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Fusarium spp. suppress germination and parasitic establishment of bean and hemp broomrapes

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Summary. Thirty-nine *Fusarium* isolates were obtained from newly emerged infected bean broomrape (*Orobanche crenata*) and hemp broomrape (*O. ramosa*) collected from infested fields of faba bean (*Vicia faba*) and tomato (*Lycopersicon esculentum*) respectively, in two governorates located in the south of Giza, Egypt. All *Fusarium* isolates were identified to species level and the effect of their culture filtrates on the germination of seeds from the two *Orobanche* species was tested *in vitro*. The inhibition of seed germination differed between the tested *Fusarium* isolates, depending on the plant part from which they were isolated, with isolates from the shoots of *Orobanche* inhibiting seed germination more than isolates from the inflorescences. The culture filtrates of *Fusarium* species from *O. crenata* were more toxic to the seeds of both *Orobanche* species than the *Fusarium* filtrates from *O. ramosa*. Seeds of *O. crenata* were more resistant to *Fusarium* culture filtrates than seeds of *O. ramosa*. The highest inhibition of *Orobanche* seed germination was achieved by six *Fusarium* isolates, one of which was identified as *F. oxysporum*, one as *F. equiseti*, whilst the other four were all *F. compactum*. Aqueous mixtures of mycelia and conidia of all the *Fusarium* isolates were directly sprayed on *O. ramosa* tubercles attached to the roots of tomato plants grown in transparent plastic bags, and were also used to infest soil in pots seeded with both faba bean and *O. crenata*. Two of the four *F. compactum* isolates (22 and 29) were significantly more pathogenic against *O. crenata* and *O. ramosa*, respectively, than the other *Fusarium* isolates tested in the pots and plastic bags. The study clearly shows the potential of biocontrol agents originating in one *Orobanche* sp. (e.g. *O. crenata*) to control another *Orobanche* sp. (e.g. *O. ramosa*), as many *Fusarium* isolates deriving from *O. crenata* were found to be more pathogenic to *O. ramosa* seeds than the isolates from *O. ramosa* themselves. This may widen the host range of these fungal pathogens, with the use of isolates from one *Orobanche* species effective against other species as well.

Key words: biocontrol agents, *Orobanche crenata*, *Orobanche ramosa*, pathogenicity, seed germination assay.

Introduction

Broomrapes (*Orobanche* spp.) are aggressive and injurious holoparasitic weeds that have a very strong impact on agriculture in East Africa, the Mediterranean region, and the Middle East (Abang *et al.,* 2007; Parker, 2009). Yield losses range from 5 to 100% depending on host susceptibility, the level of infestation, and environmental conditions (Abang *et al.,* 2007).

Crenate broomrape (*O. crenata* Forsk.) causes major crop losses in legume crops, especially faba bean (*Vicia faba* L.), lentils (*Lens culinaris* Medikus), forage legumes (Rubiales *et al.,* 2004; Abang *et al.,* 2007) and white lupine (*Lupinus albus* L.) (Fernández-Aparicio *et al.,* 2009). Hemp broomrape or branched broomrape (*O. ramosa* L.) parasitizes plants from eleven different dicotyledonous families, with hosts severely infested including tomato (*Lycopersicon esculentum* Mill.), potato (*Solanum tuberosum* L.), eggplant (*S. melongena* L.) and cabbage (*Brassica oleracea* var. *capitata* L.) (Abang *et al.,* 2007).

Orobanche ramosa together with *O. aegyptiaca* Pers. (Egyptian broomrape) infests about 2.6 million hectares (mainly in the Mediterranean area, North Africa and Asia) of solanaceous crops (Ze-

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hhar *et al.,* 2002; Boari and Vurro, 2004). In Australia, *O. ramosa* is a scheduled weed and is subject to extensive quarantine control (Panetta and Lawes, 2007; Matthews *et al.,* 2008). *Orobanche crenata*, on the other hand, has spread to the Mediterranean basin as well as to other regions with a Mediterranean climate, including Australia (Grenz and Sauerborn, 2007), with heavy yield losses in the field (Parker and Riches, 1993). Additionally, Gressel *et al.* (2004) stated that *O. crenata* is the most damaging and widespread *Orobanche* species in food legume crops in northern Africa, with the most seriously affected crop being faba bean.

Substantial yield reduction, mainly due to *O. crenata* infestation of faba bean, has also been seen in Egypt in the last five years, with yields dropping from an average of 2.7 to 0.6 tons per hectare, and a decrease of cultivated land from 17,650 hectares in 1991 to 800 hectares in 2000 (SP-IPM, 2003).

Various methods have been tried to control *Orobanche* spp. They include crop rotation (Krishnamurthy and Rao, 1976), delay in the sowing date (Perez-De-Luque *et al.,* 2004), resistant plant breeding (Nadal *et al.,* 2007), seed germination stimulants (Vurro *et al.,* 2006), soil solarization (Mauromicale *et al.,* 2001), soil sterilants (Parker and Riches, 1993), use of both catch and trap crops (Linke and Saxena, 1991), soil amendments (Westwood and Foy, 1999; Haidar and Sidahmed, 2006), chemical control by herbicides (Jurado-Expósito *et al.,* 1999; Nadal *et al.,* 2008) and biological control using fungi, especially *Fusarium* spp. (Amsellem *et al.,* 2001a, b; Dor *et al.,* 2007; Sauerborn *et al.,* 2007).

The use of phytopathogenic fungi and/or their toxic metabolites to control weeds is interesting (Evidente and Abouzeid, 2006) since biocontrol agents are highly specific (Boari and Vurro, 2004; Elzein *et al.,* 2004) and since a wide range of *Fusarium* strains has been isolated from *Orobanche* species and also from *Striga* (Sauerborn *et al.,* 2007).

