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RESEARCH ARTICLE

Effectiveness and technological feasibility of bioherbicide candidates for biocontrol of Green Foxtail (*Setaria viridis*)

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Green foxtail (*Setaria viridis*), one of the most common and troublesome weeds worldwide, is becoming very difficult to manage because of the lack of registered herbicides and the appearance of herbicide-resistant populations. Among the new and possible environment-friendly strategies, the use of biological control methods seems to have potential. *Drechslera gigantea, Exserohilum rostratum* and *Exserohilum longirostratum* have previously proved to be promising bioherbicide agents against several grass weeds in field trials. While previous studies have established the susceptibility of *S. viridis* under greenhouse conditions, so far no attempt has been made to establish the effectiveness and feasibility of these fungi as bioherbicides for green foxtail. When spore suspensions were applied as foliar sprays to green foxtail seedlings in a greenhouse, all three fungi caused severe damage by 1 day after application, and seedlings in most cases died within 1 week. The fungi were compatible with several agro-chemicals and host specific when tested against major vegetable crop species grown in the Mediterranean. The demonstrated technological feasibility of producing large amounts of quickly germinable conidia (i.e., asexual spores) on inexpensive solid media increases the potential of these fungi to be used as bioherbicides.

Keywords: weed biocontrol; bioherbicides; *Setaria viridis*; *Drechslera gigantea*, *Exserohilum rostratum*; *Exserohilum longirostratum*

Introduction

Green foxtail, *Setaria viridis* (L.) Beauv., a weed of European origin, is one of the most common and troublesome weeds worldwide. It is found in temperate zones as well as in the cooler subtropics of South and North America, Asia and Australia. It is very competitive with spring-sown crops, and has an abundant seed production (Holm, Plucknet, Pancho and Herberger 1977; Douglas, Thomas, Morrison and Maw 1985). Green foxtail is managed mainly by chemical herbicides, which is in disfavor for many reasons, among which the availability of fewer registered herbicides and the appearance of herbicide-resistant weeds appear to be the most important (Heap and Morrison 1996; Beckie, Thomas and Legere 1999). Among the new environment-friendly strategies proposed for its management, the use of biological control methods seems to have potential. Three promising bioherbicide candidates, *Drechslera gigantea*, *Exserohilum longirostratum* and *Exserohilum rostratum*, were

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previously tested, singly or in a mixture, against seven grasses, but not against *S. viridis* (Chandramohan and Charudattan 2001). In field trials conducted in Florida, the three isolates almost completely controlled the weeds (Chandramohan, Charudattan, Sonoda and Singh 2002). Preliminary observations were carried out also in Canada by applying, with encouraging results, the mixture of pathogens on green foxtail plants (Boyetchko, Peng, Sawchyn, Byer and Charudattan 2002). Considering that green foxtail is increasing in abundance and becoming troublesome in the Mediterranean area and that the Mediterranean population may be different from its American counterpart, it was of interest to investigate the efficacy of these three bioherbicide agents against an Italian population of *S. viridis* and to establish the possibility of their commercial development.

The objectives of this study were to evaluate: (1) the bioherbicidal performance of the three fungi against *S. viridis*, used alone or combined with an adjuvant or chemical herbicides at reduced rates; (2) the compatibility of some agrochemicals widely used in Mediterranean crops with fungal growth and (3) conidia germination; (4) the influence of media and light on mycelial growth and conidia production; (5) the conidia yield on low-cost solid substrates; (6) the speed and rate of conidial germination; and (7) the safety of the biocontrol agents towards common Mediterranean vegetable crop species.

Materials and methods

Fungal strains and plant seeds

Drechslera gigantea (Heald & Wolf) Ito., *Exserohilum longirostratum* (Subram.) Sivan., and *Exserohilum rostratum* (Drechsler) Leonard & Suggs were isolated by S. Chandramohan in Florida from large crabgrass (*Digitaria sanguinalis* (L.) Scop.), crowfootgrass (*Dactyloctenium aegyptium* (L.) Willd.) and johnsongrass (*Sorghum halepense* (L.) Pers.), respectively (Chandramohan and Charudattan 2001). The fungal strains were then stored in the fungal collection of the Institute of Sciences of Food Productions, Bari, Italy. They were also stored as stock cultures at 4°C in glass vials on potato dextrose agar (PDA, Oxoid). For inoculum production, *D. gigantea* and *E. rostratum* were transferred to PDA plates and grown at 25°C under fluorescent light and in the dark, respectively. *E. longirostratum* was transferred to tomato agar (TA) (composed of 180 mL tomato juice L^{-1}, $CaCO_3$ 1 g L^{-1}, agar 15 g L^{-1}, autoclaved at 0.75 atm for 20 min.) plates and grown with alternate 12 h D:12 h L at 25°C.

