The Use of *Bacillus subtilis* for Screening Fusaric Acid Production by *Fusarium* spp.

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Abstract

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Fusaric acid (FA) is one of the most important secondary metabolites produced by *Fusarium oxysporum* (Schlecht) (FO), *F. solani* (Mart.) Appel & Wollenweber, and *F. moniliforme* Sheldon. It is toxic to humans, many plants, and microorganisms and it enhances the toxicity of fumonisin and trichothecene. A simple and rapid method for fusaric acid (FA) screening in *Fusarium* isolates was developed. In this study, several strains of *Fusarium oxysporum* were tested for their ability to produce FA by using a suitable race of *Bacillus subtilis* as the bioassay. A modified method using small agar blocks with the fungus producing FA was applied in the screening test. FA standard and *F. culmorum* were used as controls. The experimental *F. oxysporum* isolates and FA standard produced transparent zones on the plates with *Bacillus subtilis*. The differences in size of the transparent zones corresponded to the quantity of FA when thin-layer chromatography was used.

Keywords: different strains; weed toxin; TLC chromatography; malt agar

Pathogenic fungi produce various biologically highly active substances (organic acids, specific polysaccharides) into the growth *milieu*, many of which are toxic to both plants and microorganisms (Bilaj 1989). Specific in this sense is fusaric acid (FA), an important nonspecific mycotoxin. Its chemicall formula is $C_{10}H_{13}O_2N$ (5-*n*-butylpyridine-2-carboxylic acid). It is a mild, weak organic acid (Gäumann 1957). FA is produced by several *Fusarium* spp. such as *F. moniliforme* Sheldon, *F. oxysporum* (Schlecht), and *F. solani* (Mart.) Appel & Wollenweber and their host-specific forms (Bilaj 1989). In treated or infected plants, FA can cause wilting, necrosis and other symptoms. Inoculated tomato seedlings showed an injurious effect (wilting and brown discoloration) at a concentration of 0.6 μg FA per plant (Ouchi *et al*. 1989). In spite of this finding, the role of FA in the pathogenesis of plants is still unknown (Remotti *et al*. 1995).

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The content of FA found in 77 strains from different sources of autoclaved maize was 177 mg/kg *in vitro*, or up to 11.97 mg/kg *in vivo* (Bacon *et al*. 1996). Fusaric acid is produced by several species strains of *Fusarium* moulds species, many of which also produce other mycotoxins which should be of concern to food and animal feed producers. Another study showed that FA enhances the toxicity of β-trichothecene, namely deoxynivalenone DON. This is because fusaric acid competes with tryptophan for binding to blood albumin and, therefore, increases the concentrations of free tryptophan in blood. The result is an increased brain uptake of free tryptophan and increased serotonin synthesis. This is the basis of the toxicological synergism between DON and fusaric acid (Smith *et al*. 1998). Several results, however, show a great diversity among *Fusarium* fungi in the formation of this phytotoxin (Mutert *et al.* 1981). In this latter study, moderate amounts (12–290 mg/g) of FA were detected by HPLC in the culture fluids of different strains of *F. oxysporum*. The effect of FA on the growth of a number of plant species and its phytotoxicity has been investicated by Kachlicki and Jedryczka (1997). According to these authors, the most severe effects were observed for the plant root growth at 0.1–10mM FA, and even 50 μm caused almost total retardation of oilseed rape (*Brassica napus* L.) and a strong inhibition of garden cress (*Lepidium sativum* L.) and lettuce (*Lactuca sativa* L.) root growth. Ten mM of FA caused almost total inhibition of seed grain germination, and at 1mM it completely retarded the growth of all plant species studied. The range of 0.1–10mM is the same range of FA reported by Chawla and Wenzel (1987) as influencing the growth of barley callus. Pavlovkin *et al.* (2004), using electrophysiological measurements, showed that at 1mM FA caused an early hyperpolarisation in the roots of *Ricinus communis* L. induced at pH 5.5, which was followed by a marked depolarisation of the membrane potential difference (Em). During this time, an increased electrolyte leakage from primary *R. communis* roots was observed. At higher pH values (6.5 and 8), the depolarisation caused by FA was immediate without the hyperpolarisation observed at pH 5.5.

In our work, we tested some isolates of *F. oxysporum* for FA production using a suitable race of *B. subtilis* as a bioindicator. Thist constitutes the first step of a screening test to separate effectively and clearly a *F. oxysporum* races into FA producers and FA non-producers. This method is simple, cost-effective, and relatively quick.

