Turk J Agric For
29 (2005) 157-164

Avenacin A-1 Content of Some Local Oat Genotypes and the In Vitro Effect of Avenacins on Several Soil-Borne Fungal Pathogens of Cereals

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Received: 07.05.2004

Abstract: Avenacins are a mixture of 4 major (avenacin A-1, B-1, A-2 and B-2) autofluorescent compounds that are accumulated in the roots of oats (*Avena* spp.), especially root tips, and that have antimicrobial properties. In this research, we screened 189 genotypes of the family *Gramineae* for autofluorescence and also quantified 35 *Avena* genotypes for avenacin A-1 content, which is the most abundant and toxic avenacin type. Screening under UV transillumination proved that none of the species, except for *Avena* spp. accumulated avenacins in their roots. We aimed to find a genotype that lacks avenacin A-1 in order to investigate fungus-oat interaction in that particular interaction. The avenacin A-1 contents of *Avena* spp. varied between 4.7 and 6.5 mg g^{-1} fresh weight of root tips. Although there was significant statistical variation in means of avenacin A-1 contents, the search for a genotype that lacks avenacin A-1 was unsuccessful. A soil-borne fungi collection from cereals (*Culvularia* sp., *Drechslera victoriae, Rhizoctonia solani* (A-6 type), *Pythium ultimum, Fusarium culmorum, F. nivale, F. oxysporum* and *F. poae*) was also included briefly in this research to assess the antifungal activity of avenacins. According to the bioassay, all fungi exhibited inhibition zones around the oat root extract with the exception of *P. ultimum*. This result suggests that avenacins might contribute to fungal disease resistance and could be used for disease resistance breeding for some major root colonizing fungi.

Key Words: oat, avenacins, phytoanticipin, saponins, disease resistance, phytoalexins

Bazı Yerli Yulaf Çeşitlerinde Avenacin A-1 İçeriği ve Bu Maddenin Tahıllarda Görülen Toprak Kökenli Bazı Funguslara Etkisi

Özet: Avenasinler yulaf (*Avena* spp.) kök uçlarında bulunan ve antimikrobiyal etkiye sahip otofloresan dört ana kimyasal maddeden (avenasin A-1, B-1, A-2 ve B-2) meydana gelmiştir. Bu çalışmada, *Gramineae* familyasına ait toplam 189 genotipi, avenasin varlığı açısından taradık ve *Avena* spp.'e ait 35 genotipte en fazla bulunan ve en toksik olan avenasin A-1'in miktarını araştırdık. UV ışık altında yapılan taramada *Avena* spp. dışında hiç bir tür üyelerinde avenasine rastlanmamıştır. Bitki-patojen ilişkisini saptamak amacıyla, *Avena* türleri arasında avenacin A-1 üretmeyen bir genopin olup olmadığını araştırdık. Yapılan çalışmada genotipler arasında avenasin A-1 miktarı 4.7-6.5 mg g^{-1} yaş ağırlık arasında ölçülmüştür. Avenasin A-1 miktar bakımından *Avena* genotipleri arasında istatistiksel olarak bir fark görülmesine rağmen, avenacin A-1 üretmeyen bir genotipe rastlanmamıştır. Avenasinin toprak kökenli funguslara antimikrobiyal etkisini araştırmak amacıyla, tahıllardan izole edilen toprak kökenli bir kaç fungus türü de (*Culvularia* sp., *Drechslera victoriae, Rhizoctonia solani* (A-6 tip), *Pythium ultimum, Fusarium culmorum, F. nivale, F. oxysporum* ve *F. poae*) bu çalışmaya dahil edilmiştir. Biyoassay sonucuna göre, *P. ultimum* hariç, tüm funguslarda ekstrakt bölgesinde inhibisyon zonu oluştuğu tespit edilmiştir. Bu sonuçlar avenasinlerin fungal patojenlere karşı dayanıklılıkta bir katkı sağladığı ve kökleri kolonize eden bu funguslara karşı dayanıklılık ıslahında kullanılabileceğini göstermektedir.