Two promising *Fusarium* strains, *F. oxysporum* Schlecht. strain E1d and *F. arthrosporioides* Sherb. strain E4a, were shown to have potential control of *O. aegyptiaca* (Amsellem *et al.,* 2001a), and the efficacy of these strains has since then been transgenically improved (Cohen *et al.,* 2002b). The activity of many fungal strains was attributed to their capacity to produce various phytotoxic metabolites which can be considered natural herbicides in parasitic weed management programs (Evidente and Abouzeid, 2006). *Fusarium* toxins have been extensively tested for their herbicidal effect against *Orobanche* and *Striga* species, and some have already been proposed as control agents, since they interfere with the seed germination processes of parasitic weeds (Zonno and Vurro, 2002; Azam *et al.,* 2003).

The objectives of the present study were (i) to screen, isolate and identify *Fusarium* isolates from diseased juvenile *O. crenata* and *O. ramosa* shoots and inflorescences collected from infested faba bean and tomato fields in Egypt, (ii) to evaluate the effect of *Fusarium* culture filtrates on seed germination of the *Orobanche* species *in vitro*, and (iii) to assess the virulence of all *Fusarium* isolates against the *Orobanche* species *in vivo*.

The main goal of the present study was to determine how well *Fusarium* isolates derived from one *Orobanche* species, suppressed seed germination and parasitic establishment in other *Orobanche* spp., so as to broaden the spectrum of controlling these weeds. Positive results here will indicate that there is a potential to obtain *Fusarium* isolates capable of suppressing a wide range of harmful weeds.

Materials and methods

Plant material

Seed of faba bean (*V. faba*) cv. Giza 3 and tomato (*L. esculentum*) cv. Castle Rock were supplied by the Agricultural Research Centre, Giza, Egypt.

Orobanche samples

One-hundred newly emerged *O. crenata* plants were separated from infested faba bean fields in El-Fayoum and Beni-suef governorates, Egypt. *Orobanche ramosa* plants (55 samples) were collected from infested tomato fields in the El-Badrashein area (central Egypt). Broomrape plants were stored in glass containers in the dark at 20±2°C prior to fungal isolation.

Isolation and identification of fungi

Fusarium isolates were recovered from 3–5-mm-sized pieces cut from the shoots and inflo-

rescences of broomrape plants. The broomrape pieces were surface-sterilized with 1% sodium hypochlorite for 4 min and plated on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) amended with 250 μg mL^{-1} chloramphenicol (Sigma Chemical Co, St. Louis, MO, USA) and 100 μg mL^{-1} streptomycin sulphate (Sigma). The plates were incubated in the dark at 25±2°C for 8 days and were examined daily for fungal growth. Pure fungal cultures were maintained on PDA agar plates and stored at 4°C for use throughout the study.

Fusarium isolates were identified macroscopically and microscopically using the specific taxonomic keys (Booth, 1971; Burgess *et al.,* 1994; Leslie and Summerell, 2006).

Preconditioning of *Orobanche* seeds

Seeds of *O. crenata* were provided by the Agricultural Research Centre, Ministry of Agriculture, Giza, Egypt, and seeds of *O. ramosa* were obtained from mature plants grown in the El-Badrashein area and dried for two weeks at ambient conditions. *Orobanche* seeds were preconditioned using the method of Kebreab and Murdoch (1999). Seeds (5 mg of each species) in Miracloth (Calbiochem, San Diego, CA, USA) were immersed in 70% ethanol for 3 min, then twice in 1% sodium hypochlorite containing a drop of the wetting agent Tween 80 (polyoxyethylene sorbitan monooleate) (Sigma) for 4 min. Seeds were rinsed three times with sterile distilled water, left to dry in the dark at 22±2°C for 24 h, and were then spread on wet glass microfiber filters (GF/A) (2.4 cm in diameter; Whatman International Ltd, Springfield Mill, Maidstone, UK) containing drops of MES buffer (*N*-morpholino ethane sulfonic acid) (GFS Chemicals Inc., Columbus, OH, USA) adjusted to pH 5.8 in glass Petri dishes. The dishes were wrapped in aluminum foil and incubated in the dark at 22±2°C for three weeks. Filters were cut later into four pieces, each containing about 1 mg *Orobanche* seeds.

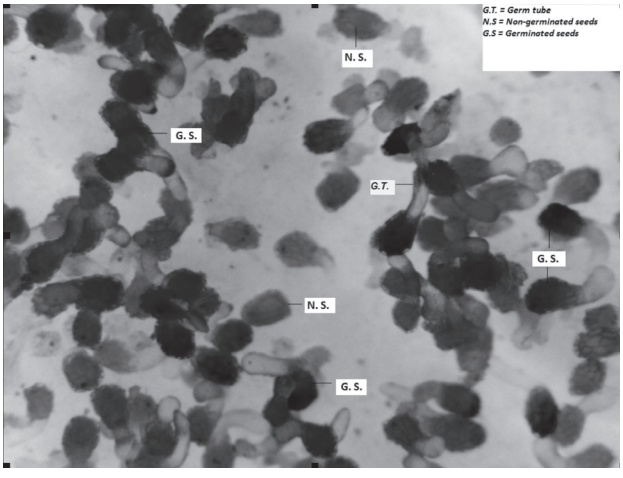

Figure 1. Microscopic view showing germinated *Orobanche ramosa* seeds with well developed protruding germ tubes.

Pattern of activity of *Fusarium* culture filtrates in relation to the germination of *Orobanche* seeds

Fusarium isolates were grown in a stationary potato dextrose broth (PDB) culture and incubated in the dark at 25±2°C for 5 days. Filtrates (5 mL each) were transferred under aseptic conditions, and were filter-sterilized using sterile millipore membranes (pore size 0.22 μm, Millipore Corporation, Billerica, MA, USA) to remove cell debris. Glass microfiber filters (Whatman) in glass Petri dishes were moistened with 2 mL of each test filtrate. Additionally, 2 mL of the synthetic germination stimulant Nijmegen-1 (2 μg mL^{-1}) (Benvenuti *et al.,* 2004) was used. The synthetic germination stimulant was prepared by dissolving 10 mg of Nijmegen-1 in 10 mL acetone (Sigma) and then diluting 500 fold with distilled water.

