OAT ROOT EXTRACT AFFECTS IN VITRO GROWTH OF SEVERAL SOIL-BORNE CEREAL FUNGAL PATHOGENS

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Abstract

The oat root tips are known to contain saponins called avenacins that have antimicrobial activity. We tested antifungal activity of oat root extract against fungal pathogens isolated from cereal crops to investigate whether the extract could inhibit the germination, germ tube growth and colony development of the fungi in vitro conditions. Seven fungal species isolates causing root and crown rot in cereals included in this study were Drechlera victoriae, Culvularia sp., F. oxysporum, F. accuminatum, F. poae, F. avenaceum, and F. moniliforme. The germination at 18 hours after treatment (hat) in control plugs was more than 96% in all fungal isolates; however it varied greatly among the isolates treated with oat root extract. A total inhibition of Culvularia sp. conidia was observed in treated plugs, whereas the least sensitive isolate against oat root extract was F. avenaceum; 90% of the fungus's conida germinated at 18 hat. No germ tube growth of Culvularia sp. and F. moniliforme was visible at any time point. Germ tubes of the other fungal isolates extended to some degree. Four different oat tip extract concentration was incorporated into potato dextrose agar medium: 1, 2, 5 and 10 oat root tip/ml agar medium. The most sensitive fungal isolate was Culvularia sp., whereas the least sensitive was F. avenaceum. The results prove that antifungal compounds found in plants can be used as an effective control measure by either classical breeding or gene transformation into economically important crops.

Key words: *oat, spooning, avenacins, disease resistance, fungal pathogens, cereal*

INTRODUCTION

Plants accumulate diverse secondary metabolites. Some of these metabolites have anti-microbial activity against pathogens. Some of these secondary metabolites are absent from healthy plants and accumulated only following pathogen attack or abiotic stress, these compounds are called "phytoalexins". Alternatively, some other secondary compounds can be present during normal development of the plants, these compounds are called "phytoanticipins" (Mansfield, 2000).

Phytoalexins have attracted more attantion since the concept developed by Müller and Börger (1940). There are some research indicated that phytoalexins accumulated at the at the infection site in only resistant plants (but not susceptible) which proves a strong relationship between resistance of the plants (Mansfield, 1983; Bailey and Mansfield, 1982). However there is some evidence that phytoalexins can accumulate in both susceptible and resistant plants (Mert-Türk et al., 2003a; Mert-Türk et al., 2003b). Although many studies have been done about phytoalexins, only a few works have been done about phytoanticipins and their roles in resistance against pathogens (review by Morrisey and Osbourn, 1999). Avenacins are a well-characterized phytoanticipins found in oat root tips and constitute of four structurally related compounds (avenacin A-1, avenacin B-1, avenacin A-2 and avenacin B-2) (Crombie and Crombie, 1986a). *Gaeumannomyces graminis* var. *tritici* attacks roots of wheat and barley causing the important disease called take-all, but does not cause an infection of oats. A close correlation between the presence of avenacins and resistance to *G. graminis* var. *tritici* has been confirmed (Papadopoulou et al., 1999).

Increasing use of pesticides in agriculture against crop pest has impact on human beings as well as environment. Therefore, using crops that contain disease-fighting compounds in sustainable agriculture is important. We, therefore, aimed to investigate the inhibition rates of several soil-borne fungal pathogens of cereal crops by oat root tip extract that contains those four structurally related antimicrobial compounds.

MATERIAL AND METHODS

Cereal fungal pathogens

The fungus collection was isolated from diseased cereal plant roots. The fungi included in this research were *Fusarium oxysporum, F. accuminatum, F. avenaceum, F. moniliforme, F. poae, Drechlera victoriae* and *Culvularia* sp.

Plants

Because of high yield of avenacin A-1 obtained (Mert-Türk et al., 2004), Ankara 76 variety of *Avena sativa* was used during the entire experiment.

Oat Root Tip Extract Preparation

The extraction method was adopted from Osbourn et al. (1991). All the extraction procedures were made under sterile conditions to avoid contamination. The seeds were dehulled and surface sterilized with 1% NaOCl solution for 5 min. They were then rinsed with several changes of sterile distilled water for an hour at room temperature. They were then transferred into sterile Petri dishes containing moist filter paper and sealed with parafilm in order to avoid microbial contamination. The Petri dishes were kept at 4^oC refrigerator for vernalisation and then allowed to germinate at 23^oC for 2 days. The bulks of 100 root tips (0.5 cm) were removed with an aid of scissors and transferred into an eppendorf tube. The root tips were meshed with microhomogineser and centrifuged at 10 000 r.p.m. for 10 min. The supernatant was removed for whether to place it on the plugs or incorporate into PDA growth medium (Merck, Germany) as follows.