Anahtar Sözcükler: yulaf, avenasinler, fitoantisipinler, saponinler, hastalıklara dayanıklılık, fitoaleksinler

Introduction

Most plants produce antimicrobial secondary products, and most of these compounds can inhibit the growth of microorganisms in vitro. Some of these antimicrobial compounds are activated to high concentrations after a pathogen infection or abiotic stress. These compounds are called phytoalexins. Some others, so-called phytoanticipins, may be present

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constitutively in healthy plants, and they act as chemical barriers against pathogen attack (Mansfield, 2000).

The majority of research so far has focused on phytoalexins, because these molecules are actively synthesized as part of the battery of induced defense responses associated with disease resistance. There are some reports that prove that phytoalexins not only accumulate at the site of infection but do so, following penetration by microorganisms, quickly enough and in sufficiently high concentrations to inhibit microbial growth (Lyon and Wood, 1975; Rossall et al., 1980; Long et al., 1985). However, examples of phytoalexin accumulation during compatible interactions have also been reported (Mert-Türk et al., 2003a, 2003b).

Pre-formed antimicrobial compounds, phytoanticipins, however, have attracted less attention, although they seem to be the first antimicrobial barrier to pathogens (Osbourn, 2001). Saponins are an important group of preformed plant secondary metabolites and they are known to protect plants against microbial attack. Tomato plants contain α-tomatine found in tomato leaves in approximately 1 mM concentrations. This concentration is enough to inhibit many non-pathogenic fungi. However, many fungi that have an economic impact, such as *Septoria lycopersici*, *Fusarium oxysporium* f. sp. *lycopersici* and *Alternaria solani*, produce enzymes that degrade saponin (Sandrock and Van Etten, 1998).

Avenacins are a family of 4 structurally related triterpenoid saponins, avenacin A-1, B-1, A-2 and B-2, found in roots of oat (*Avena* spp.) (Crombie and Crombie, 1986a). Both avenacin A-1 and B-1 fluoresce under UV irradiation. It has been reported that avenacins protect oat roots against *Gaeumannomyces graminis* var. *tritici* infection (Crombie and Crombie, 1986a, 1986b; Osbourn et al., 1991), but not against *G. graminis* var. *avenae*, as this fungal strain has an ability to degrade avenacins (Osbourn, 1994). Papadopoulou et al. (1999) generated mutants of *Avena strigosa* that do not produce avenacins, and found that these mutants were more susceptible than wild types to *G. graminis* var. *tritici* infection. Carter et al. (1999) obtained 161 fungal isolates from the surface sterilized roots of field-grown oats and wheat plants to investigate the nature of the root-colonizing fungi supported by these 2 cereals. They found that almost all the fungi isolated from the oat roots were avenacin A-1 resistant.

The aim of this research was to screen the members of the family *Gramineae* for avenacin contents in the hope of finding a genus that produces avenacins like oats, to quantify the content of avenacin A-1 (more abundant and fungitoxic than other avenacins) in local oat genotypes for any oat genotype that lacks avenacin A-1, and to investigate briefly the antifungal effects of avenacins in some soil-borne fungal pathogens of cereals obtained from diverse geographical areas of Turkey.

Materials and Methods

Plant Material. The cereal species screened for avenacin contents under UV illumination were *Avena* spp., *Aegilops* spp., *Triticum* spp., *Hordeum* spp. and *Secale* spp.

Screening of Some Genotypes of the Family *Gramineae* for Avenacin Contents. This research was carried out entirely at Çanakkale Onsekiz Mart University, Çanakkale. Avenacin A-1 fluoresces under UV illumination, a property that is extremely rare among saponins. We used this feature to exploit avenacin presence in *Gramineae* genotypes. Seeds were surface sterilized with 1% NaOCl for 3 min and rinsed in sterile distilled water 5 times. They were transferred into Petri dishes containing 2 layers of moist filter paper, then chilled at 4 °C in a refrigerator for 48 h and allowed to germinate at 20 °C for 2-4 days, depending on the species. The roots were approximately 2-5 cm long at that stage. Ten seedlings per genotype were then put in a UV cabin, and visualized under 302 nm UV light. The genotypes were classified according to whether their root tips fluoresced bright blue under UV illumination. The experiment was repeated on 3 different occasions.