Filter pieces containing hundreds of preconditioned *O. crenata* or *O. ramosa* seeds were placed over the glass microfiber filters in each dish. Dishes were sealed and incubated in the dark at 22±2°C for 7 days and the germination percentage of seeds was determined using a binocular microscope. Seeds were considered germinated if their radical penetrated the coat and had protruding germ tubes that were at least twice the seed length (Figure 1), whilst non-germinated seeds either did not form any germ tubes or had shorter germ tubes (Figure 1). Non-germinated seeds were counted and expressed as a percentage of the number of germinated seeds in relation to the total number of seeds compared to the controls for each replicate (170 seeds of *O. crenata* and 190 seeds of *O. ramosa*).

The toxicity levels (TL) of *Fusarium* isolates against seed germination of the two broomrapes were scored as follows: low toxicity isolates (L),

0–29% seed inhibition; medium (M), 30–59% seed inhibition; high (H), >60% seed inhibition. The experiment was conducted three times with four independent replicates for each isolate. Dishes containing seeds of both *Orobanche* species and moistened with sterile distilled water (2 mL) and Nijmegen-1 (2 mL) served as controls.

Pathogenicity test against *O. crenata* in pots

Large glass beakers were filled with 500 g naturally infested soil brought from Beni-suef governorate. Five mg of surface-sterilized seeds of *O. crenata* (approximately 860 seeds) were mixed with the soil of each beaker before surface-sterilized seeds of *V. faba* (4 seeds per beaker) were sown as described by Bernhard *et al*. (1998). Faba bean and *O. crenata* seeds were surface-sterilized in 70% ethanol for 3 min, followed by immersion in 1% sodium hypochlorite for 4 min. Surface-sterilized seeds were then washed ten times with sterile distilled water. The beakers were kept at 23±2°C with 14 h day (under 80 μmol $m^{-2}s^{-1}$ fluorescent light) and watered when needed.

To ensure a high infestation level, after three weeks, the contents of the beakers were transferred to small 25-cm-diameter free-draining plastic pots filled with 6 kg of soil containing an additional 20 mg of *O. crenata* seeds. The soil was mixed with inoculum (100 mL) from each *Fusarium* isolate (Table 1) as a suspension of mycelium and conidia in sterile distilled water.

Inoculation of the *Fusarium* isolates in pots was done according to the method of Ghannam *et al*. (2007). The fungal biomass was prepared by gently transferring 5-mm mycelium plugs from two-week-old fungal colonies of *Fusarium* isolates to autoclaved flasks (three plugs per flask) containing 100 mL of PDB amended with 250 mg L^{-1} chloramphenicol (Sigma). The flasks were shaken for 14 days at 28±2°C in the dark. Spores from cultures were washed free of mycelia through Miracloth (Calbiochem), centrifuged at 16,000 *g* for 20 min, and the supernatant was decanted. The spores were re-suspended in sterile distilled water, centrifuged again, decanted, and the concentrations were adjusted to 10^8 conidia mL^{-1} using a haemocytometer. The inoculum was prepared by incorporating both the fungal mycelium and fungal conidia, which were separately suspended in sterile distilled water. A mixture of 5 g fungal mycelium with 5×10^8 mL^{-1} conidia was then suspended in 100 mL 1 M sucrose solution. The suspension was homogenized by a Polytron PT 1200C homogenizer (Kinematica AG, Luzern, Switzerland) for one min at 6000 rpm.

Pots were kept for one month in an evaporatively cooled greenhouse at 25±2°C. The pots were watered daily to container capacity and were fertilized weekly with Thrive® inorganic liquid fertilizer (Arthur Yates & Co Limited, Milperra, NSW, Australia) at the manufacturer's recommended rate. The experiment was conducted three times with four independent replicates per isolate; control pots did not receive any fungal inoculum. Underground tubercles parasitizing the host roots were gently dusted to remove the soil and cleaned under tap water to record the progress of the color of infection and any changes in tubercle consistency.

The disease severity (DS) produced by each *Fusarium* isolate was expressed as a disease index scored on a 0–3 scale (Table 1). To fulfill Koch's postulates, random pieces of diseased tissue were surface-sterilized and the pathogen recovered as described above.

Effect of *F. compactum* (isolate No. 22) on the growth characteristics of faba bean

The aim of this experiment was to evaluate what the effect of the most antagonistic *Fusarium* isolate, No. 22, identified as *F. compactum* in soil infested with *O. crenata* was on the growth of faba bean. Thirty-cm-diameter free-draining pots were filled with 8 kg of soil each. Three treatments were applied: 1, control (without *O. crenata*); 2, soil with *O. crenata*; 3, soil with *O. crenata* + *F. compactum*. The fungal inoculum was prepared by incorporating both fungal mycelium and fungal conidia, which were separately suspended in distilled water. A mixture of 5 g fungal mycelium with 5×10^8 mL^{-1} conidia was then suspended in 100 mL 1 M sucrose solution. The suspension (100 mL) was then homogenized as above and thoroughly mixed into the soil with a cement mixer.

Faba bean seeds were surface-sterilized as above and sown in the pots. Each treatment was replicated five times with three plants per replicate, and the pots were placed in an evaporatively cooled greenhouse and maintained at 25 ±2°C.

The pots were watered daily to container capacity and were fertilized weekly with inorganic

liquid fertilizer as above. Plant growth was monitored by recording the fresh and dry weight of the roots and shoots, the length of the roots and shoots, and the number of pods per plant at the time of harvest (18 weeks after sowing).

Pathogenicity test against *O. ramosa* using plastic bags

The method used was similar to that of Parker and Dixon (1983), and modified by Amsellem *et al*. (1999), to grow *Orobanche* on pre-planted hosts on glass microfiber filters.

Glass microfiber filter sheets (Whatman) (14×12 cm) were introduced in plastic bags (25×18 cm) and 50 mg surface-sterilized seeds (approximately 188 seeds mg^{-1}) of *O. ramosa* were spread out on the filter sheets. Tomato plants at the second-leaf stage (bearing 2–3 primary leaves) were transferred to the plastic bags with the shoot system out and the washed roots fixed over the paper sheet inside the bags. Drops of Nijmegen-1 (5 μg mL^{-1}) were applied to the tomato roots to accelerate the natural host-produced stimulants as recorded by Cohen *et al*. (2002a).

