

SHORT COMMUNICATION

DIFFERENTIAL ACCUMULATION OF ANTIFUNGAL COMPOUNDS DURING ONION SEED GERMINATION AND UPON INFECTION BY *ASPERGILLUS NIGER* VAN TIEGHEMN. Özer¹, G. Chilosi² and P. Magro²¹Department of Plant Protection, Agriculture Faculty, Trakya University, 59030 Tekirdag, Turkey²Dipartimento di Protezione delle Piante, Università degli Studi della Tuscia, Via S. Camillo De Lellis, I-01100 Viterbo, Italy

SUMMARY

Extracts from germinating seeds of two onion cultivars differing in susceptibility were fractionated by TLC for their content of antifungal compounds following inoculation with two *Aspergillus niger* isolates characterised by different degrees of virulence. Three antifungal fractions absorbing ultraviolet light were detected from healthy germinating seeds of the less susceptible cv. 'Rossa Savonese' whereas no fractions were observed from tissues of the more susceptible cv. 'Bianca Agostana'. Inoculation with both isolates resulted in the induction of several fungitoxic compounds. The antifungal profile of 'Rossa Savonese' appeared to be more complex, suggesting a possible causal relationship between antifungal compound profile and resistance. Detected onion fractions exerted antifungal activity against both isolates. Our results suggest a role of fluorescent compounds in resistance to *A. niger* during onion seed germination, when the tissues are particularly exposed to attack by seed- and soilborne pathogens.

RIASSUNTO

ACCUMULAZIONE DIFFERENZIALE DI COMPOSTI ANTIFUNGINI DURANTE LA GERMINAZIONE DEL SEME DI CIPOLLA ED A SEGUITO DI INFEZIONE CON *ASPERGILLUS NIGER* VAN TIEGHEM. È stato verificato, mediante TLC, il profilo dei composti antifungini presenti nei semi germinanti di due varietà di cipolla caratterizzate da diversa suscettibilità ad *Aspergillus niger*. Nei germinelli della cv. "Rossa Savonese" (meno suscettibile) sono stati osservati tre composti antifungini fluorescenti in luce ultravioletta che non erano presenti nella cv. "Bianca Agostana" (più suscettibile). L'inoculazione con due isolati di *A. niger* ha indotto diversi composti con attività fungitossica. Il profilo antifungino di "Rossa Savonese" è risultato essere più complesso, indicando una

possibile relazione tra composti antifungini e resistenza. I composti separati da semi di cipolla hanno presentato attività inibitoria nei confronti di entrambi gli isolati. I risultati ottenuti indicano che questi composti possono avere un ruolo nella resistenza durante la fase di germinazione, allorché i tessuti sono particolarmente esposti al potenziale attacco di patogeni.

Key words: antifungal activity, *Aspergillus niger*, onion, resistance.

Water-soluble phenols and flavones are important antifungal compounds expressed in red or yellow pigmented onion bulb scales (Walker and Stahmann, 1955). These compounds, diffusing into infection droplets, prevent spore germination and penetration of potential pathogens, and appear to constitute an important resistance factor. In addition, it has been observed that the inner fleshy scales of white onion are more resistant to infection caused by *Botrytis allii* than those from the red onion; this is associated with more rapid accumulation of phenolic compounds in the early stages of infection and faster activation of peroxidase (Magro *et al.*, 1983). Two cyclopentane phytoalexins, termed tsibulins, have been found to differentially accumulated in onion bulb scales during resistant and susceptible reactions (Dmitriev *et al.*, 1990). Despite these extensive studies on the role of antimicrobial compounds effective in resistance mechanisms in onion bulbs, to our knowledge no information is available on the involvement of these compounds in the protection of resting onion seeds which lack an active defence system, and during early germination, when the tissues are particularly exposed to attack by soilborne pathogens.

Aspergillus niger van Tieghem is a seed- and soilborne pathogen that causes black mould of onion, one of the most serious diseases caused by this fungus. Contaminated seeds and soil appear to constitute the principal inoculum source (Hayden and Maude, 1992; Hayden *et al.*, 1994; Köycü and Özer, 1997). We have previously reported that *A. niger* isolates differ in virulence and, both qualitatively and quantitatively, in polygalacturonase production (Özer *et al.*, 1999). This suggests

Corresponding author: P. Magro
Fax: +39.0761.357473
E-mail: magro@unitus.it

that particular polygalacturonases may contribute to virulence during onion seed colonisation. In addition, different degrees of susceptibility among onion cultivars were shown to occur.

The aim of this study was to investigate the expression of antifungal compounds during early onion seed germination and their influence on infection by *A. niger* isolates An6 (more virulent) and An14 (less virulent).