Extraction of Avenacin A-1. The seeds were prepared and germinated as described above. The terminal 0.5 cm of the main root of each seedling was harvested with a razor blade, and root tips were transferred into an Eppendorf tube in batches of 10. Each batch of tips was ground in 100 µl of 50% methanol (Merck, Germany) using a micro pestle (Eppendorf, Germany). The material was pelleted by centrifugation and re-extracted until all fluorescent material had been recovered. The methanol suspension was vacuum-dried. Dry extract was stored at -20 °C until further evaluation. The experiment was repeated on 5 occasions.

Thin Layer Chromatography and Spectrophotometry. The residue was resuspended in 20 µl of absolute methanol and loaded onto 0.25 mm thick silica TLC plates (Merck, Germany). The plates were developed in a chromatography tank containing chloroform:methanol:water (13:6:1) as described by Osbourn et al. (1991). The chromatograms were air-dried for 10 min prior to UV illumination.

Quantification of Avenacin A-1. Of among the 4 avenacins, avenacin A-1 is the major and brightest under UV illumination. The R_f value of avenacin was 0.48, as described by Osbourn (1994). The bands corresponding to avenacin A-1 were scraped off and transferred into an Eppendorf tube containing 1 ml of 50% ethanol (Merck, Germany). The suspension was then vortexed for 1 min and centrifuged for 10 min at 14,000 r.p.m. The ethanol suspension was then transferred into a clean Eppendorf tube. The suspension was completed to 1 ml and quantified at 223 nm in spectrophotometry as described by Crombie and Crombie (1986a).

F test of ANOVA was performed for the analysis of data. The avenacin A-1 content of each genotype was, however, compared to that of the others using the Duncan test.

Fungal Pathogens. The fungi used in this experiment were provided by Prof. Dr. Erkol Demirci (Atatürk University, Erzurum) and specialist Filiz UÇAR (Bornova, İzmir) who had isolated them from cereals previously. The fungi used in this research were *Culvularia* sp., *Drechslera victoriae*, *Rhizoctonia solani* (A-6 type), *Pythium ultimum*, *Fusarium culmorum*, *F. nivale*, *F. oxysporum* and *F. poae.* A disk from each fungus isolate was placed in the center of Petri dishes containing PDA (Merck, Germany) and incubated until the colonies were approximately 3-5 cm in diameter.

Effects of Avenacin on Fungal Colony Development. The seeds were prepared and germinated as described above. The extraction process was performed under sterilized conditions. The oat root tips (0.5 cm) were removed with the aid of scissors and put into an Eppendorf tube. The root tips were mashed with micro pestles. They were vortexed for 1 min and centrifuged at 10,000 r.p.m. for 10 min. The oat root tip extract was removed into a clean Eppendorf tube. Approximately 5 µl of pure extract was dropped onto PDA, approximately 1 cm from the end of each developing colony. The control plates received 5 µl of sterile distilled water. The Petri dishes were kept in the dark at 20 °C in an incubator until the colony radial extended above the drop; this usually took 2-7 days, depending on the fungus. Six Petri dishes were used for each fungus isolate.