Two plants were placed in each bag and the experiment was conducted three times with four independent replicates for each *Fusarium* isolate. Polyethylene bags were placed upright in a black box so that plant roots were in the dark and their shoots projected into the light above the box. Other plastic bags were used to cover the plants so that they would retain moisture. The boxes were placed for 15 days in a growth chamber set at 23±2°C with a 14 h day under fluorescent light (80 μmol $m^{-2}s^{-1}$). The bags were replenished with 20 mL of 1/4 strength Hoagland's modified salt mixture (MP Biochemicals, Irvine, CA, USA) as needed.

Orobanche seed germination, radical attachment and development were recorded periodically on either 2×2 cm root sections or the whole root system using a stereoscopic microscope (×10 – ×60).

As described by Amsellem *et al*. (2001b), the bags were opened after 15 days and the tomato roots with small tubercles (6 mm) of *O. ramosa* were sprayed with inoculum (5×10^8 CFU mL^{-1}) of each *Fusarium* isolate (Table 2) prepared as described above. The bags were checked daily for up to 20 days for disease development, and disease severity (DS) produced by each *Fusarium* isolate was expressed as a disease index rated on a 0–3 scale (Table 2). Bags sprayed with sterile distilled water containing Tween 80 (0.02%, v:v) served as the control. To fulfill Koch's postulates, random pieces of diseased tissue were surface-sterilized and the pathogen recovered as above.

Statistical analysis

All experiments were arranged in a completely randomized block design. Percentage data were arcsine-transformed before ANOVA. To evaluate the effect of the treatments on seed germination and on *Orobanche* tubercles, all data were subjected to ANOVA, and significant differences between the means were determined using Fisher's Protected LSD Test at P=0.05. Superanova® (Abacus Concepts, Inc., Berkeley, CA, USA) was used for all analyses. Since repeated independent pot and plastic bag experiments showed similar trends and there were no significant differences between them, the data were combined for analysis.

Results

Fusarium isolates recovered from bean and hemp broomrapes

Seventy-six fungal isolates, mostly belonging to the three genera *Fusarium*, *Aspergillu*s and *Alternaria*, were recovered during the survey of the mycoflora associated with infected young broomrape plants. Thirty-nine *Fusarium* isolates were recovered from the two broomrape species, 21 from *O. ramosa* and 18 from *O. crenata*. Higher populations were found on the shoots (22 isolates) than on the inflorescences (17 isolates). The 39 *Fusarium* isolates belonged to nine species, with 23 isolates (60%) belonging to just two species, *F. solani* Mart. (12 isolates) and *F. oxysporum* (11 isolates) (Tables 1 and 2). Four isolates (two from each *Orobanche* species) representing 10.25% of the total, were identified as *F. compactum sensu* Gordon (Tables 1 and 2).

Pattern of activity of *Fusarium* culture filtrates in relation to the germination of *Orobanche* seeds

Two-way ANOVA detected that the two species of *Orobanche* and the different *Fusarium* isolates and their interaction (P<0.001) very significantly inhibited germination of broomrape seeds (Table 3 and Figure 2a, b). *O. ramosa* was inhibited to

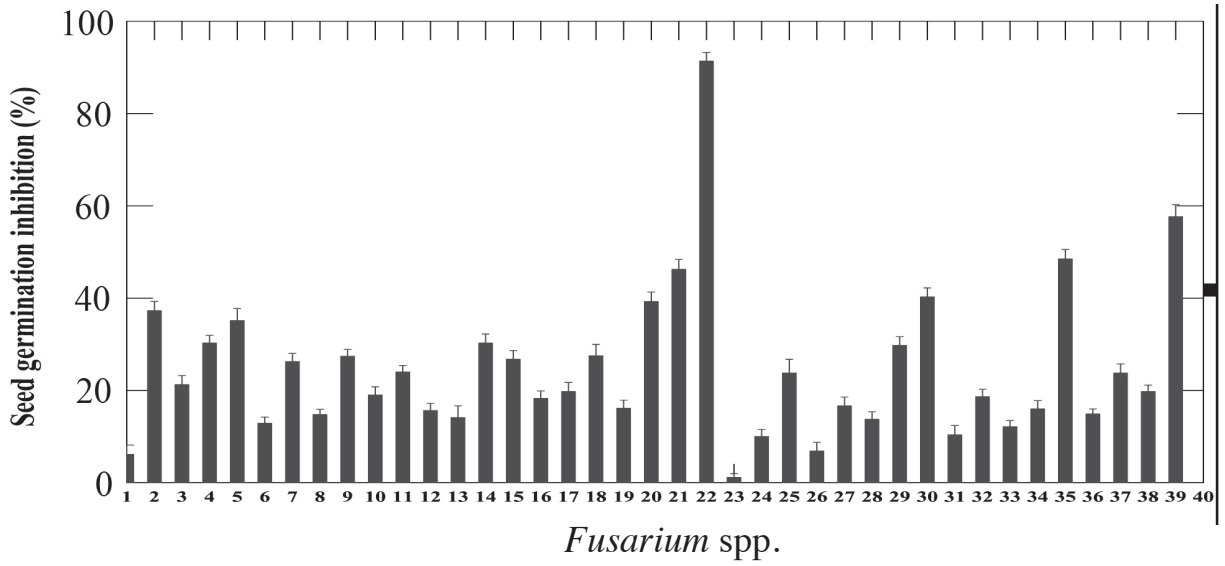

Figure 2a. Toxicity of the *Fusarium* isolates on the seed germination of *Orobanche crenata*. The numbering of the 39 *Fusarium* isolates is as in Tables 1 and 2. Error bars are standard errors.