Effects of Extracts on Conidium Germination and Germ Tube Growth

The conidium germination method was adopted from Antonov *et al.* (1997). Water agar (Agar agar, Merck) was poured into Petri plates 15-20 ml/plate. Agar plugs were cut with a sterile cork borer (10 mm diameter) and placed into Petri dishes (7 plugs/dish). Then, 20 µl/ plug of oat tip extract was applicated on the surface of the plugs, spread gently over the surface of the plugs and let to evaporate for 15-20 min. An aliquot of 20 µl conidium suspension of each isolate was then pipetted onto the surface of each agar plug which had been applicated with oat extract previously. Control plugs received 20 µl /plug of sterile distilled water. Different isolates were tested in separate Petri dishes to prevent cross-contamination. The Petri dishes were sealed with parafilm and incubated at 22ºC. Six and 18 hours after treatment (hat), percentage germination of conidia was determined from counts of 100 conidia selected from a random sweep across the diameter of each agar plug. A conidium was considered germinated if the germtube length was just greater once the length of the conidium.

Average germ tube length was determined on the basis of random measurement of ten germtubes on each agar plug using microscope eyepiece graticule at 6 and 18 hat. Non-germinated conidia were not included in measurement data. If there was not at least 10 germinated conidia across the whole plug in all seven plugs, then the data for germ tube growth was ignored, therefore, not analyzed.

Effects of Extracts on Mycelial Growth

Four different doses of oat root extract were incorporated into Potato Dextrose Agar (PDA): 1 root tip/ml, 2 root tips/ml, 5 root tips/ml and 10 root tips/ml medium. The extract were prepared as above and incorporated into PDA at 45^0C and shacked gently. Then they were poured into Petri dishes at a volume of 15-20 ml. Control plates received only sterile distilled water.

Discs of 0.7 cm of young cultures were cut from the growing margins of subcultures on PDA and placed in the centre of the medium in each Petri dishes. The cultures were incubated at 22^0C. The

measurement of each colony was made when at least one control plate was covered entire the Petri dish for that particular fungal isolate. Each treatment was replicated 10 times. Inhibitory effects of different oat root extract were determined by comparing treatment plates with control plates.

Statistical Analysis

Analysis of variance (ANOVA) was carried out to compare the treated plates with control plates of the same fungal isolate. Comparison of means was applied to the experimental data as following: Z (proportion) test for germination, T test for germ tube growth and Tukey test for colony development.

RESULTS

Conidium Germination

The effect of oat root extract on conidium germination varied greatly among the fungal isolate tested exhibiting in most cases a highly significant decrease ($P<0.01$) in germination (Table 1). Most of the conidia of *Culvularia* sp. and *D. victoriae* were germinated on control plugs at 6 hours after treatment (hat), 92.6 and 97.1%, respectively, whereas the percentages of the germination of both fungal isolates were 0 and 0.3% on oat root extract applicated plugs, respectively. The only *Fusarium* isolate to be germinated in control plugs was *F. poae,* although only 0.6% of the conidia on the applicated plugs were germinated. Almost none of the conidia of other *Fusarium* isolates, *F. avenaceum*, *F. acuminatum*, *F. moniliforme* and *F. oxysporum*, were germinated at 6 hat time point.

Germination rates of the fungal isolates increased at 18 hat in control plugs; at least 96% conidia germination was observed on all control plugs. However germination rate differed greatly among the isolates on treated plugs. *Culvularia* sp. seemed to be the most affected from the treatment. In that treatment, a total inhibition of germination was observed. *F. moniliforme* and *D. victoriae* followed it with a rate of 0.6 and 3.7% germination of all conidia, respectively, comparing 96.1 and 98% germination on the control plugs of the same fungi. Germination rates of *F. oxysporum* and *F. acuminatum* were 19.4 and 21.7%, respectively, in treated plugs. *F. avenaceum* seemed to be the least affected by the oat root extract treatment: 90 % of conidia germinated.

Effects on Germ Tube Growth Rates

As mentioned in the previous section, no conidium germination of any fungal isolate was observed at 6 hat in plugs treated with oat root extract and even in control plugs of *F. avenaceum, F. moniliforme* and *F. oxysporum* (Table 2). Therefore no germ tube growth of those fungi on treated plugs was visible at this stage. The germ tube length of *D. victoriae*, *Culvularia* sp. and *F. poae* in control plugs was approximately 131, 58 and 42 μm, respectively.