Setaria viridis seeds were collected from naturally infested fields near Bari.

Experiment 1: Bioherbicidal efficacy

Green foxtail seeds were surface sown in 10 cm diameter plastic pots containing a commercial potting medium, watered at sowing with water and 0.2% potassium nitrate (KNO_3) as mild fertilizer and then watered regularly with water only (or with water plus KNO_3). Pots were placed on a bench in a growth chamber at 25°C and 12 h photoperiod. Two-week-old plants were used for the experiment.

For inoculum, conidia suspensions were prepared as follows: fungal plates were prepared as described in the previous section. Conidia were then harvested by

flooding the plates with distilled water, gently scraping the culture surface with a rubber spatula to detach conidia and then filtering supernatant with a double-layer cheesecloth. The conidia concentration was finally adjusted to 10^6 conidia mL^{-1}.

For each of the three fungi, the following treatments were carried out with three replicate pots for each treatment:

Treatment 1: Conidial suspension.
Treatment 2: Conidial suspension combined with a wetting agent (Biopower® – Bayer CropScience or, in case of *E. longirostratum*, Astrol nuovo™ – DowAgroScience).
Treatment 3: Conidial suspension combined with a grass herbicide at reduced rate (Targa® Flo, Bayer CropScience).
Treatment 4: Water control without conidia.
Treatment 5: Water plus the respective wetting agent without conidia.
Treatment 6: Water plus the herbicide without conidia.

Biopower® and Astrol nuovo™ were used at the label rate (3.3 mL L^{-1} and 1 mL L^{-1}, respectively), while Targa® Flo was used at low rate (LR = 1/5 of the label rate, i.e.: 3.75 g L^{-1}).

The test was carried out in a greenhouse by spraying 2-week-old seedlings with a chromatography sprayer (Ecospray, Roth). For each treatment, 3 mL per pot were uniformly applied on the leaves to run-off. The inoculated plants were first held in a dew chamber for 12–14 h in the dark at 25°C and then maintained in a growth chamber, completely randomized, at the same temperature with 12 h photoperiod. Pots were watered every 2 days.

The plants were observed daily and 15 days after treatment (DAT), the fresh and dry weights of plants in each pot were recorded and expressed as average biomass.

Experiment 2: Compatibility with agrochemicals

Eight agrochemicals (Table 1), chosen from those recommended for use in Mediterranean horticultural or fruit crops, were dissolved into sterile molten PDA and then poured into 9 cm Petri dishes. Three replicates were prepared for each treatment. A control consisting of unamended PDA plates was also prepared. The plates were inoculated centrally with a 6 mm mycelial plug taken from growing colonies and incubated in a completely randomized design at 25°C under fluorescent light.

The growth of each fungal colony was recorded 4 days after inoculation (DAI) for *E. rostratum* and *E. longirostratum* and 7 DAI for *D. gigantea*, due to the different growth speeds. Two perpendicular diameters were measured per plate and the average value was expressed in mm.

Experiment 3: Effect of selected agrochemicals on conidial germination

For each strain, a conidial suspension of approximately 10^4 conidia mL^{-1} was amended with the eight agrochemicals used in the previous experiment (Table 1) in accordance with the recommended rates. Sterile dew chambers consisting of 3-well glass slides were prepared, and a 120 μL droplet of conidial suspension containing the pesticide was poured into each well. Each treatment had three replicates.

Table 1. Agrochemicals used for the compatibility tests (Experiments 1 and 2).