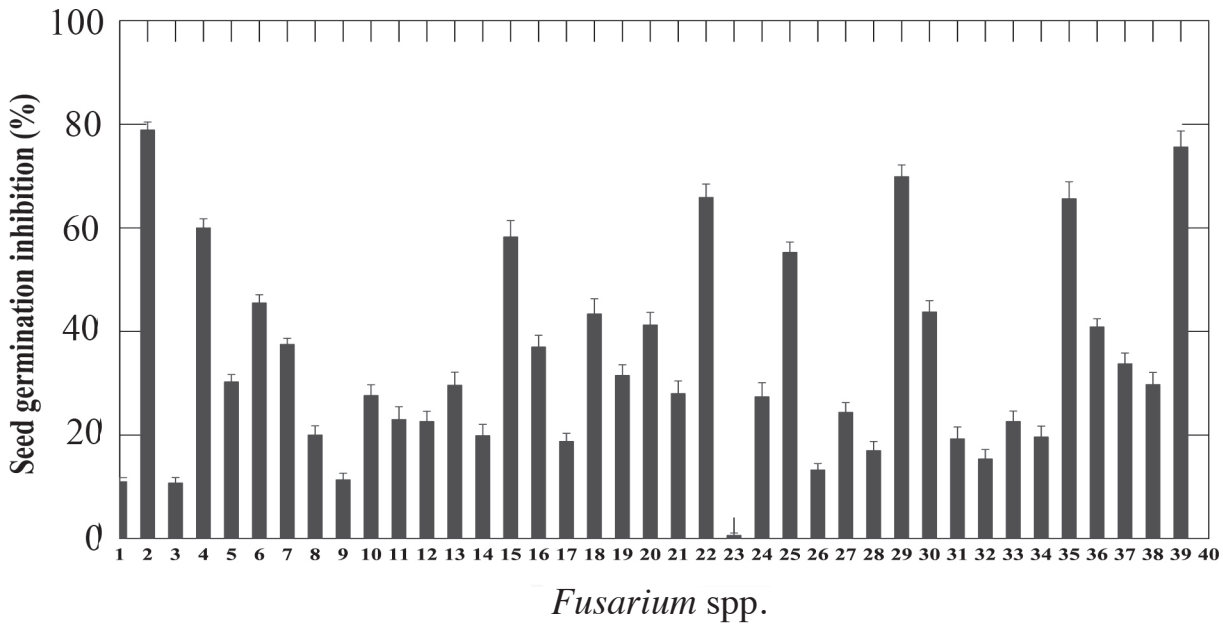

Figure 2b. Toxicity of the *Fusarium* isolates on the seed germination of *Orobanche ramosa*. The numbering of the 39 *Fusarium* isolates is as in Tables 1 and 2. Error bars are standard errors.

Table 1. Toxicity levels (TL) of *Fusarium* culture filtrates on the seed germination of bean broomrape (*Orobanche crenata*), and disease severity (DS) of *Fusarium* isolates on *O. crenata* in an experiment conducted in pots under greenhouse conditions. One-way ANOVA showed a highly significant difference between the 39 isolates (F=92.2, $P<0.001$).

Host	*Fusarium* species (one isolate per species)	Isolate code	TL[a]	DS[b]
O. ramosa shoots	*Fusarium* spp.	#1	L	0
	F. compactum	#2	M	0
	F. verticillioides	#3	L	0
	F. equiseti	#4	L	0
	F. proliferatum	#5	M	0
	F. solani	#6	L	0
	F. solani	#7	L	0
	F. oxysporum	#8	L	0
	F. oxysporum	#9	L	0
	F. oxysporum	#10	L	0
O. crenata shoots	*Fusarium* spp.	#11	L	0
	Fusarium spp.	#12	L	0
	F. oxysporum	#13	L	0
	F. oxysporum	#14	L	0
	F. oxysporum	#15	L	0
	F. oxysporum	#16	L	0
	F. solani	#17	L	0
	F. solani	#18	L	0
	F. solani	#19	L	0
	F. solani	#20	M	0
	F. solani	#21	M	0
	F. compactum	#22	H	3
O. ramosa inflorescences	*F. acuminatum*	#23	L	0
	F. equiseti	#24	L	0
	F. solani	#25	L	0
	F. solani	#26	L	0
	F. solani	#27	L	0
	F. solani	#28	L	0
	F. compactum	#29	L	0
	F. proliferatum	#30	M	0
	F. oxysporum	#31	L	0
	F. oxysporum	#32	L	0
	F. oxysporum	#33	L	0
O. crenata inflorescences	*F. sambucinum*	#34	L	0
	F. oxysporum	#35	M	1
	F. solani	#36	L	0
	F. avenaceum	#37	L	0
	F. avenaceum	#38	L	0
	F. compactum	#39	M	2

[a] L, 0–29% seed inhibition; medium (M), 30–59% seed inhibition; high (H), >60% seed inhibition.
[b] DS, disease severity, expressed as a disease index rated on a 0–3 scale where 0, no infection (0% browning); 1, light tissue browning (<30% browning); 2, 30–70% tissue browning; 3, 75–100% browning and possible tissue wet rot.

Table 2. Toxicity levels (TL) of *Fusarium* culture filtrates on the seed germination of hemp broomrape (*Orobanche ramosa*) and disease severity (DS) of *Fusarium* isolates on *O. ramosa* in an experiment conducted in plastic bags. One-way ANOVA showed a highly significant differences between the 39 isolates (F=97.6, $P<0.001$).