MATERIALS AND METHODS

Isolates of *Fusarium oxysporum* (Schlecht) were tested for their capacity to produce metabolites, namely FA, which is toxic to *B. subtilis*. These were obtained from naturally infected ears of winter wheat (*Triticum aestivum* L.) cultivars Agra, Mara, and BU-25, and from the seeds and roots of infected lentils (*Lens culinaris* L.). As a control, we used *F. culmorum* (W.G.Sm.) Sacc., an isolate from the ears of wheat (*T. aestivum* L.) cv. Agra, and *F. oxysporum* f.sp. *lycopersici* isolates No. 485 and No. 545 from Czech Collection of Microorganisms in Brno. These strains, which were isolated from tomato (*Solanum lycopersicum* Mill.), are known to be hosts of *F.oxysporum* and are FA producers (Katan & Auser 1974).

The fungi were cultivated on malt agar (2% agar malt medium, IMUNA, Šarišské Michaľany, Slovakia) and liquid brewer malt medium (300 ml malt and 200 ml distilled water) at 25 ± 1°C, for 21 days. *F. oxysporum* isolates No. 485 and No. 545 were used for testing FA production during ontogenesis and were cultivated on Czapek-Dox agar for 14 days.

Suitable race of *Bacillus subtilis* as the bioassay was maintained on a solid medium (6 g peptone, 1.5 g beef extract, 3.0 g yeast extract, 25 g agar) at 37 ± 2°C for one day. Before sterilisation, the pH of the medium was adjusted to 6.0. After this cultivation period, agar blocks (0.5 mm^2) with the fungus were transferred to plates with *B. subtilis* (Betina & Pilátová 1958). We selected *Bacillus subtilis* strain No. 8/58 (ATCC, Rockville, USA) from a range of different microorganisms. This strain is commonly used in the plant protection. In the case of FA, a sheet of paper (0.5 mm^2) was immersed in 200 and 300 mg/l solutions of standard FA, then placed into the culture medium containing *B. subtilis.*

We estimated the active substances produced by each fungal race according to the zones of inhibition of the growth of *Bacillus subtilis*. We measured the size of the zone (in mm) after different periods of the fungus cultivation ranging from 6 to 21 days. The effect of the tested fungi on *B. subtilis* was compared with standard FA. Pure FA was obtained from Sigma Chemical Co. (St. Louis, USA). The

quantity of FA produced was estimated using the chromatographic method of Barna *et al.* (1983), which we modified as follows.

The samples were ground, extracted with CH_2Cl_2, and analysed using a modified TLC method. Briefly, after evaporating CH_2Cl_2 to dryness, the residue was re-dissolved in 3 ml of EtOH (UV-grade) and applied to silufol plates (Silufol UV 254, HPTLC Kieselgel 60, Merck, Darmstadt, Germany) together with the FA standard. The silufols plates were developed in a butanol: ethyl acetate: acetic acid: water (3:2:2:2 v/v) solution. The plates were dried in a thermostat at 80°C. FA was detected under UV light and its content was quantified spectrophotometrically using a Specord M40 spectrophotometer (Carl Zeiss, D-Jena, Germany) at 270 nm, based on the calibration curve of FA standard.

RESULTS

Testing of isolates producing FA by *B. subtilis*

The efficiency of the *F. oxysporum* isolates tested could be well demonstrated by the formation of inhibition zones of *B. subtilis* growth. For example, the zones formed by the isolate *F. oxysporum* Agra were very clearly limited and transparent and formed more clearly than the zones around the FA standard (Figures 1 and 2). The diameter of the zones ranged from 5 to 20 mm on liquid malt medium (Table 1) and from 7 to 24 mm on solid malt agar (Table 2). The most stable activity was observed with the isolate from the infected roots of lentil on malt medium, merely from 7 to 13 mm. Very small zones were obtained with the isolate from infected seeds of lentil (Table 1). On the other hand, the isolate from the seeds of lentil was more active when compared with the same isolate on liquid malt medium. The zones of inhibition reached a maximum of 18 mm or 16 mm on average for wheat cv. Mara and Agra, respectively (Table 2). No zones were formed by the isolate of BU-25 from the ears of wheat and around the mycelium of *F. culmorum*.