The germination of *F. moniliforme*, *D. victoriae* and *Culvularia* sp. conidia was completely inhibited on plugs containing oat root extract at 18 hat (Table 2), therefore no measurement could be done. A very strong inhibitory effect of oat root tip extract (10.3% control germ tube growth) was observed in germ tube growth of *F. oxysprorum* at 18 hat. Strong inhibitory effects were also observed for *F. poae* and *F. avenaceum* (26% and 33%) during 18 hours incubation. The inhibition potency of oat root extract on the germ tube growth of *F. acuminatum* was the least compared to other isolates.

Effects of Extracts on Mycelial Growth

A collection of six fungal species isolates was assessed by measuring the effects of oat root extract on the growth of the fungal colonies. As indicated in material and method section, four different concentrations of oat root tip extract (1, 2, 5 and 10 root tip/ml PDA) were incorporated into the PDA growth medium, and colony growth of each pathogen isolate was measured when the fungus in control plates covered entire the surface. The effects of the oat root extract on the colony growth of fungi

varied among the four extract concentrations and among six fungal species isolates tested exhibiting in most cases a highly significant decrease (Table 3).

Strong antifungal activity of oat root tip extract was observed for all the fungal isolates tested especially at high concentrations. Inhibitory effects of 1 root tip extract/ml concentration on the growth of *Culvularia* sp. was the highest (33% of the control plates) among the other fungal isolates. *Culvularia* sp.'s colony diameter reduced to 27, 11 and 2% of control plates at 2, 5 and 10 oat root tip concentrations, respectively. Because the growth of *D. victoriae* on agar medium was very slow, we did not include it to this experiment.

The reaction of the *Fusarium* isolates to oat root extract was different from each other. The inhibition rate decreased for all the isolates in all concentrations. Strong inhibition activity of the extract was visible for *F. oxysporum*, *F. moniliforme* and *F. acuminatum* (16%, 24% and 33%, respectively) at 10 root tip/ml concentration.

The mean of colony diameter of *F. poae* at 1 root tip/ml concentration was statistically similar to control plates, however, when the concentration increased in the medium, the inhibition rate proportionally increased, too. Similar results apply to *F. avenaceum*. The inhibition rate of the colony growth increased by concentration. However the colony diameters of *F. poae* and *F. avenaceum* were 48% and 52% of control, respectively, at the highest concentration 10 root tip/ml. These percentages are higher than those observed in other species isolates.

DISCUSSION

Avenacins, like many other saponins, have potent antifungal activity (Crombie et al., 1984; Osbour, 2001). In this research we aimed to assess antifungal effect of these compounds on germination, germ tube growth and colony development of fungi that had been isolated from cereal crops.

A dramatic decrease in the germination rates of the fungal conidia following oat root extract application was visible. *Culvularia* sp., *D. victoriae* and *F. moniliforme* were the most sensitive fungal isolates, whereas *F. avenaceum* and *F. poae* seemed to be the most resistant isolates against to oat root extract (Table 1). Similar results obtained from the effects of oat root extract on germ tube growth rates (Table 2). While reduction on germ tube growth was visible in all fungal conidia applicated with oat root extract, the most resistant isolates appeared to be *F. avenaceum* and *F. acuminatum*. Significant reduction in mycelial growth was also observed in most fungal isolates tested especially when the concentration of extract increased in PDA medium. The colony of *Culvularia* sp. did almost not extend beyond the fungal discs on the medium surface at 10 root tip/ml concentration. The colony diameter of *F. avenaceum* on PDA medium incorporated 10 root tip/ml concentration was 52% of the control plates (Table 3).

It has been reported that concentration of avenacin A-1 causing 50% (EC_{50}) inhibition of growth of *F. avenaceum* after 6 days were more than 200 μg/ml (Crombie et al., 1986b; Osbourn et al., 1991). The conidia and germ tubes of *F. avenaceum* also found to be resistant against to oat root extract in our experiment, too. Crombie et al. (Crombie et al., 1986b) also reported that the inhibition concentrations for *G. graminis* var. *tritici* and *G. graminis* var. *avenae* were 1.5-3 μg/ml and 5-80 μg/ml, respectively, depending on the isolates of the fungal varieties. In the same report, the concentrations of other avenacins (avenacin A-1, avenacin B-1 and avenacin B-2) required for EC_{50} inhibition of *F. avenaceum* were the same as avenacin A-1. Having used whole extract, we included four avenacins together in our assays. In general, some *Fusarium* isolates (e.g. *F. avenaceum*, *F. acuminatum* and *F. paoe*) may be evolved with the oat plants to compromise the effects of avenacins, *D. victoriae* and *Culvularia* sp. may, however, not develop similar mechanisms for the same purpose. The ability of a pathogen to infect oat roots is greatly dependent on the ability to degrade avenacins with an enzyme, so-called avenacinase, which especially deglucosylates avenacin to less toxic forms (Osbourn et al., 1994). Carter et al., (1999) reported that the majority of the avenacin-resistant fungi were able to degrade avenacin A-1.Therefore, avenacin A-1 is likely to influence the development of fungal

communities within oat roots Carter et al., (1999) and possibly also around the roots (Gibson and Krasnoff, 1999).