Host	*Fusarium* species (one isolate per species)	Isolate code	TL[a]	DS[b]
O. ramosa				
shoots	*Fusarium* spp.	#1	L	0
	F. compactum	#2	H	1
	F. verticillioides	#3	L	0
	F. equiseti	#4	H	1
	F. proliferatum	#5	M	0
	F. solani	#6	M	0
	F. solani	#7	M	0
	F. oxysporum	#8	L	0
	F. oxysporum	#9	L	0
	F. oxysporum	#10	L	0
O. crenata				
shoots	*Fusarium* spp.	#11	L	0
	Fusarium spp.	#12	L	0
	F. oxysporum	#13	L	0
	F. oxysporum	#14	L	0
	F. oxysporum	#15	M	0
	F. oxysporum	#16	M	0
	F. solani	#17	L	0
	F. solani	#18	M	0
	F. solani	#19	M	0
	F. solani	#20	M	0
	F. solani	#21	L	0
	F. compactum	#22	H	1
O. ramosa				
inflorescences	*F. acuminatum*	#23	L	0
	F. equiseti	#24	L	0
	F. solani	#25	M	0
	F. solani	#26	L	0
	F. solani	#27	L	0
	F. solani	#28	L	0
	F. compactum	#29	H	2
	F. proliferatum	#30	M	0
	F. oxysporum	#31	L	0
	F. oxysporum	#32	L	0
	F. oxysporum	#33	L	0
O. crenata				
inflorescences	*F. sambucinum*	#34	L	0
	F. oxysporum	#35	H	0
	F. solani	#36	M	0
	F. avenaceum	#37	M	0
	F. avenaceum	#38	M	0
	F. compactum	#39	H	1

[a] See Table 1.
[b] See Table 1.

Table 3. Results of two-way ANOVA showing the effects of two species of *Orobanche*, of different *Fusarium* isolates, and of their interaction, on inhibition of broomrape seed germination.

Source	df	Mean-Square	F-ratio	*P*
Orobanche spp. (O)	1	1.557	411.816	<0.001
Fusarium spp. (F)	38	0.581	153.592	<0.001
O × F	38	0.139	36.736	<0.001
Error	546	0.004		

a greater extent than *O. crenata* (Figure 2a, b). The interaction between the two *Orobanche* species and the *Fusarium* isolates indicated that the *Fusarium* isolates differed significantly in the degree to which they inhibited seed germination of the two *Orobanche* species ($P<0.001$). For example, *O. ramosa* seed germination was significantly inhibited by *Fusarium* isolates (#2 and 29) but *O. crenata* seeds were inhibited much less with the same isolates. On the other hand, *O. crenata* seed germination was inhibited more strongly with *Fusarium* isolate #22, which was less effective against *O. ramosa* seeds (Figure 2a, b).

Culture filtrates of *Fusarium* isolates from *O. crenata* were more toxic to both *O. crenata* and *O. ramosa* seeds than the culture filtrates from *O. ramosa* isolates (Tables 1 and 2), and seeds of *O. crenata* were more resistant to *Fusarium* culture filtrates than seeds of *O. ramosa* (Tables 1 and 2 and Figure 2a, b).

Most *Fusarium* isolates, whether from *O. crenata* or *O. ramosa* had either low TL (L) or moderate TL (M) on the seed germination of both *Orobanche* species. Culture filtrates of six of the *Fusarium* isolates (#2, 4, 22, 29, 35 and 39) strongly inhibited germination of *O. ramosa* seeds (TL=H) (Table 2 and Figure 2 b), and the filtrate of one of these isolates, #22, also had the highest inhibition of *O. crenata* seed (TL=H) (Table 1 and Figure 2a). Only *Fusarium* isolate #23, identified as *F. acuminatum* did not inhibit germination of seeds of either *Orobanche* species (zero inhibition) (Tables 1 and 2).

Specifically, of the isolates with moderate or high inhibition of the seed germination of *O. ramosa*, five isolates (#2, 4, 5, 6 and 7) were obtained from *O. ramosa* shoots, and only three isolates (#25, 29 and 30) from *O. ramosa* inflorescences. Similarly, of the isolates inhibiting *O. ramosa* seed germination, six (#15, 16, 18, 19, 20 and 22) were obtained from the shoots of *O. crenata*, and of the isolates inhibiting *O. ramosa* seed germination, five (#35, 36, 37, 38 and 39) came from *O. crenata* inflorescences (Tables 1 and 2).

Certain *Fusarium* isolates recovered from *O. crenata* (#15, 18, 35, 36 and 37) were more toxic to *O. ramosa* seeds than to seeds of the *O. crenata* host from which they came.

Of the isolates examined, those isolates identified as *F. compactum* (*Fusarium* isolates #2, 22, 29 and 39) seemed most promising as inhibitors of *Orobanche* seeds. Three of these isolates (#2, 22 and 39) had a TL=M or H, and isolate #22 (from *O. crenata*) scored the highest virulence against *O. crenata*. The only exception was *F. compactum*, *Fusarium* isolate #29, which had a TL=L on *O. crenata* (Tables 1 and 2).

Biocontrol experiments in pots and in plastic bags

Only three of the *Fusarium* isolates, *F. compactum* (#2 and 39) and *F. oxysporum* (#35) were virulent against tubercles of *O. crenata* in the pot trials (Table 1). *F. compactum* derived from *O. crenata* shoots (#22) was the most virulent, causing tubercle rot by the end of the evaluation period. *Fusarium* species were recovered from all the diseased tissues investigated.

In the plastic bag assay, five isolates (#2, 4, 22, 29 and 39) were virulent to *O. ramosa* tubercles, and one of these (#29) which was identified as *F. compactum*, caused severe blackening of tissues (Figure 3) but without changing tubercle consistency. The other four isolates caused only a light browning of the tubercles (Table 2). Here too, *Fusarium* species were recovered from all the diseased tissues investigated.

