Resistance to Fusarium Basal Rot of Onion in Greenhouse and Field and Associated Expression of Antifungal Compounds

N. Özer,*[,1] N.D. Köycü,[1] G. Chilosi[2] and P. Magro[2]

Greenhouse and field evaluations of onion for resistance to Fusarium basal rot caused by *Fusarium oxysporum* f.sp. *cepae* were conducted on cultivars 'Akgün 12' and 'Rossa Savonese' previously described as resistant at the seedling stage. In the greenhouse experiments inoculations were carried out on seeds or soil; in the field experiments evaluation was performed on onion sets from plants grown in naturally infested soils. Akgün 12 and to a lesser extent Rossa Savonese were resistant to the disease at the bulb stage in all experiments. Results were also consistent with those obtained from a previous screening at the seedling stage. Onion sets were also extracted and fractionated by thin layer chromatography to determine their content of antifungal compounds. Extracts were characterized by the expression of distinct antifungal components, which may be involved in resistance to the pathogen.
KEY WORDS: *Allium cepa* L.; antifungal compounds; *Fusarium oxysporum* f.sp. *cepae*; greenhouse tests; field experiments; resistance.

INTRODUCTION

Fusarium basal rot caused by *Fusarium oxysporum* Schlechtend.: Fr. f.sp. *cepae* (Hans.) Snyder & Hansen (*FOC*) represents one of the most serious seed- and soilborne diseases of onion (*Allium cepa* L.) worldwide (1,4,5,8,17). Resistant onion cultivars offer one of the best nonchemical means for controlling this fungal pathogen. Resistance to Fusarium basal rot has been reported in several onion cultivars and lines (1,3,5,7,9,10,12,16,17,19,20). However, there is still interest in obtaining onion genotypes with higher resistance to *FOC* than the cultivars released to date. Improvements in screening procedures and in the understanding of the mechanisms governing resistance to *FOC* represent important tools in the development of resistant onion genotypes. We have recently developed a screening procedure for resistance to *FOC* at the seedling stage that could predict the performance in the field under a natural disease epidemic. We have also found that the constitutive expression and the differential induction of antifungal compounds absorbing UV light are important factors regulating the resistance to *FOC* infection in onion during the early stage of germination (15). The objectives of this study were to evaluate during the onion set development under greenhouse and field conditions (i) the resistance level of two onion cultivars, 'Akgün 12' and 'Rossa Savonese', previously

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[1]Dept. of Plant Protection, Faculty of Agriculture, University of Trakya, 59030 Tekirdağ, Turkey. *Corresponding author [e-mail nurayozer@hotmail.com].
[2]Dept. of Plant Protection, Faculty of Agriculture, University of Tuscia, 01100, Viterbo, Italy.

found to be resistant in screening at the seedling stage (15); and (ii) the expression and the induction of antifungal ultraviolet (UV) light-absorbing compounds from set tissues of these cultivars.

MATERIALS AND METHODS

Inoculation procedures The experiment was conducted under greenhouse and field conditions with two cultivars (Akgün 12 and Rossa Savonese) resistant to Fusarium basal rot during seedling development (15). The yellow cv. Akgün 12 was obtained from Yalova Horticultural Research Institute (Turkey) and the red cv. Rossa Savonese from a local market.

The greenhouse experiments consisted of two different inoculation procedures using two cultivars. Inoculations were carried out using FOC8 and FOC16 isolates, respectively, for the seed and soil inoculations. These isolates had been obtained previously from onion seeds (8) and were chosen since FOC8 was found to be the more aggressive by seed inoculation, and FOC16 the more aggressive by the soil inoculation (14). The former inoculation procedure compared the two cultivars for resistance to basal rot following seed inoculation with the FOC8 isolate. Seeds were surface-sterilized by immersion in sodium hypochlorite solution (1%) for 3 min, rinsed for 5 min in sterile distilled water, and dried on sterile filter paper. These seeds were inoculated by soaking in a suspension of conidia containing 1×10^6 spores ml^{-1} for 1 h. In the latter inoculation procedure the cultivars were compared after soil inoculation. In this case, a sterilized soil mixture of 1/3 field soil + 1/3 manure + 1/3 sand was inoculated with FOC16. A conidial suspension (1×10^6 spores ml^{-1}) was added to a sterilized soil mixture at the rate 1:2.5, mixed thoroughly, dried and kept in a greenhouse. Prior to inoculation, seeds were surface-sterilized as described above. Non-inoculated seeds and soil were used for controls. For each procedure, four replicates of 50 seeds were sown in plastic pots (15×30×12cm). After sowing, pots were maintained in the greenhouse.