Product	Manufacturer	Active ingredients (w/w)	Use	Formulation	Label rate
ASTROL ™ NUOVO	Dow AgroSciences	12% sorbitan mono oleato etossilato	Wetting agent for pesticides	Soluble liquid	100 mL hl^{-1}
BIOPOWER®	Bayer CropScience	6.7% 3,6-dioxaeicosylsulphate sodium salt 20.2% 3,6-dioxaoctadecylsulphate sodium salt	Adjuvant for herbicides	Suspension concentrate	1 L ha^{-1}
BUTISAN® S	Basf	43.1% metazachlor	Broadleaved weed residual herbicide	Suspension concentrate	1.5 l ha^{-1}
DECIS®	Bayer	2.8% deltamethrin	Broad spectrum insecticide	Emulsifiable concentrate	30 mL hl^{-1}
OMITE® 57 EW	Sipcam	57% propargite	Miticide	Oil/water emulsion	100 mL hl^{-1}
RIDOMIL® GOLD M.Z.	Syngenta	64% mancozeb 3.9% metalaxil-m	Fungicide	Soluble granules	2.5 kg ha^{-1}
TARGA® FLO	Bayer	5.2% quizalofop-ethyl isomer D	Grass weed herbicide	Suspension concentrate	1 L ha^{-1}
TITUS®	DuPont	25% rimsulfuron	Broadleaved and grass weeds herbicide	Soluble granules	60 g ha^{-1}

Controls consisted of conidial suspensions without the agrochemical. The slides were incubated, completely randomized, for 24 h (8 h for *E. longirostratum*) in the dark at 25°C. The germination rate was determined under a microscope.

Experiment 4: Effects of media and light on fungal growth and conidia production

For inoculum production, a small mycelial plug was transferred from the stock culture to sterile 9 cm plastic plates containing fresh media. The plates were incubated for 3 days until adequate colony growth was observed. These plates served as the source for uniform mycelial plugs used in this test.

Four different media were used:

1. PDA (as above).
2. MA (malt extract agar, Oxoid, 50 g L^{-1}, without correction of pH, sterilized at 0.75 atm for 10 min).
3. TA (as above).
4. Czapek Agar (Czapek-Dox broth, Difco, 35 g L^{-1} plus agar technical No. 3 Oxoid, 12 g L^{-1}, autoclaved at 1 atm for 15 min).

A 6 mm diameter mycelial plug was taken from the margin of the growing PDA colonies using a cork borer, and placed centrally upside down in each Petri dish containing 16 mL of medium.

For each of the four media mentioned above, four different light conditions were used:

1. Continuous fluorescent light.
2. Continuous darkness.
3. Alternate 12 h dark/light.
4. Continuous Wood's lamps (near UV).

Fungal growth was recorded by measuring two perpendicular colony diameters in each plate every 2 days, starting at 2 DAI, up to 12 DAI, or until the full colonization of the plates.

A completely randomized design with three experimental factors (media, lighting and time) and three replicates was used.

For evaluation of conidial production, plates were prepared and the fungi were grown as previously described. Conidia were collected 15 DAI as described above. Conidia suspension was adjusted to a final volume of 10 mL. Conidia concentration was determined with the aid of a hemocytometer and expressed as number of conidia mL^{-1}.

The two experimental factors (media and lighting) were combined in a completely randomized design with three replicates.

Experiment 5: Effects of solid media on conidia production

In order to obtain large amounts of conidia by using an inexpensive, eco-friendly method, 10 media were tested by placing different amounts in each flask, according to the density of each product as reported in Table 2. The solid media were chosen considering the cheapness and the easiness of sources of supply, such as kernels, raw materials or food-industry wastes.

Remains of olive tree winter pruning, including cut up leaves and branches, were air-dried for 15 days before use. Winery dregs were oven-dried for 48 h at 60°C and stored under vacuum in a refrigerator until use. Crushed olive kernels were a by-product of olive oil extraction. Tomato skins, the waste product of tomato sauce preparation (Titian, Perfect peel and HLY 19 varieties), were freeze-dried (Labconco FreeZone freeze-dryer) and stored under vacuum at 5°C until use.

The media were placed in flasks (250 mL) with 30% (w/w) distilled water, left overnight at room temperature and then autoclaved for 20 min at 1 atm.

Flasks were inoculated with 1 mL of conidia suspension (containing 10^6 conidia) obtained as described above. The inoculated flasks were arranged in a randomized design in the dark at 25°C and incubated for 35 days. The cultures were shaken vigorously every 3 days to prevent lumps.

Conidial yield was measured 35 DAI. The number of conidia per gram of substrate was calculated by rinsing the culture with distilled water, filtering through a double layer of cheesecloth, and then quantifying the conidia by using a hemocytometer.

Experiment 6: Speed and rate of conidial germination

For each isolate, a conidia suspension (120 μL, approximately 10^4 conidia mL^{-1}) obtained as previously described, was placed in each well of a 3-well sterile glass slide

Table 2. Solid media used (Experiment 5).