Onion (*Allium cepa* L.) seeds of the red cultivar 'Rossa Savonese' (RS) less susceptible to this pathogen and the white cultivar 'Bianca Agostana' (BA) more susceptible, were inoculated with *A. niger* isolates and incubated for 24 h at 25°C following the procedure of Özer *et al.* (1999). One g of seed for each cultivar was used.

Tissues from inoculated and healthy germinating seeds were separately collected and homogenised in 95% ethanol using 2 ml g⁻¹ seed. After 24 hours extraction at 25°C in the dark, mixtures were filter-sterilised through a 0.22 µm membrane (Millipore). Similarly, ethanolic extracts from healthy germinating onion seeds and *A. niger* spores were prepared as controls. The extracts from germinating seeds (50 µl each sample) were separated by thin-layer chromatography (TLC) as described by Sutton and Deverall (1984) on silica gel (TLC plates 60 F₂₅₄, Merck) using chloroform:methanol (10:1) as the developing solvent. The different compounds separated by chromatography were visualised by UV fluorescence when examined in a Spectro-line Model cc-80 cabinet at long (365 nm) and short (254 nm) wavelengths. The fractions corresponding to different compounds were marked and their Rf values calculated. The separated fractions, which were not found from fungal spores, were isolated by extraction with 0.5 ml ethanol (Merck), added to the silica gel scraped from the TLC plate at a location corresponding to each compound. Silica gel was removed by centrifugation at 12,000 g for 20 min. Spectral analysis of each band isolated by TLC was carried out in a Perkin-Elmer Lambda 15 UV/VIS spectrophotometer. The absorbance spectra of the different compounds were obtained between 200 and 400 nm and compared for a preliminary characterisation of compounds in healthy and infected germinating onion seeds.

Fractions from TLC plates, dissolved in ethanol, were allowed to dry in a laminar flow hood and the residues were dissolved in 500 µl sterile distilled water to determine their effects on germination and germ tube length of *A. niger* spores. These were bioassayed for antifungal activity as described by Stewart and Mansfield (1985) with some modifications. The assay mixtures were prepared by mixing the fractions and spore suspensions (10⁶ spores ml⁻¹ distilled water) to obtain a rate of 75% for each fraction and a final spore

concentration of 250,000 spores ml⁻¹. To each mixture 10 µl sucrose (1%) were added to stimulate spore germination. In the controls, sterile water was used instead of the fraction solution. Mixtures were placed on microscope slides and incubated for 24 hours at 30°C in the dark. Germination was then stopped by adding 10 ml lactophenol, and germ tube elongation and percentage of germination were evaluated. Inhibition was expressed as a percentage of the control.