Two onion locations in Tekirdağ Province which were known to be naturally infested with *FOC*, were selected for the field experiment. The seeds of each cultivar, which were not contaminated with potential pathogens (8), were used in these experiments. To check for the absence of these pathogens, the seeds were aseptically placed on Potato Dextrose Agar and on sterile filter paper (Blotter method) moistened with sterile distilled water in 9 cm Petri dishes and incubated at 20°C. Sixteen replicates of 25 seeds were used for each media. None of these pathogens grew under these conditions. Both field experiments were arranged in a completely randomized design with four replicates of 1 m^2 plot size. Onion seeds were sown in each plot, using 5 g seeds m^{-2}.

Daily average temperatures were 23° and 14.7°C in the greenhouse and field experiments, respectively, and the daily average relative humidity ranged between 71% and 55%.

Disease incidence Four months after sowing, bulbs of 25 plants from each replicate in the pot and field experiments were collected at random, the base part of the onion set was cut horizontally, and the stem plate was examined macroscopically for brown discoloration. Tissues dissected from stem plates of each set were cultured on PDA and disease incidence was recorded as the percentage of basal rotted sets caused by *F. oxysporum* f.sp. *cepae*. The data were subjected to analysis of variance and mean ranking values were tested using the Tukey-Kramer Test (*P*=0.05).

Extraction of UV-absorbing compounds from onion sets and antifungal activity Onion sets developing from *FOC*-inoculated and control seeds in the pots and from the seeds in naturally infested soil were homogenized and separated by thin-layer chromatography (TLC) as described previously (18) on silica gel (TLC plates 60 F_{254}, Merck, KGaA, Darmstadt, Germany) using chloroform: methanol (10:1) as the developing solvent. Additionally, ethanolic samples of pure 0.1 M catechol (Sigma Chemical Co., St. Louis, IL, USA) and 0.1 M protocatechuic acid (Sigma) were co-chromatographed as standards. The different compounds were visualized by their fluorescence when examined in a UV fluorescence analysis cabinet (Spectroline Model CC-80, Spectronics Corporation, Westbury, NY, USA) at long (365 nm) and short (254 nm) wavelength after development. 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. The spectral study of each band isolated by TLC was carried out in a PerkinElmer Lambda 15 UV/VIS spectrophotometer (PerkinElmer, Shelton, CT, USA). The absorbance spectra of the different compounds were obtained between 200 and 400 nm and compared for a preliminary characterization of compounds in healthy and infected onion sets.

Antifungal activity of each separated fraction was measured as described previously (13) by mixing the fractions and spore suspensions to obtain a rate of 75% for each fraction and a final spore concentration of 2.5×10^5 spores ml^{-1}. The mixtures were placed on microscope slides and incubated for 24 h at 30°C in the dark, after which percent germination was determined for four replicates of each treatment. Fungal inhibition was expressed as IA_{50} (absorbance value required for 50% inhibition of spore germination).

RESULTS

Disease incidence The percentage of rotted onion sets was recorded along with the occurrence of *F. oxysporum* f.sp. *cepae* in greenhouse and field screenings (Table 1). From rotted sets the constant occurrence of *FOC* was found, thus indicating that infection was due to this pathogen. No *FOC* infection was recorded from healthy material. Akgün 12 was found to be highly resistant to the pathogen in both greenhouse and field experiments, with only a limited number of rotted sets. In both experiments the level of resistance displayed by Rossa Savonese was significantly lower than that of Akgün 12. However, the sets of Rossa Savonese exhibited a lower incidence of basal rot in the field experiment than in the greenhouse experiment.

Antifungal compounds Following seed and soil inoculation in greenhouse experiments, different compounds absorbing UV light were found to be present in the extracts from inoculated and control tissues of both cultivars (Table 2). The fractions separated by TLC were grouped according to their increasing R_f values, ranging from 0.07 to 0.76. Due to the similarity of each of the R_fs to those described during a screening at the seedling stage (15), the same nomenclature was used apart from the novel fraction I_a. Fractions I_a (R_f 0.07), II (R_f 0.17), IV (R_f 0.30) and IX (R_f 0.63) were found to be constitutively expressed from control onion sets of both cultivars. On sets of both cultivars raised from seed inoculation, I_a, II and X (R_f 0.76) bands were detected. On onion sets of Akgün 12 from inoculated seeds, a distinct band was also observed (Fraction VI, R_f 0.41). Sets of both cultivars raised from soil inoculation showed the expression of a number of fractions that were not found