Results

Screening Roots of Several Genotypes for Fluorescence Material. After inspection of roots of germinated seedlings of 189 genotypes, including *Avena sativa*, *A. fatua*, *A. sterilis*, *A. byzantina*, *Aegilops biuncialis*, *A. columnaris*, *A. umbellulata*, *A. tauschii*, *A. vavilovii*, *A. speltiodes* var. *ligustica*, *A. speltoides* var. *aucheri*, *Triticum durum*, *T. aestivum*, *T. monoccocum* ssp. *urartu*, *T. monococcum* ssp. *boeticum*, *T. dicoccoides*, *T. monococcum* ssp. *monococcum*, *T. turgidum* ssp. *durum*, *T. aestivum* ssp. *aestivum*, *T. turgidum* ssp. *turgidum*, *T. turgidum* ssp. *dicoccoides*, *Hordeum vulgare*, *H. spontaneum, H. vulgare* ssp. *distichon*, *H. vulgare* ssp. *vulgare*, *H. vulgare* ssp. *spontaneum*, *H. spontaneum*, *Secale cereale* and *S. montanum* under UV illumination, only *Avena* genotypes in the collection were identified as having fluorescence (Figure 1). No fluorescent root material was found in any other genotypes tested.

Quantification of Avenacin A-1. The *Avena* genotypes used in quantification were *A. sativa* genotypes Antalya Serik, Coker 227, Coker 1214, Salem, CI5922, CI8357, Century, PA-7904-151, Ankara 802, Ankara 84, Yerli Ilgın-inan, Yerli Ilgın, Yerli beyaz yulaf, Apak, Cheokota, 8381 Cheokota, Y-1779, Y-330, Ankara 76, TR 39575, TR53291, TR53295, TR53298, TR63368 and TR35491; *A. byzantina* genotypes TR37417,

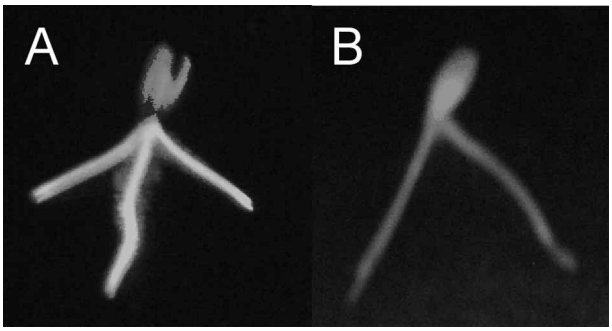

Figure 1. Fluorescenced root tips of *Avena sativa* (A) and *Triticum turgidum* ssp. *durum* (B) under UV transilluminator (302 nm).

TR37433, TR37 450, TR 37481 and TR 40756; *A. sterilis* genotypes TR42457, TR42464 and TUR00356; and *A. fatua* genotypes TR22594 and TR48231. All *Avena* genotypes fluoresced under UV illumination. Root extracts were fractionated on TLC and avenacin A-1 was visible under UV light as a bright bluish purple with an R_f value of 0.48 from the samples obtained from *Avena* genotypes only. Only a single genotype from other cereals including *Triticum durum*, *Hordeum vulgare*, *Secale serale* and *Aegilops biuncialis* developed on the TLC. However, no bands corresponded to avenacin A-1 on the TLC plates. This proved once more that these species do not produce detectable levels of avenacins in their root tips.

We quantified avenacin A-1 in *Avena* genotypes to find a genotype that lacks avenacin A-1 in order to calculate plant-pathogen interaction in these pathosystems. All genotypes tested, however, contained avenacin A-1. We found significant statistical differences in means of avenacin A-1 contents among the genotypes ($P < 0.01$); the amount varied between 4.7 and 6.5 mg g fw^{-1} root tips (Table 1). Table 2 shows the differences among the *Avena* genotypes according to the Duncan multiple comparison test.

Table 1. The quantification of avenacin A-1 in *Avena* genotypes. The experiment was repeated 5 times.