Chemical measurement of FA production

All tested isolates from plants in which *B. subtilis* zonation formed, i.e. in which FA production occurred, were chromatographically tested and proved. The highest levels of FA were found on the 9th day (43.3 mg/l) in the isolate Agra, and in the isolate Mara 158.0 mg/l cultivated on malt medium (Table 1). The content of FA on solid malt agar was

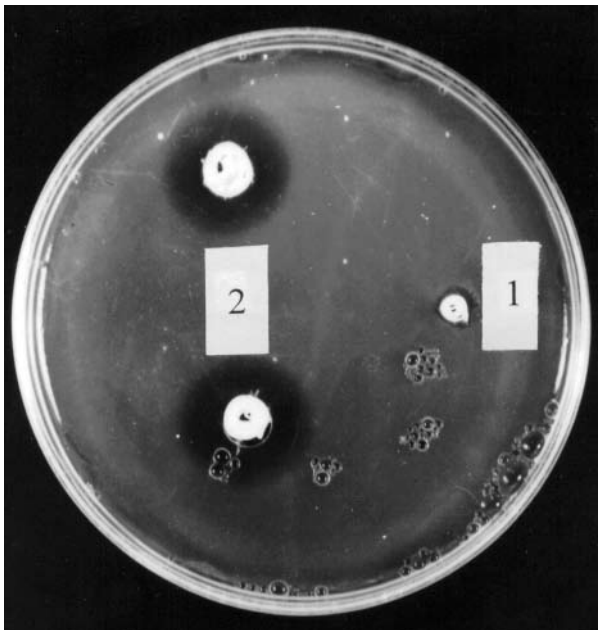

Figure 1. The formation of zones in the plate with *Bacillus subtilis* around 1 (standard fusaric acid (FA) 20 mg/l) and 2 (*F. oxysporum* isolate Agra) cultivated on liquid malt agar forming highly transparent zones

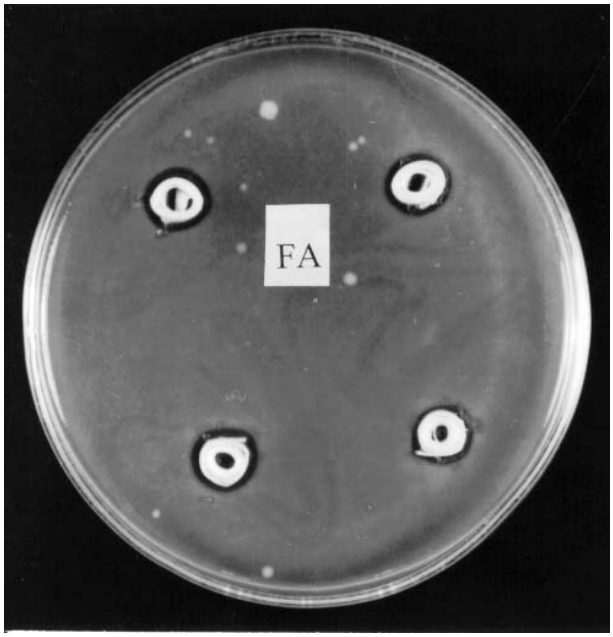

Figure 2. The formation of zones in plate with liquid malt agar by *Bacillus subtilis* around standard fusaric acid (FA) 200 mg/l.

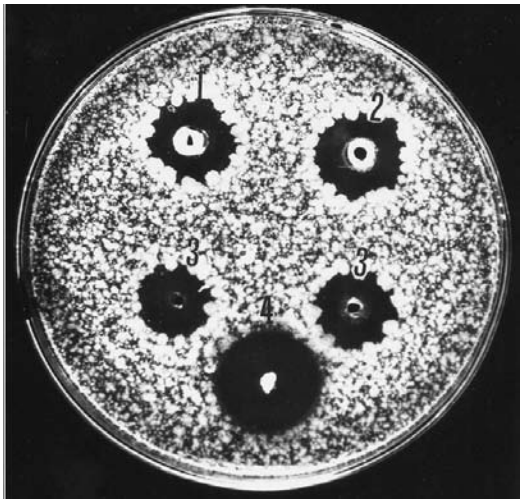

Figure 3. Transparent zones on plate with precultivated *Bacillus subtilis* by isolates of *F. oxysporum* (Schlecht) cultivated on solid malt medium. Isolates from (1) lentil seed, (2) lentil root, (3) isolate No. 545, and 3) isolate No. 485 and from (4) wheat cv. Agra

the highest in the isolate from lentil roots on the 13th day (273.0 mg/l) (Table 2). This was the highest value of all isolates from infected plants tested. A high value (136.0 mg/l) was also found in the isolate Mara implying the same level as *B. subtilis*.