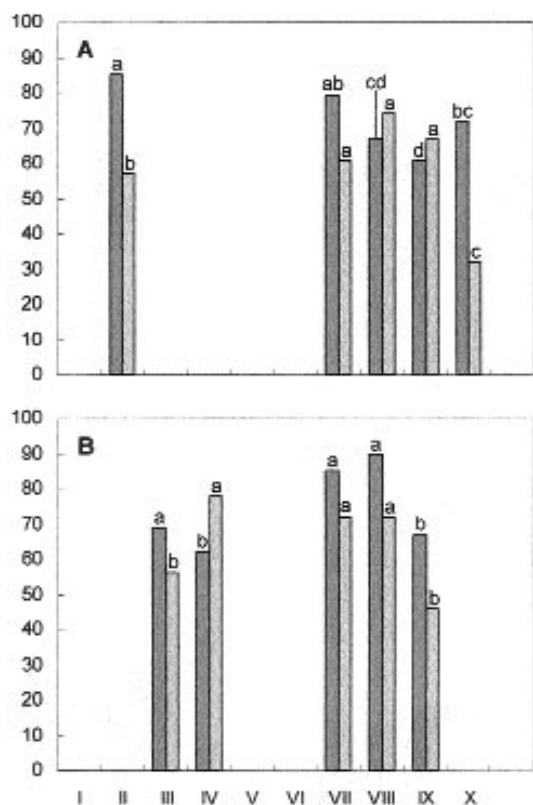
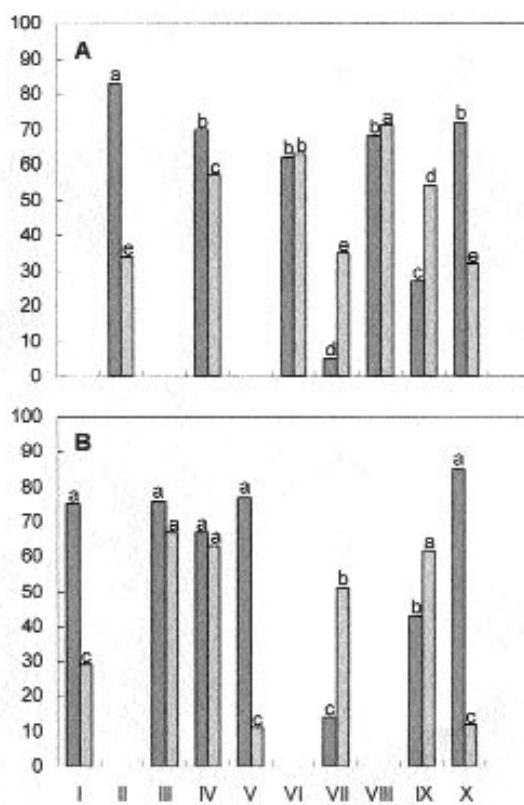
Following seed inoculation, the extracts from control and inoculated tissues of both cultivars contained appreciable amounts of compounds absorbing ultraviolet light. The fractions separated by TLC were grouped according to their increasing Rf values, ranging from 0.06 to 0.90 (Table 1). Three fractions, II (Rf 0.09, λ_{max} 224.0), IX (Rf 0.83, λ_{max} 203.1) and X (Rf 0.90, λ_{max} 202.5) were detected from control germinating seeds of RS whereas no bands were observed from corresponding tissues of BA. When RS was inoculated with the more virulent isolate An6, five bands were detected including fractions II, IX, X and two new bands, VII (Rf 0.67, λ_{max} 228.9) and VIII (Rf 0.78, λ_{max} 228.1). On germinating RS seeds infected with An14 fractions II, IV (Rf 0.28, λ_{max} 230.4), VI (Rf 0.54, λ_{max} 202.2), VII-X were observed. On BA infected with An14 fractions I (Rf 0.06, λ_{max} 204.0), III (Rf 0.11, λ_{max} 203.2), IV, V (Rf 0.47, λ_{max} 226.3), VII, IX, X were detected. When BA was inoculated with An6 five bands were observed (III, IV, VII, VIII, IX). The compounds were measured by their maximum absorbance at λ_{max}. Amounts of antifungal compounds constitutively expressed in RS increased in tissues infected with both isolates. The maximum absorbance values (abs max) of induced compounds varied, indicating possible differences in amount and chemical composition.

The ability of the detected compounds to inhibit growth of two *A. niger* isolates was examined. The compounds tested from control tissues (data not shown) and those inoculated with An6 (Fig. 1) and An14 (Fig. 2) exerted antifungal activity determined as inhibition of both germination and hyphal growth. Inhibition of hyphal growth and branching occurred following the exposure of spores to the separated compounds.

The results of the present study indicate that different antifungal compounds were expressed in germinating onion seeds and were differentially induced upon infection with *A. niger*. Susceptibility of the two onion genotypes could be a consequence of the accumulation of particular antifungal compounds. Three preformed antifungal compounds, fractions II, IX, X were expressed in germinating seeds of RS but not BA. In addition, during *A. niger* infection antifungal compounds were found to be differentially induced in BA and RS.

Table 1. Antifungal compounds separated by TLC from germinating seeds of onion cultivars 'Rossa Savonese' and 'Bianca Agostana' after inoculation with *A. niger* isolates An6 and An14.

Fraction number	Rf value	An6				An14				Control			
		Rossa Savonese		Bianca Agostana		Rossa Savonese		Bianca Agostana		Rossa Savonese		Bianca Agostana	
		λ_{\max} nm	abs ¹	λ_{\max} nm	abs	λ_{\max} nm	abs	λ_{\max} nm	abs	λ_{\max} nm	abs	λ_{\max} nm	abs
I	0.06	–	–	–	–	–	–	204.0	0.262	–	–	–	–
II	0.09	224.0	0.045	–	–	224.0	0.062	–	–	224.0	0.036	–	–
III	0.11	–	–	203.2	0.361	–	–	203.2	0.367	–	–	–	–
IV	0.28	–	–	232.0	0.261	230.4	0.195	232.0	0.140	–	–	–	–
V	0.47	–	–	–	–	–	–	226.3	0.267	–	–	–	–
VI	0.54	–	–	–	–	202.2	0.126	–	–	–	–	–	–
VII	0.67	228.9	0.203	228.9	0.255	228.9	0.268	228.9	0.285	–	–	–	–
VIII	0.78	228.1	0.436	228.1	0.219	228.1	0.426	–	–	–	–	–	–
IX	0.83	204.0	0.835	204.0	0.873	204.0	0.875	204.0	0.883	203.1	0.295	–	–
X	0.90	202.9	0.377	–	–	202.8	0.203	202.8	0.268	202.5	0.081	–	–