		Amount of Avenacin A-1 (mg g fw^{-1})			
Avena spp.	Genotypes	Min.	Max.	Mean	Std error
A. sativa	Antalya Serik	5.90	7.40	6.50	0.33
A. sativa	Coker 227	5.40	7.30	6.30	0.32
A. sativa	Coker 1214	5.60	6.80	6.10	0.20
A. sativa	Salem	5.70	6.60	6.10	0.16
A. sativa	CI5922	5.70	6.50	6.10	0.15
A. sativa	CI8357	5.90	6.90	6.30	0.21
A. sativa	Century	5.50	6.90	6.30	0.24
A. sativa	PA-7904-151	6.10	6.90	6.50	0.14
A. sativa	Ankara 802	5.90	6.60	6.20	0.12
A. sativa	Ankara 84	5.50	6.10	5.90	0.11
A. sativa	Yerli Ilgın-inan	5.60	6.50	6.10	0.16
A. sativa	Yerli Ilgın	6.00	6.60	6.20	0.10
A. sativa	Yerli beyaz yulaf	4.50	4.90	4.70	0.06
A. sativa	Apak	6.20	6.90	6.50	0.15
A. sativa	Cheokota	5.00	5.30	5.10	0.05
A. sativa	8381 Cheokota	5.00	5.90	6.30	0.16
A. sativa	Y-1779	5.10	5.60	5.40	0.10
A. sativa	Y-330	5.00	5.40	5.20	0.07
A. sativa	Ankara 76	5.70	6.00	5.90	0.05
A. sativa	TR 39575	6.10	6.60	6.40	0.08
A. sativa	TR 53291	5.00	5.30	5.10	0.05
A. sativa	TR 53295	4.70	5.10	4.90	0.06
A. sativa	TR 53298	5.80	6.20	6.00	0.07
A. sativa	TR 63368	5.90	6.20	6.10	0.05
A. byzantina	TR 35491	4.90	5.20	5.00	0.05
A. byzantina	TR 37417	6.10	6.40	6.30	0.05
A. byzantina	TR 37433	5.80	6.30	6.10	0.10
A. byzantina	TR 37 450	5.60	7.00	6.20	0.23
A. byzantina	TR 37481	5.10	7.10	6.30	0.40
A. sterilis	TR 40756	4.80	5.60	5.10	0.13
A. sterilis	TR 42457	4.10	5.10	4.70	0.21
A. sterilis	TR 42464	4.50	5.40	5.00	0.16
A. sterilis	TUR 00356	4.50	5.60	5.00	0.22
A. fatua	TR 22594	5.10	5.90	5.40	0.14
A. fatua	TR 48231	4.50	6.00	5.10	0.25

Table 2. The result of the Duncan multiple comparison test.

		Subset for alpha = 0.05				
Avena spp.	Genotype	1	2	3	4	5
A. sativa	Yerli beyaz yulaf	4.70				
A. sterilis	TR 42457	4.70				
A. sativa	TR 53295	4.90	4.90			
A. byzantina	TR 35491	5.00	5.00			
A. sterilis	TR 42464	5.00	5.00			
A. sterilis	TUR 00356	5.00	5.00			
A. sativa	Cheokota	5.10	5.10			
A. sativa	TR 53291	5.10	5.10			
A. sterilis	TR 40756	5.10	5.10			
A. fatua	TR 48231	5.10	5.10			
A. sativa	Y-330	5.20	5.20			
A. sativa	8381 Cheokota		5.30			
A. sativa	Y-1779		5.40	5.40		
A. fatua	TR 22594		5.40	5.40		
A. sativa	Ankara 84			5.90	5.90	
A. sativa	Ankara 76			5.90	5.90	
A. sativa	TR 53298				6.00	6.00
A. sativa	Coker 1214				6.10	6.10
A. sativa	CI5922				6.10	6.10
A. sativa	Yerli Ilgın-inan				6.10	6.10
A. sativa	TR 63368				6.10	6.10
A. byzantina	TR 37433				6.10	6.10
A. sativa	Salem				6.10	6.10
A. sativa	Ankara 802				6.20	6.20
A. sativa	Yerli Ilgın				6.20	6.20
A. byzantina	TR 37 450				6.20	6.20
A. sativa	Coker 227				6.30	6.30
A. byzantina	TR 37417				6.30	6.30
A. sativa	CI8357				6.30	6.30
A. sativa	Century				6.30	6.30
A. byzantina	TR 37481				6.30	6.30
A. sativa	TR 39575				6.40	6.40
A. sativa	Antalya Serik					6.50
A. sativa	PA-7904-151					6.50
A. sativa	Apak					6.50
	Significance (P)	0.09	0.09	0.06	0.10	0.10

Note: Means for groups in homogeneous subsets are displayed. There is not statistically significant difference among genotypes under the same subsets.