TABLE 1. Basal rot incidence on sets of two onion cultivars following artificial inoculations of seeds or soil with *Fusarium oxysporum* f.sp. *cepae* isolates FOC8 and FOC16, respectively, in greenhouse experiments and from field experiments conducted in naturally infected fields at two different locations

Cultivar	Greenhouse experiment		Field experiment	
	Inoculation procedure (isolate)	Basal rot incidence (%)	Location	Basal rot incidence (%)
Akgün 12	Seed (FOC8)	4.0 b[z]	I	14.0 bc
	Soil (FOC16)	15.0 b	II	10.0 c
Rossa Savonese	Seed (FOC8)	57.0 a	I	35.0 ab
	Soil (FOC16)	71.0 a	II	39.0 a

[z]Within column, means followed by a common letter do not differ significantly (*P*=0.05) according to the Tukey-Kramer test.

TABLE 2. Compounds absorbing UV light, separated by thin layer chromatography from sets of two onion cultivars raised from seeds or soil inoculated with *Fusarium oxysporum* f.sp. *cepae* isolates FOC8 and FOC16, respectively

Fraction	R_f	Isolate	Cultivar			
			Akgün 12		Rossa Savonese	
			λmax (nm)	*A*max[z]	λmax (nm)	*A*max
		FOC8	203.8	0.670	253.1	0.335
I_a	0.07	FOC16	-	-	-	-
		Control[y]	204.4	1.022	203.2	0.751
		FOC8	-	-	-	-
I	0.10	FOC16	204.4	0.809	202.6	0.484
		Control	-	-	-	-
		FOC8	202.8	0.454	202.5	0.372
II	0.17	FOC16	-	-	-	-
		Control	202.4	0.369	202.4	0.363
		FOC8	-	-	-	-
III	0.26	FOC16	201.8	0.343	202.3	0.326
		Control	-	-	-	-
		FOC8	-	-	-	-
IV	0.30	FOC16	201.8	0.305	202.5	0.354
		Control	203.3	0.449	202.6	0.401
		FOC8	202.6	0.280	-	-
VI	0.41	FOC16	-	-	-	-
		Control	-	-	-	-
		FOC8	-	-	-	-
IX	0.63	FOC16	201.7	0.308	202.3	0.356
		Control	202.3	0.399	202.3	0.323
		FOC8	202.5	0.246	202.9	0.230
X	0.76	FOC16	202.6	0.391	203.4	0.476
		Control	-	-	-	-

[z]Maximum absorbance values (A_{max}) were measured at respective λ_{max}.
[y]Controls were represented by sets of plants grown from healthy seeds in uninoculated soil.

in the sets from seed inoculation. These were fractions I (R_f 0.10), III (R_f 0.26), IV and IX. Fraction X was also found in the sets from soil inoculation. The ability of the detected absorbing UV light compounds to inhibit spore germination of two different *F. oxysporum* f. sp. *cepae* isolates was examined (Table 3). The compounds tested from onion sets of both cultivars raised from inoculated seeds exerted more antifungal activity than those grown in infested soil. In particular, the distinctive fraction VI from Akgün 12 was characterized

TABLE 3. Inhibition of spore germination of *Fusarium oxysporum* f.sp. *cepae* isolates FOC8 and FOC16 exerted by fluorescent fractions isolated from sets of two onion cultivars raised from seeds or soil inoculated with FOC8 and FOC16, respectively, and from control sets. Data are presented as IA_{50}, the absorbance values required for 50% inhibition of spore germination

Fraction	Isolate	Cultivar	
		Akgün 12	Rossa Savonese
	FOC8	0.38	0.20
I_a	FOC8 control[z]	0.89	0.71
	FOC16	-	-
	FOC16 control	-	-
	FOC8	-	-
I	FOC8 control	-	-
	FOC16	0.55	0.45
	FOC16 control	-	-
	FOC8	0.23	0.22
II	FOC8 control	0.36	0.28
	FOC16	-	-
	FOC16 control	-	-
	FOC8	-	-
III	FOC8 control	-	-
	FOC16	0.31	0.36
	FOC16 control	-	-
	FOC8	-	-
IV	FOC8 control	-	-
	FOC16	0.26	0.23
	FOC16 control	0.47	0.45
	FOC8	0.19	-
VI	FOC8 control	-	-
	FOC16	-	-
	FOC16 control	-	-
	FOC8	-	-
IX	FOC8 control	-	-
	FOC16	0.33	0.43
	FOC16 control	0.37	0.40
	FOC8	0.16	0.15
X	FOC8 control	-	-
	FOC16	0.30	0.61
	FOC16 control	-	-

[z]For controls, the IA_{50} values were obtained from fractions from control required for 50% inhibition of spore germination against respective isolates FOC8 and FC16.

by a consistent antifungal activity. When the seeds of both cultivars were sown in a field naturally contaminated with *FOC*, six bands (I_a, I, II, III, IV, VI) were detected from the extracts of onion sets (Table 4). Only fraction I_a was found to be expressed from the sets of both cultivars in both experimental fields. In both locations, fraction I from Rossa Savonese as well as fractions II and VI from Akgün 12 were distinctive. All fractions isolated exerted consistent antifungal activity towards the two *FOC* isolates tested (Table 5).