Plants evolved an impressive array of defensive tools. At the same time, pests and pathogens developed mechanisms to compromise plant resistance mechanisms. Natural resistance has several obvious advantages over the use of pesticides, such as genetic permanency, negligible cost once cultivars developed and high efficacy. The genes responsible from the avenacins biosynthesis may have potential for the development of improved disease resistance in cultivated cereals. Furthermore more plants should be screened for any form of pre-formed antimicrobial contents to be used for the same purpose.

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Table 1. The effect of the oat root extract on conidium germination of cereal pathogenic fungi at two different time occasions. The numbers show the percentage of germination on control or oat root extract application.

	Percentage of germination at two different times			
	6 hours after treatment		18 hours after treatment	
Isolates	Control	Oat Extract	Control	Oat Extract
Culvularia sp.	92.6±3.6	0.0	99.7±0.8	0.0
Drechlera victoriae	97.1±2.5	0.3±0.8	98.0±1.4	3.7±3.9
Fusarium poae	47.6±6.7	0.6±1.0	99.7±0.8	45.4±14.6
Fusarium oxysporum	0.3±0.8	0.0	100.0	19.4±9.2
Fusarium avenaceum	0.0	0.0	99.1±1.1	89.7±2.9
Fusarium acuminatum	0.0	0.0	97.4±1.9	21.7±4.6
Fusarium moniliforme	0.0	0.0	96.1±2.6	0.6±1.0

There is significant difference from control in means of conidium germination in all control and treatment groups according to Z (proportion) test ($P<0.01$). Two sets of data were compared to each other for each isolate.

Table 2. The germ tube growth of the fungal pathogens at 6 and 18 hours after treatment on plugs treated with oat root extract compared to those on control plugs. The numbers in parenthesis indicates percentage of control.

	Length of germ tube (mμ) at two specific time occasions			
	6 hours after treatment		18 hours after treatment	
Isolates	Control	Oat extract	Control	Oat ext
Culvularia	58.4±11.1	-	238.7±41.8	-
D. victoriae	130.6±15.9	-	631.8±151.2	-
Fusarium poae	41.6±1.9	-	151.3±18.6	40.21±6.52 (26.6%)
F. oxysporum	-	-	296.0±93.6	30.5±2.1 (10.3%)
F. avenaceum	-	-	139.7±12.4	98.0±17.6 (33.1%)
F. acuminatum	-	-	210.7±21.3	122.7±12.4 (58.2%)
F. moniliforme	-	-	97.1±5.1	-

There is statistically difference in means of germ tube growth of the isolate groups isolates in treated plugs compared to control plugs according to T test (*P<0.01*).

* The measurement of germ tubes was performed from only germinated conidia. Non-germinated conidia were ignored.

** No germ tube measurement was performed in plugs where fewer than ten germinated conidia observed.

Table 3. The effect of oat root extract on the colony growth of seven pathogenic fungi.

	Mean colony diameter (cm) and percentage control (in parenthesis)				
Isolates	Control	1 root tip/ml	2 root tip/ml	5 root tip/ml	10 root tip/ml
***Culvularia* sp.**	8.15±0.054a	2.70±0.056b (33)	2.21±0.053c (27)	0.92±0.055d (11)	0.19±0.059e (2)
Drechlera victoriae	-*	- -	- -	- -	- -
Fusarium poae	8.25±0.034a	7.95±0.114ab (96)	7.34±0.088c (89)	5.80±0.060d (70)	4.02±0.070e (48)
F. oxysporium	8.25±0.027a	6.36±0.123b (77)	4.83±0.058c (58)	4.00±0.150d (48)	1.35±0.054e (16)
F. avenaceum	8.27±0.021a	7.57±0.148b (92)	7.14±0.043c (86)	5.40±0.126d (65)	4.31±0.094e (52)
F. acuminatum	7.94±0.146a	6.32±0.215b (79)	5.01±0.133c (63)	4.27±0.100d (53)	2.65±0.092e (33)
F. moniliforme	8.21±0.041a	7.43±0.177b (90)	5.15±0.096c (63)	3.70±0.068d (45)	1.95±0.031e (24)

Different letters in the same line indicate statistically differences from the control according to Tukey test (*P<0.01*).

* No recorded data.