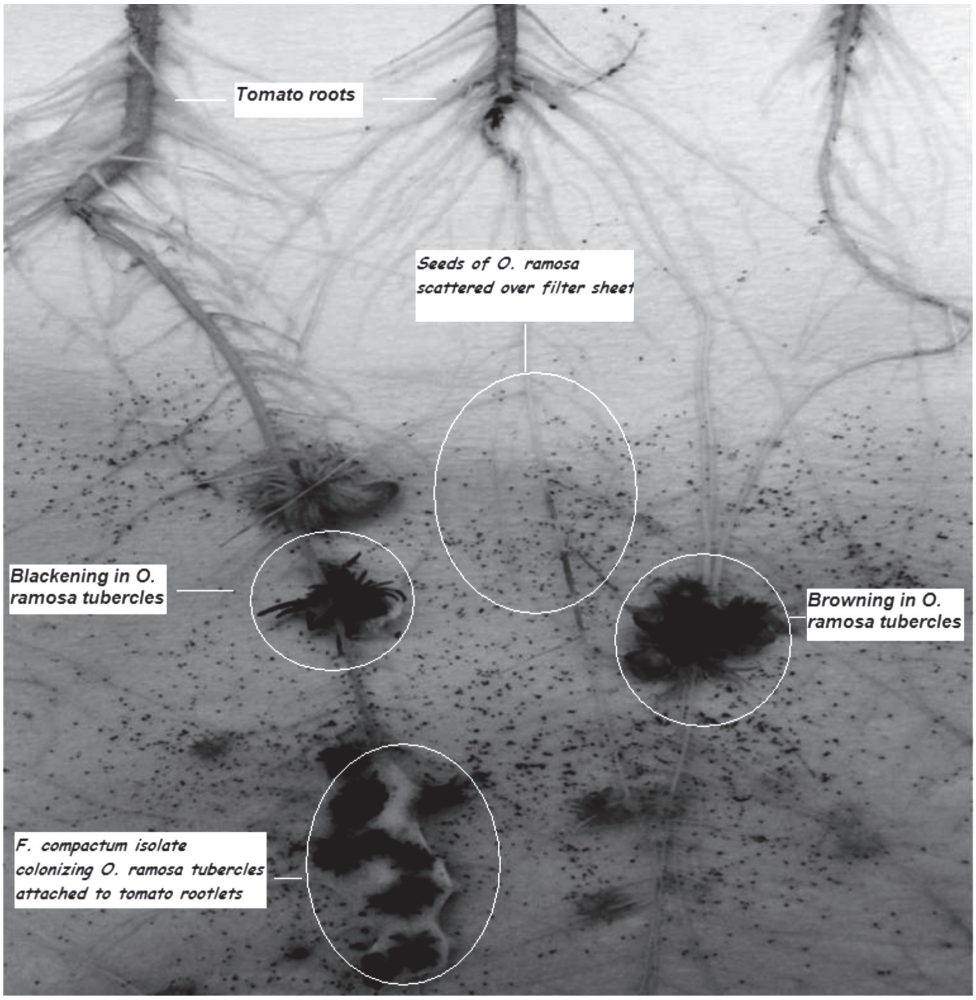

Figure 3. Pathogenicity of *Fusarium compactum* (*Fusarium* isolate 29) on the seeds and tubercles of *Orobanche ramosa* formed on tomato roots grown in plastic bags.

Table 4. Effect of the application of *Fusarium compactum* (*Fusarium* isolate 22) and/or *Orobanche crenata* on the growth characteristics of faba bean (*Vicia faba*) plants grown in an evaporative-cooled greenhouse maintained at 25±2°C.

Treatment [a]	Shoot length (cm) [b]	Root length (cm) [b]	Total fresh weight (g) [b]	Total dry weight (g) [b]	Number of pods per plant [b]
Control	56.16 a	24.39 a	484.18 a	82.05 a	3.36 ab
O. crenata	47.80 b	19.40 b	416.25 b	70.48 b	1.80 b
O. crenata + *F. compactum*	51.05 c	21.66 c	438.46 c	74.95 c	2.26 a

[a] Plants were harvested after 18 weeks. Values are means of 15 replicates per treatment from three independent experiments (3 plants pot^{-1}).
[b] Values with the same letter within a column are not significantly (P>0.05) different according to Fisher's protected LSD test.

Effect of *F. compactum* (isolate #22) on the growth characteristics of faba bean

Application of *F. compactum* (#22) to soil infested with *O. crenata* (treatment 3) significantly ($P<0.05$) enhanced the growth of faba bean in the greenhouse experiment as compared with soil containing only *O. crenata* (treatment 2) (Table 4). *F. compactum* increased the fresh and dry weight of faba bean plants by 5.1% and 5.9%, and the length of the shoots and roots by 6.3 and 10.4% respectively, compared to plants grown in soil infested only with *O. crenata*. There was also an increase of 20.3% in the number of pods per plant (Table 4).

Discussion

A large number of microbial biological control agents have been, or are being, evaluated for their effectiveness against parasitic weeds. Despite the number of micro-organisms surveyed, only some are considered effective candidates to control broomrape, but so far none of them have reached large-scale application in the field. Clearly, finding a weed pathogen is only a preliminary step in a long process leading to the development of an effective and safe bioherbicide. The protocol for developing such a bioherbicide includes a number of steps such as: a survey of the weed pathogens, their isolation, identification and classification, screening for efficacy (pathogenicity testing), testing for host specificity and safety, inoculum mass production, preliminary field testing, formulation, and the mode of delivering the formulated agents to the target weed (Elzein and Kroschel, 2003).

In view of the importance of tomato and faba bean as major cash crops for farmers in Egypt, and also of the reduction in areas under faba bean cultivation in Upper Egypt, such as Beni-Suef in which the area under faba bean cultivation fell from 17,650 hectares to only 800 hectares (SP-IPM, 2003), a screening program was set up to isolate the fungal flora (mainly *Fusarium*) from the two *Orobanche* species in one of the main areas of production for each crop (Beni-Suef for faba bean and the Badrashein for tomato). This was intended to be an important step to incorporate any results obtained into the knowledge of *Fusarium* candidates that already exists.

Fungi were isolated from the shoots and inflorescences of broomrape at the earliest sign of disease symptoms to identify the primary *Fusarium* pathogens of the broomrape. This is in agreement with earlier studies that examined *Fusarium* species commonly isolated from diseased *O. ramosa* plants (Abouzeid *et al.,* 2004) as well as from *Striga* and other *Orobanche* species (Sauerborn *et al.,* 2007). In the present study, *F. solani* and *F. oxysporum* together with *F. compactum*, comprised 70% of the total fungal population isolated from the shoots and inflorescences of *O. crenata* and *O. ramosa*. Seven of the isolated *Fusarium* species (*F. acuminatum* Ellis & Everhart, *F. avenaceum* [Fr.] Sacc., *F. equiseti* [Corda] Sacc., *F. oxysporum*, *F. sambucinum* Fuckel, *F. solani* and *F. verticillioides* [Sacc.] Nirenberg) are among the *Fusarium* species most frequently found in Egyptian soils (Moubasher, 1993).