In particular, high values of FA production were found in races of *F. oxysporum* isolates No. 485 and No. 545 (Figure 3). Especially on solid malt agar (Table 2), the maximum quantity of FA was 870.0 mg/l in the isolate of *F. oxysporum* No. 545, which was the highest value in all isolates during the test period. Much lower values with a maximum of only 172.2 mg/l FA were found in *F. oxysporum* No. 485.

DISCUSSION

The isolates from naturally infected plants from different sources showed transparent inhibition

Table 1. Inhibition zone of growth of precultivated *Bacillus subtilis* by isolates of *F. oxysporum* (Schlecht) cultivated on liquid malt medium

Isolate	Days of cultivation *F. oxysporum*/FA concentration (mg/l)							
	6/mg		9/mg		13/mg		21/mg	
	zone	means ± S.D.	zone	means ± S.D.	zone	means ± S.D.	zone	means ± S.D.
Weat cv. Agra	++	139.5 ± 2.0	+	143.3 ± 1.2	+++	79.0 ± 0.8	++	85.0 ± 0.7
Size (mm)		15 ± 0.3		15 ± 0.4		20 ± 3		15 ± 0.2
Weat cv. Mara	+++	81.6 ± 1.5	+++	158.0 ± 0.5	++	87.0 ± 1.0	+	133.0 ± 0.1
Size (mm)		6 ± 0.0		10 ± 0.5		10 ± 0.2		10 ± 0.1
BU-25	+	0	No zones were formed					
Size (mm)								
Weat *F. culmorum*	mycelium was digested by BS							
Size (mm)								
Lentil seed	++	96.3 ± 0.2	no zones were formed					
Size (mm)		5 ± 0.2						
Lentil root	+++	82.5 ± 0.4	+++	76.5 ± 0.8	+++	75.0 ± 0.5	+++	100.0 ± 0.5
Size (mm)		7 ± 0.5		13 ± 0.6		11 ± 0.2		
485 CCM	++	120.0 ± 0.1	+	131.0 ± 0.2	++	1136.2 ± 0.4	+	152.1 ± 0.4
Size (mm)		15 ± 0.2		12 ± 0.5		11 ± 0.5		9 ± 0.1
545 CCM	++	1130.0 ± 0.1	++	150.0 ± 0.4	++	170.0 ± 0.5	++	172.2 ± 0.4
Size (mm)		12 ± 0.3		15 ± 0.3		9 ± 0.3		9 ± 0.3
FA	+++	200.0 ± 0.4	Not evaluated					
Size (mm)		20 ± 0.2						

+ no distinct zone; ++ distinct zone; +++ transparent zone

Table 2. Inhibition of *Bacillus subtilis* growth by isolates of *F. oxysporum* (Schlecht) cultivated on malt agar

Isolate	Days of cultivation *F. oxysporum*/FA concentration (mg/l)							
	6/mg		9/mg		13/mg		21/mg	
	zone	means ± S.D.		means ± S.D.		means ± S.D.		means ± S.D.
Weat cv. Agra	+	105.8 ± 1.0	+++	138.5 ± 0.5	+++	176.0 ± 0.4	++	80.0 ± 0.2
Size (mm)		18 ± 0.2		18 ± 0.4		17 ± 0.2		10 ± 0.3
Weat cv. Mara	++	91.8 ± 0.1	+	81.6 ± 0.1	+	136.0 ± 0.2	++	72.0 ± 0.7
Size (mm)		15 ± 0.2		18 ± 0.3		17 ± 0.1		8 ± 0.3
W.BU-25	no zones were formed							
Size (mm)								
Weat *F. culmorum*	mycelium was digested by BS							
Size (mm)								
Lentil seed	+	96.3 ± 0.2	++	76.6 ± 0.4	++	206.0 ± 0.3	+	191.0 ± 0.2
Size (mm)		12 ± 0.3		17 ± 0.2	1	5 ± 0.1		
Lentil root	++	86.5 ± 0.3	+	105.0 ± 0.4	++	273.0 ± 0.5	+	270.0 ± 0.2
Size (mm)		15 ± 0.2		15 ± 0.1		7 ± 0.2		7 ± 0.2
485 CCM	++	78.0 ± 0.4	++	98.6 ± 0.3	++	452.0 ± 0.2	++	830.0 ± 0.4
Size (mm)		15 ± 0.2		12 ± 0.4		17 ± 0.3		8 ± 0.2
545 CCM	++	362.0 ± 0.4	+++	356.0 ± 0.4	+++	575.0 ± 0.1	+++	870.0 ± 0.2
Size (mm)		22 ± 0.2		21± 0.3		24 ± 0.2		23 ± 0.5
FA	+++	300.0 ± 0.2	mot evaluated					
Size (mm)		22 ± 0.3						