The highest level of avenacin A-1 was obtained from *A. sativa* genotypes Antalya Serik, PA-7904-151 and Apak. The amount of avenacin A-1 was 6.5 mg g fw^{-1} in root tips, while the minimum amount of avenacin A-1 was observed in the samples extracted from the *A. sterilis* genotype TR 42457. This genotype accumulated 4.7 mg g fw^{-1} in root tips.

The Effects of Avenacins on the Colony Growth of Cereal Root Pathogens. The fungi were allowed to grow in Petri dishes containing PDA until their colonies reached 3-4 cm in diameter and 5 µl of oat root tip extract was placed carefully on the PDA, approximately 1 cm from the edge of each colony. This work was carried out to illustrate that avenacins can indeed inhibit a broad range of soil-borne fungal pathogens.

Most of the fungi tested including *Culvularia* sp., *Drechslera victoriae*, *Rhizoctonia solani* (A-6 type), *F. culmorum*, *F. nivale*, *F. oxysporum* and *F. poae* exhibited inhibition zones where the drops were initially placed (Figure 2). The inhibition rate of *D. victoriae* around the root tip extract was smaller compared to that of the others. *P. ultimum* seemed to be quite resistant to the oat root extract. No inhibition was observed around the wheat extract or sterile distilled water.

Discussion

One hundred eighty cereal genotypes were screened for autofluorescence to determine whether any of them, other than oats, contain avenacins. Among the genotypes tested, only *Avena* spp. seemed to produce avenacins. The aim of this part of the research was to investigate any genotype containing avenacins, like oats, and to compare their resistance against cereal fungal pathogens and perhaps to use it for breeding into wheat. It is known that oats are more resistant to root colonizing fungi than wheat. Wheat is the most grown and consumed cereal worldwide as well as in Turkey, and it is affected by diverse soil-borne fungal pathogens. Breeding the genes responsible for avenacin biosynthesis into wheat would be worthwhile. Unfortunately we did not find any genotype that produces avenacins other than oats. However, we will endeavor to screen more cereals, mostly from wild-type genotypes, in the future, for this purpose.

Our second goal in this research was to find an oat genotype that lacks avenacin A-1. Therefore we screened 35 genotypes belonging to *Avena sativa*, *A. sterilis*, *A. fatua* and *A. byzantina*. The roots of the genotypes seemed to fluoresce the same under UV light, and we did not observe any significant differences among them, in means of autofluorescence in roots. The quantification process, however, revealed statistical differences among the genotypes. However, the attempt to find an avenacin-deficient genotype was unsuccessful.