¹ absorbance value at λ_{\max} .**Fig. 1.** Percentage of spore germination inhibition (■) and hyphal growth inhibition (□) of *A. niger* isolate An6 exerted by fluorescent fractions from germinating onion seeds cultivar 'Rossa Savonese' (less susceptible) (A) and 'Bianca Agostana' (more susceptible) (B) inoculated with isolate An6. Each set of columns bearing different letters are significantly different according to the Chi-square test (germination inhibition) and T-test (hyphal growth inhibition) ($P < 0.05$).**Fig. 2.** Data as in Fig. 1 but referring to *A. niger* isolate An14.

Fractions II, VI, VIII and X may represent components that confer additional resistance on RS during early seed germination when the seedling is exposed to *A. niger* attack. The less virulent isolate An14 determined the induction of a more complex antifungal profile than An6, represented by fractions I and V on BA, fraction VI on RS.

The components detected exerted antifungal activity against the isolates considered. The isolates appeared to differ in sensitivity when assayed for antifungal activity with fractions characterised by same Rf. Thus, the fungitoxicity of fractions considered may contribute to the action of different resistance factors that limit fungal invasion in onion such as the defence-related proteins previously described in onion (Favaron *et al.*, 1993; Cammue *et al.*, 1995). As reported for different host-pathogen interactions (Nicholson and Hammerschmidt, 1992; León *et al.*, 1993) antifungal compounds such as phenols can be considered as important natural compounds involved in active resistance of onion seeds; thus, further experiments are needed for a complete chemical characterisation of the antifungal fractions separated from onion seeds.

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REFERENCES

- Cammue B.P.A., Thevissen K., Hendriks M., Eggermont K., Goderis I.J., Proost P., Van Damme J., Osborn R.W., Guerbet F., Kader J.C., Broekaert W.F., 1995. A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. *Plant Physiology* **109**: 445-455.
- Dmitriev A.P., Tverskoy L.A., Kozlovsky A.G., Grodzinsky D.M., 1990. Phytoalexins from onion and their role in disease resistance. *Physiological and Molecular Plant Pathology* **37**: 235-244.
- Favaron F., Castiglioni C., Di Lenna P., 1993. Inhibition of some rot fungi polygalacturonases by *Allium cepa* L. and *Allium porrum* L. extracts. *Journal of Phytopathology* **139**: 201-206.
- Hayden N.J., Maude R.B., 1992. The role of seed-borne *Aspergillus niger* in transmission of black mould of onion. *Plant Pathology* **41**: 573-581.
- Hayden N.J., Maude R.B., Proctor F.J., 1994. Studies on the biology of black mould (*Aspergillus niger*) on temperate and tropical onions 1. A comparison of sources of the disease in temperate and tropical field crops. *Plant Pathology* **43**: 562-569.
- Köycü N.D., Özer N., 1997. Determination of seed-borne fungi in onion and their transmission to onion sets. *Phytoparasitica* **25**: 25-31.
- León J., Lawton L., Raskin I., 1993. Phenolic acids and the biosynthesis of defence-related compounds. In: Schultz J., Raskin I. (eds.). Plant signals in interactions with other organisms. Current topics in plant physiology: an American society of plant physiologists series, Vol. 11, pp. 65-78. Rockville U.S.A.
- Magro P., Marciano P., Di Lenna P., 1983. Metabolic alteration induced by *Botrytis allii* in two onion cultivars with different resistances to neck rot. *Plant Pathology* **32**: 295-302.
- Nicholson R.L., Hammerschmidt R., 1992. Phenolic compound and their role in disease resistance. *Annual Review of Phytopathology* **30**: 369-389.
- Özer N., Chilosi G., Magro P., 1999. Polygalacturonase production by *Aspergillus niger*: expression in onion seeds and possible involvement in virulence. *Journal of Plant Pathology* **81**: 17-24.
- Stewart A., Mansfield J.W., 1985. The composition of wall alterations and appositions (reaction material) and their role in the resistance of onion bulb scale epidermis to colonization by *Botrytis allii*. *Plant Pathology* **34**: 25-37.
- Sutton D.C., Deverall B.J., 1984. Phytoalexin accumulation during infection of bean and soybean by ascospores and mycelium of *Sclerotinia sclerotiorum*. *Plant Pathology* **33**: 377-383.
- Walker J.C., Stahmann M.A., 1955. Chemical nature of disease resistance in plants. *Annual Review of Plant Physiology* **6**: 351-364.

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