DISCUSSION

Results obtained indicate that a reliable screening procedure at the seedling stage which reveals differences in Fusarium rot resistance among different onion genotypes and corresponding results with field performance, can improve the success of selection of resistant genotypes in a relatively short time. In our recent experiments (15), screenings on seedlings raised from inoculated seeds showed that onion genotypes react differently to

TABLE 4. Compounds absorbing UV light, separated by thin layer chromatography from sets of two onion cultivars grown in naturally infected soil at two different locations

Fraction	R_f	Location I				Location II			
		Cultivar							
		Akgün 12		Rossa Savonese		Akgün 12		Rossa Savonese	
		λmax (nm)	Amax[z]	λmax (nm)	Amax[z]	λmax (nm)	Amax[z]	λmax (nm)	Amax[z]
I_a	0.07	202.8	0.152	202.9	0.295	203.8	0.402	203.3	0.461
I	0.10	-	-	204.4	0.903	-	-	203.7	0.668
II	0.17	204.0	0.612	-	-	204.9	0.973	-	-
III	0.26	-	-	202.9	0.246	202.6	0.221	202.2	0.313
IV	0.30	202.9	0.247	202.8	0.295	-	-	202.7	0.362
VI	0.41	202.8	0.263	-	-	203.1	0.321	-	-

[z]Maximum absorbance values (A_{max}) were measured at respective λ_{max}.

TABLE 5. Inhibition of spore germination of *Fusarium oxysporum* f.sp. *cepae* isolates FOC8 and FOC16 exerted by fluorescent fractions isolated from sets of two onion cultivars grown in naturally infected soil in two locations. Data are presented as IA_{50}, the absorbance values required for 50% inhibition of spore germination.

Fraction	Isolate	Location I		Location II	
		Cultivar			
		Akgün 12	Rossa Savonese	Akgün 12	Rossa Savonese
	FOC8	0.13	0.27	0.28	0.55
I_a	FOC16	0.10	0.64	0.29	0.55
	FOC8	-	0.57	-	0.37
I	FOC16	-	0.80	-	0.60
	FOC8	0.63	-	0.65	-
II	FOC16	>1	-	0.84	-
	FOC8	-	0.16	0.16	0.17
III	FOC16	-	0.27	0.21	0.33
	FOC8	0.16	0.17	-	0.19
IV	FOC16	0.24	0.34	-	0.36
	FOC8	0.16	-	0.22	-
VI	FOC16	0.18	-	0.28	-

the disease, enabling one to perform a preliminary selection. The optimized greenhouse screening methods reported here greatly increased the consistency and reliability of previous results. Moreover, they were confirmed by field tests. Akgün 12 confirmed the high level of resistance observed at the seedling stage (15) also at the bulb stage, whereas Rossa Savonese displayed lower disease incidence.

Different UV-absorbing compounds were expressed in onion sets and were differentially induced with pathogenic *FOC* isolates under greenhouse and field conditions. There were fewer fractions than found at the seedling stage (13,15). R_fs of isolated fractions coincided with those previously reported, apart from fraction I_a, expression of which is probably developmentally regulated. As found in inoculated onion seedlings (15), none of the separated fractions coincided with catechol or protocatechuic acid, which represent important fungitoxic compounds in the dry uninfected bulbs of colored cultivars (11). All the fractions tested were characterized by antifungal activity against the *FOC* isolates tested. The level of antifungal activity may have been due to the specific sensitivity of *FOC* isolates and to the variations in amount and chemical composition of fractions, as revealed by the differences in the maximum absorbance values recorded. The level of resistance of the two onion genotypes is reflected by the expression and induction of particular fractions. Fraction VI was induced in onion sets of Akgün 12 following seed inoculation as well as fractions II and VI in field experiments. Nevertheless, in field tests fraction I was specifically induced in Rossa Savonese. Although the role of single fractions cannot be proven, induced fractions may be considered as components of a coordinated battery of defenses mounted in resistant onion cultivars against *FOC*, such as the defense-related proteins described previously in onion (2,6).

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