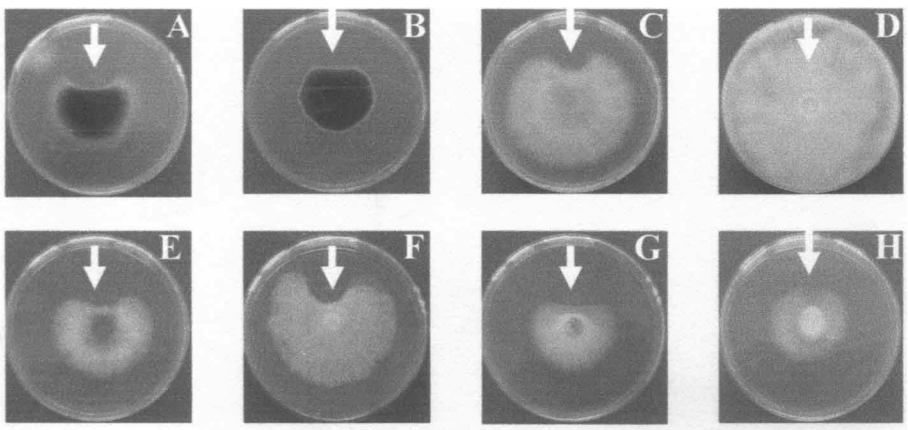

Figure 2. The inhibition zones around oat root extract drops from several soil-borne fungal pathogens of cereals. In order to assess the antifungal effect of the oat root extract, 20 µg of pure extract was dropped approximately 1 cm apart from the developing colony of each fungus. An inhibition zone was observed where the drop was placed in all fungi tested, except *Pythium ultimum*. (A) *Culvularia* sp; (B) *Drechslera victoriae*; (C) *Rhizoctonia solani* (A-6 type); (D) *P. ultimum*; (E) *Fusarium culmorum*; (F) *F. nivale*; (G) *F. oxysporum*; (H) *F. poae.*

Evidence that avenacins are likely to protect oat roots against fungal attack is also emerging from studies of oat genotypes that lack avenacins. Although there is little natural variation in avenacin content among *Avena* genotypes, one diploid oat species (*A. longiglumis*) that does not contain avenacins has been identified previously (Osbourn et al., 1994). This species is susceptible to infection by *G. graminis* var. *tritici*, which is avenacin sensitive and is normally unable to infect oats. Our goal in that special part of the research was also similar, but the target organisms would be fungi other than *G. graminis* var. *tritici*, such as *Culvularia* spp., *Drechslera victoriae*, *Rhizoctonia solani*, *P. ultimum*, *F. culmorum*, *F. nivale*, *F. oxysporum* and *F. poae*. Efforts to find avenacin-deficient oat genotype will continue.

The term "phytoanticipin" is proposed to distinguish preformed antimicrobial compounds from phytoalexins (Van Etten et al., 1994). They include some saponins (e.g., avenacins and α-tomatine) that are stored in plant cells and are readily activated by the enzymes that are always present in healthy plants. The major mechanism of the antifungal activity of saponins is due to their ability to complex with sterols in fungal membranes and to cause a loss of membrane integrity, although the precise mechanism is not fully understood (Nishikawa et al., 1984; Keukens et al., 1995).

We tested several cereal root colonizing fungi (*Culvularia* sp., *Drechslera victoriae*, *Rhizoctonia solani* (A-6 type), *P. ultimum*, *F. culmorum*, *F. nivale*, *F. oxysporum* and *F. poae*) in vitro to see whether oat root tip extract would inhibit growth. We observed that all fungi, except for *P. ultimum,* exhibited an inhibition zone around the extract spot. The mechanism used by avenacins is to complex with sterols in the fungal membrane (Arneson and Durbin, 1968). *P. ultimum* did not exhibit an inhibition zone around the extract drop. The resistance of *P. ultimum* to avenacin could be associated with the lack of membrane sterols in this fungus.

Although it is thought that fungi that infect saponin-containing plants are often more tolerant to host plant saponins in vitro than are non-pathogens of that particular plant, suggesting that saponin resistance is a prerequisite for infection (Morrissey and Osbourn, 1999), we observed inhibition of cereal fungi by the avenacins. Papadopoulou et al. (1999) reported at least 7 genes responsible for the biosynthesis of avenacin A-1, using artificial mutagenesis in the oat species *A. strigosa*, which normally produces an abundant amount of avenacin A-1. This fact limits the possibility of transferring the genes into wheat. However, as phytoanticipins are the first biochemical barrier against fungal pathogens, it is worthwhile investigating any pre formed antimicrobial compounds in other plants for breeding or transferring into economically important crops in the future.

Acknowledgments

We thank the Scientific and Technical Research Council of Turkey (TÜBİTAK, project no: TOGTAG-2979) for its financial support. We thank Prof. Dr. Erkol Demirci (Atatürk Üniversitesi) and specialist Filiz Uçar (Bornova Crop Protection Institute) for providing the collection of soil-borne fungal pathogens of cereals. The seed used in this project was kindly provided by the Bahri Dağdaş International Cereal Research Center, Konya, the Ege Agricultural Research Institute (Gene Bank), İzmir, and the Crop Plants Central Research Institute, Ankara.

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