+ no distinct zone; ++ distinct zone; +++ transparent zone

zones practically from the beginning of the test period. The highest values of FA production were found in the isolates of *F. oxysporum* races from tomato, especially on solid malt agar with all isolates during the test period. Moreover, out of all the antagonists used, *Trichoderma viride* and *Bacillus subtilis* were found to be the most effective in suppressing the growth of *F. oxysporum* f.sp. *lycopersici in vitro* (Islam & Goswami 2002). The most stable activity was demonstrated by the isolate from the infected roots of lentil on solid malt medium despite its relatively low inhibitory values. On the other hand, the isolate from the seeds of lentil was more active when compared with the same isolate on liquid malt medium. The *in vitro* experiments showed that *Bacillus subtilis* filtrate, whether on solid or in liquid media, had a strong inhibitory activity on the spore germination and mycelial growth of *F. oxysporum* f.sp. *lentis* (Gowen *et al*. 2004). Distinct and transparent zonation of the growth inhibition of *B. subtilis* was formed by the isolates from wheat cvs. Mara and Agra. A positive correlation between the pathogenicity of wheat rhisosphere and the production of FA has been found for many strains of *F. oxysporum* (Davis 1969). In his experiment, the isolates of *F. oxysporum* without FA production did not show any disease symptoms. How these antibiotic mechanisms exist is the theme for another study. In contrast, the mycelia of *F. culmorum* were digested by *B. subtilis* cultures. This may be the reason why some *B. subtilis* zones are used in biological control of fungal diseases (Kůdela 1989). There was a great diversity (Landa *et al*. 2002) in the response of the bacterial strains to FA; however, as a group, *Bacillus* spp. and *Paenibacillus macerans* were much more sensitive to FA than *Pseudomonas* spp. *Bacillus subtilis* is a common saprophytic inhabitant of soils and is thought to contribute to nutrient cycling due to the variety of proteases and other enzymes which the members of this species are capable to produce (Landa *et al*. 2002).

The formation of a transparent zone indicates the degradation of pathogenic factors of the pathogen such as toxins and it means that the whole contents of *Bacillus subtilis* cells are lysed, a process described by Grant *et al*. (1991). The growth of *F. oxysporum* on heat-killed *B. subtilis* cells was accompanied by the loss of bacterial cytoplasm contents, and this 'cytolysis' could be catalysed in the heat-treated bacteria by the fluids from fungal culture. The appearance of cytolytic activity in cultures was paralleled by the production of proteolytic activity in them. Grant *et al*. (1991) claimed that viable bacteria, however, were not attacked by either of the concentrated culture fluids. Later, Shariffi-Tehrani and Ramezani (2003), using biochemical, physiological, and morphological tests, identified the isolates from soil as *Bacillus* spp. which produced volatile metabolites inhibiting the mycelia growth of *F. oxysporum*. In greenhouse studies, Shariffi-Tehrani *et al.* (2005), using isolates of *Bacillus subtilis,* reduced the intensity of charcoal rot (caused by *Tiarosporella phaseolina* (Tassi) Van der Aa) of soybean by 59% and 66%, respectively.

As evident from our results, the method using *Bacillus subtilis* as a bioindicator is extremely simple, clear, and effective for screening the fusaric acid production.

CONCLUSION

A very simple, rapid and easy-to-screen method for isolates of *Fusarium* if produced the toxin fusaric acid. In this study we tested some isolates of *F. oxysporum* for FA production by using a suitable race of *Bacillus subtilis* as the bioassay. As a practical applications because fusaric acid competes with tryptophan for binding to blood albumin and, uptake of free tryptophan and increased serotonin synthesis. This is the basis of the synergism between toxins deoxynivalenone and fusaric acid a base for healts safety.

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