The strain of *F. oxysporum* Schlecht. f. sp. *orthoceras* (Appel & Wollenw.) Bilay, isolated by Amsellem *et al.* (2001a), attacked only sunflower broomrape (*O. cumana* Wallr.) and not any other *Orobanche* species. By contrast, the four *F. compactum* isolates of our study (#2, 22, 29 and 39) inhibited seed germination and were pathogenic to both *Orobanche* species.

Fusarium isolates (#15, 18, 35, 36 and 37) were more toxic to *O. ramosa* seeds than to seeds of *O. crenata* though they had been isolated from this latter species. This opens up the prospect of broadening the host range of these pathogens, enabling agents from one *Orobanche* species to be used for the control of others. In any case, it would be practically desirable to have isolates with the widest possible host range among the weeds. Two strains of *F. oxysporum* (*F. oxysporum* strain E1d and *F. oxysporum arthrosporioides* strain E4a) have previously been reported pathogenic to three *Orobanche* species, *O. aegyptiaca*, *O. crenata* and *O. ramosa* (Amsellem *et al.,* 2001a).

Seeds of the two *Orobanche* species, like those of other root parasitic plants, were responsive to Nijmegen-1 only after a preconditioning period under moist conditions, as reported by Joel *et al.* (1995). Also, the response of *Orobanche* seeds to high concentrations of Nijmegen-1 was similar to those described by Zonno and Vurro (2002), who applied high concentrations of another synthetic stimulant GR24. In the present study, the germination pattern for synthetically stimulated *Orobanche* seeds was uniform and Nijmegen-1 proved to be non-toxic to the pathosystem.

In the *in vitro* tests with culture filtrates from the *Fusarium* isolates, the isolates were considered active only if they inhibited germination of at least 60% of the *Orobanche* seeds. The TL of *Fusarium* isolates from *O. crenata* was higher than the TL of isolates from *O. ramosa*, although the isolates from *O. ramosa* were more numerous. In our study, the culture filtrates from certain *Fusarium* isolates associated with infected samples of the two *Orobanche* species varied in the degree to which they inhibited seed germination. For example, *F. compactum* (isolates 2 and 29) significantly inhibited seed germination of *O. ramosa*, but they inhibited *O. crenata* seed germination much less. Conversely, *F. compactum* (isolate 22) inhibited *O. crenata* seed germination more than *O. ramosa* seed germination.

When the number of active *Fusarium* isolates from the shoots and the number of isolates from the inflorescences are compared between the two broomrapes, it is possible that there is a relationship between isolate activity and the site from which the isolate was recovered. This subject needs further study to determine whether the isolation site of the pathogen plays a role in its biological activity.

The inhibitory effect of the six promising isolates (2, 4, 22, 29, 35 and 39) (Tables 1 and 2) was most evident on the growth of the germ tubes from treated *O. ramosa* seeds. This inhibitory effect was subsequently confirmed in the *in vivo* pathogenicity tests. The capacity of the *F. compactum* culture filtrates to inhibit *Orobanche* seed germination was positively correlated with its virulence against the broomrapes, in the pathogenicity tests, although previous studies did not report that there was such a correlation between the phytotoxicity of any fungal isolates (mainly *Fusarium*) and their virulence in *in vivo* tests (Cohen *et al.,* 2002a; Abouzeid *et al.,* 2004).

In the present study, the pathogenicity test in pots showed that *Fusarium* isolate 22 (*F. compactum*), obtained from diseased *O. crenata* plants, caused significant browning as well as tissue softening (rot) in the tubercles that formed. It also appeared that this *F. compactum* isolate was not pathogenic to faba bean, and the improvement in faba bean growth achieved with this isolate was probably due both to the reduction in infection foci that it caused and possibly to the enhanced plant growth resulting from plant growth regulators produced by *F. compactum*, as by many Fusaria (Thakur and Vyas, 1983). In our study, the pathogenic fungal isolates were re-isolated from the infected tissues of *Orobanche* tubercles, meeting Koch's postulates for primary pathogens.

In the present study, *F. compactum* (isolate 22), was a potent mycoherbicidal agent and it could be useful to manage the two *Orobanche* species, especially *O. crenata*, but further research is still required to develop a suitable formulation of this organism in the field. Various *Fusarium* species have either been evaluated as bio-control agents of *O. ramosa* (Abouzeid *et al.,* 2004; Boari and Vurro, 2004) or they have actually been successfully applied in pot trials to control other broomrape species (Amsellem *et al.,* 2001b; Ghannam *et al.,* 2007).

Amalfitano *et al.* (2002) stated that two phytotoxins, fusaric and dehydrofusaric acid, produced by *Fusarium nygamai* Burgess & Trimboli, were potential mycoherbicides to control witch weed, *Striga hermonthica* (Delile) Benth. *F. compactum* species have been investigated to determine whether they produce toxins such as neosolaniol monoacetate (Lamprecht *et al*., 1989), a trichothecene which is already known to be produced by various other species of *Fusarium* (Lansden *et al*., 1978), and which was isolated for the first time from liquid cultures of a *F. compactum* strain infecting tissues of *O. ramosa* and evaluated as a means to control the seed germination of parasitic plants (Andolfi *et al*. 2005).

In conclusion, a promising approach in broomrape control seems to be to isolate fungal pathogens from one broomrape species in order to control other species. This possibility should therefore be borne in mind when looking for biological control agents to control different species within the same weed family, and it may also open the door for a broader survey to deal with other weed families. However, field trials under common agricultural practices should now be undertaken to evaluate how effective the *F. compactum* strains are against the two broomrapes and against other *Orobanche* species.

Very little is known about the formulation of *F. compactum* for use as a mycoherbicidal agent, since previous studies in this field have mostly concentrated on *F. oxysporum*, especially *F. oxysporum* f.

sp. *orthoceras* (FOO), which has been promoted as a likely candidate for weed management.

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