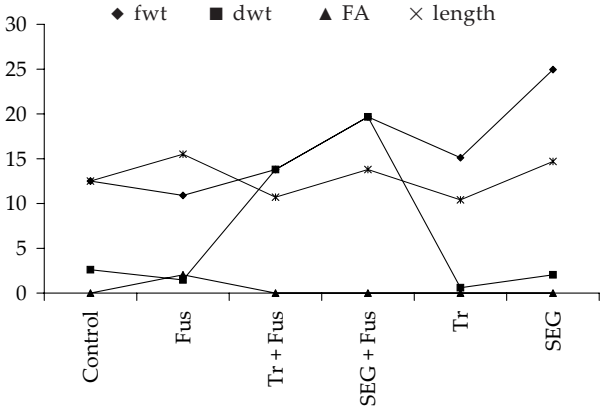

Figure 1. Productivity parameters of maize leaves (susceptible cv. Pavla) with root infected by *F. verticillioides* and treated with *Trichoderma* and polysaccharide SEG First evaluation (7 days): fwt and dwt in g, length in cm, FA – fusaric acid in μg/g

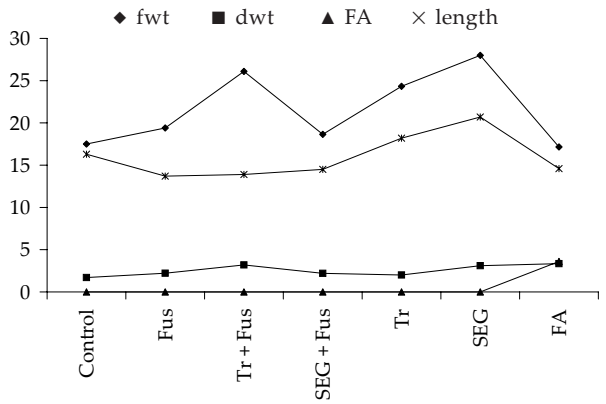

Figure 2. Productivity parameters of maize leaves (susceptible cv. Pavla) with root infected by *F. verticillioides* and treated with *Trichoderma* and polysaccharide SEG Second evaluation (14 days): fwt and dwt in g, length in cm, FA – fusaric acid in μg/g

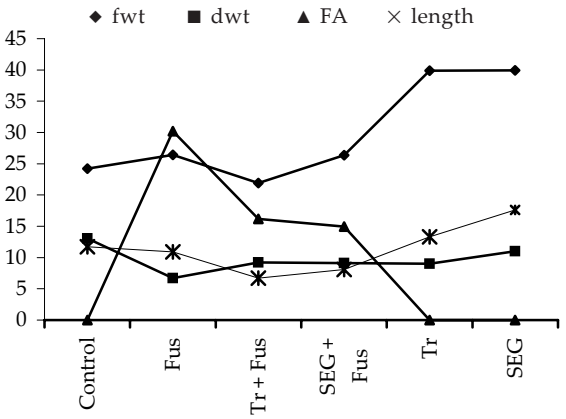

Figure 3. Productivity parameters of maize roots (susceptible cv. Pavla) with root infected by *F. verticillioides* and treated with *Trichoderma* and polysaccharide SEG First evaluation after 7 days: fwt and dwt in g, length in cm, FA – fusaric acid in μg/g

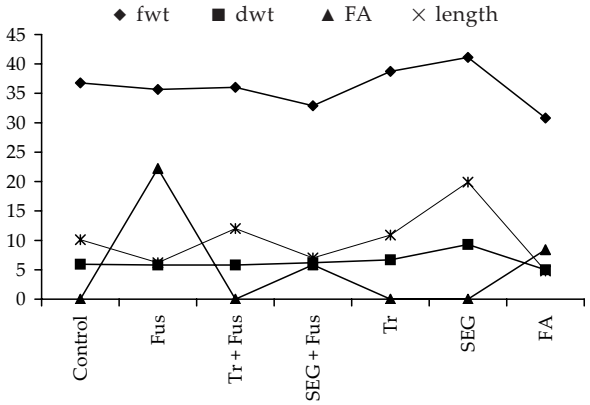

Figure 4. Productivity parameters of maize roots (susceptible cv. Pavla) with root infected by *F. verticillioides* and treated with *Trichoderma* and polysaccharide SEG Second evaluation after 14 days: fwt and dwt in g, length in cm, FA – fusaric acid in μg/g

Table 1. Productivity parameters of maize plants (susceptible cv. Pavla) with root infected by *F. verticillioides* (Fus) and treated with *Trichoderma* (Trch) and sulphoethylglucans (SEG); two evaluations (7/14 days) are presented: fwt and dwt in g, length in cm, FA – fusaric acid in μg/g; disease severity index (DSI) on 14 day; FA^{++} in 8.4^{++} μg/ml concentration in maize roots added into control agents (SEG) on 7 day

		Control	*Fusarium*	Trch + Fus	SEG + Fus	Trch	SEG	FA + SEG
Roots	fwt	24.2*	20.4*	27.9	26.3	36.9*	38.9*	
		36.8*	25.6*	36.0	27.9	38.7*	41.1*	30.8
	dwt	13.0*	6.7*	9.2	9.0	9.0	11.0*	
		8.9*	5.8*	6.8	6.2	6.7	9.3*	5.0
	FA^{++}	0.0	30.2*	16.2	15.0*	trace	0.0	
		0.0	22.2	n.d.	5.8	0.0	0.0	8.4^{++}
	length	11.7*	10.9*	8.7	6.1*	13.3	17.6*	
		14.4	8.2*	12.0	7.0*	11.0	19.9*	14.8
Leaves	fwt	12.5*	10.9*	13.8*	19.7	15.1	24.9*	
		17.5*	19.4*	26.1	18.6*	24.3	28.0*	17.1
	dwt	2.6*	1.5*	1.7	1.5	0.6*	2.0 *	
		2.9	1.2*	3.2*	2.2	2.0	3.1*	3.4
	FA	0.0	2.0	0.0	0.0	0.0	0.0	
		0.0	0.0	0.0	0.0	0.0	0.0	3.6
	length	12.5	10.5*	10.7*	13.8	10.4*	14.7*	
		16.3	12.7*	13.9	14.5	18.2*	20.7*	14.6*
DSI		0.3*	3.3*	1.1	4.2*	1.6*	0.1	1

*mean values significantly different according to *LSD* ($P < 0.05$), compared among control, disease or agents

pathogen. Another possible explanation is the effect of hormones used in our experiment which due to stimulation of callus formation (Liu et al. 1992) may also stimulate growth of both organisms (plant and pathogen), and maize plants with SEG are not under FA phytotoxicity, because of its detoxification. More sophisticated and direct experiments should be done to answer the question if the fusariotoxins are bind by SEG, or the pathogen growth is more intensive or it produces more enzymes by which relationship plant-pathogen-SEG is affected.

The productivity parameters in control plants reached those treated by both of control agents, where the higher values (fwt, dwt, and length) were still observed in *Trichoderma* and SEG variants. Several mechanisms, by which *Trichoderma* influences plant development, have been suggested, such as the production of growth hormones (Windham et al. 1989), solubilization of insoluble minor nutrients in soil and increased uptake and translocation of less-available minerals (Harman 2000). The reduction in fwt and dwt is a result caused by reduced water uptake or more probably due to the electrolyte leakage, a reaction in stress condition commonly known as chilling (Takáč 2004) or heavy metals (Pavlovkin and Mistrik 1999), but especially in toxins treated plants (Vurro and Ellis 1997). There, after a long cultivation *in vitro*, besides FA other fusariotoxins with known high phytotoxicity (Abbas et al. 1991) may be produced in infested plants such as e.g. moniliformin and fumonisins (FB). From this point of view, Vurro and Ellis (1997) reported, that some toxins could directly suppress specific defence responses in plant and especially – FB_1 strongly inhibited growth and respiration.

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ABSTRAKT

***Trichoderma* a sulfoetylglukan snižují u kukuřice napadení kořenovou hnilobou a obsah kyseliny fusariové**

Klíčící kořeny kukuřice (citlivý cv. Pavla) byli infikovány houbou *Fusarium verticillioides* (10^5/ml) a kultivovány na médiu Murashige-Skoog (MSM, Sigma, USA) obsahujícím $CaCl_2$, IAA a kinetin. Do roztoku byl zároveň přidán antagonistický kmen houby *Trichoderma*, resp. sulfoetylglukan (SEG) izolovaný z buněčných stěn *Saccharomyces cerevisiae*. Rostliny byly vyhodnoceny dvakrát, po 7 a 14 dnech experimentu. Byly sledovány produkční parametry (PP), stupeň napadení (DSI) a obsah kyseliny fusariové (FA). Použití houby *Trichoderma* i SEG částečně zvýšilo produkční

parametry infikovaných rostlin a udržovalo je na úrovni kontroly v průběhu 14denního pokusu. Houba *Trichoderma* snížila hodnoty DSI, SEG je naopak zvýšil. Hodnoty DSI byly přímo úměrné koncentraci FA. Koncentrace FA po sedmi dnech kultivace byla nižší ve variantách infikovaných rostlin a také ve variantách ošetřených SEG a *Trichodermou*. Po 14 dnech kultivace jak houba *Trichoderma*, tak SEG redukovaly koncentraci FA od 50 % až do neměřitelného množství ve variantě s houbou *Trichoderma*. Ve variantě s pozitivní kontrolou a SEG byla FA snížena na polovinu.

Klíčová slova: *Fusarium verticillioides*; biologická ochrana; *in vitro*; produkční parametry; napadení; sulfoetylglukan

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