Medium	Quantity (g)	Source
Bran	18	Grocery store
Broken corn grains	50	Grocery store
Broken wheat	50	Grocery store
Crushed olive kernels	50	Oil mill
Oat	50	Grocery store
Remains of olive trees winter pruning	13	Olive farm
Rice	50	Grocery store
Straw	3	Grocery store
Tomato skins	13	Tomato canning factory
Winery dregs	8	Winery

and incubated in the dark at 25°C. Conidial germination was determined after 2, 4, 6, 8, 10, and 24 h of incubation and expressed as percentage of germinated conidia. A conidium was considered germinated if the germ tube length was at least equal to that of the conidium. Approximately 200 conidia were counted for each well, with three replicates.

Experiment 7: Specificity of the potential bioherbicides

The specificity of the fungal strains was tested on some common Mediterranean vegetable crop species, which were not tested in the previous host-range study (Chandramohan and Charudattan 2001):

- *Brassica oleracea* var. *botrytis* (cauliflower);
- *Cichorium endivia* L. var. *latifolium* Lam. (endive) var. Gross Bouelle 1815;
- *Cichorium intybus* L. (chicory);
- *Citrullus lanatus* (Thunb.) Matsumara & Nakaivar (watermelon) var. Crimson Sweet;
- *Petroselinum crispum* (parsley) var. Novas;
- *Solanum melongena* L. (eggplant) var. Top bell.

Seedlings were purchased from a nursery, transplanted individually in 10 cm diameter plastic pots containing a commercial potting medium. The pots were placed in a growth chamber at 26°C and a 12 h photoperiod, and watered daily. For each species and for each biocontrol agent, 7 replicates were used.

Conidial suspensions prepared as described above (*D. gigantea* and *E. longirostratum*: 10^5 conidia mL^{-1}; *E. rostratum*: 10^6 conidia mL^{-1}) were applied evenly over the leaf surface using a chromatography sprayer. Depending on the size and number of leaves, between 2 and 5 mL of suspension was used per plant. Plants sprayed with distilled water were used as control. After inoculation, the plants were kept in a dew chamber at 26°C in the dark for 15 h in a completely randomized design, then placed in a growth chamber bench and exposed to a photoperiod of 12 h. Plants were monitored daily for about 20 days for the eventual appearance of disease symptoms.

Statistical analysis

All data collected were analysed by ANOVA. The means were compared using the least significant difference (LSD) test or Duncan's multiple range test.

Results

Experiment 1: Bioherbicidal efficacy

In treatment containing conidia, all three fungi caused light-brown necrotic spots with undefined margin on leaves 1 DAT. The spots became darker and well delineated in the following 2 days, especially in Treatment 1 (conidia alone) (Figure 1a). Finally the necrosis covered and blighted the entire leaf.

From symptomatic lesions on leaves treated with conidia of *D. gigantea*, *E. longirostratum* and *E. rostratum*, the respective fungus was isolated and the Koch's postulates were fulfilled.

D. gigantea

The application of conidia alone or conidia with the herbicide LR caused first symptoms of disease 1 DAT, which consisted of wilted leaves and necrosis (Figure 1b). The symptoms due to treatment with conidia alone became more severe 7 DAT, causing the plants to decline (Figure 1c). Conidia plus herbicide caused all plants to die at 15 DAT. Conidia alone caused a significant decrease in both fresh (data not shown) and dry (2.2 mg) weights of the plants (Figure 2a) compared to the water control (6 mg). The weight from conidia plus wetting agent and conidia plus herbicide were not significantly different from their corresponding control (Figure 2a).

E. longirostratum

Symptoms appeared 3 DAT; in particular, conidia plus wetting agent seriously compromised plant health, and conidia alone and conidia plus herbicide caused the leaves to wither and necrotize. After 2 weeks, while the control plants in the water only treatment continued to grow normally (Figure 1d), with a final dry weight of 2.1 mg, those treated with the conidia alone had reduced height, were severely damaged, and had a dry weight of 1.5 mg (Figure 2b). The combined application of conidia with the wetting agent Astrol nuovo further improved the efficacy of conidia as most plants were almost completely dried (Figure 1d). The fresh weight was significantly lower than that of plants treated with conidia alone as well as the water control (data not shown). The combined use of conidia plus the grass herbicide LR had better efficacy than all the other treatments, causing death and total desiccation of plants (Figure 1d) and significantly lowering the fresh weight compared to the other treatments (data not shown).

E. rostratum

As stated above, following treatments containing conidia (Treatments 1, 2 and 3), light-brown necrotic spots with undefined margin appeared on leaves 1 DAT. The

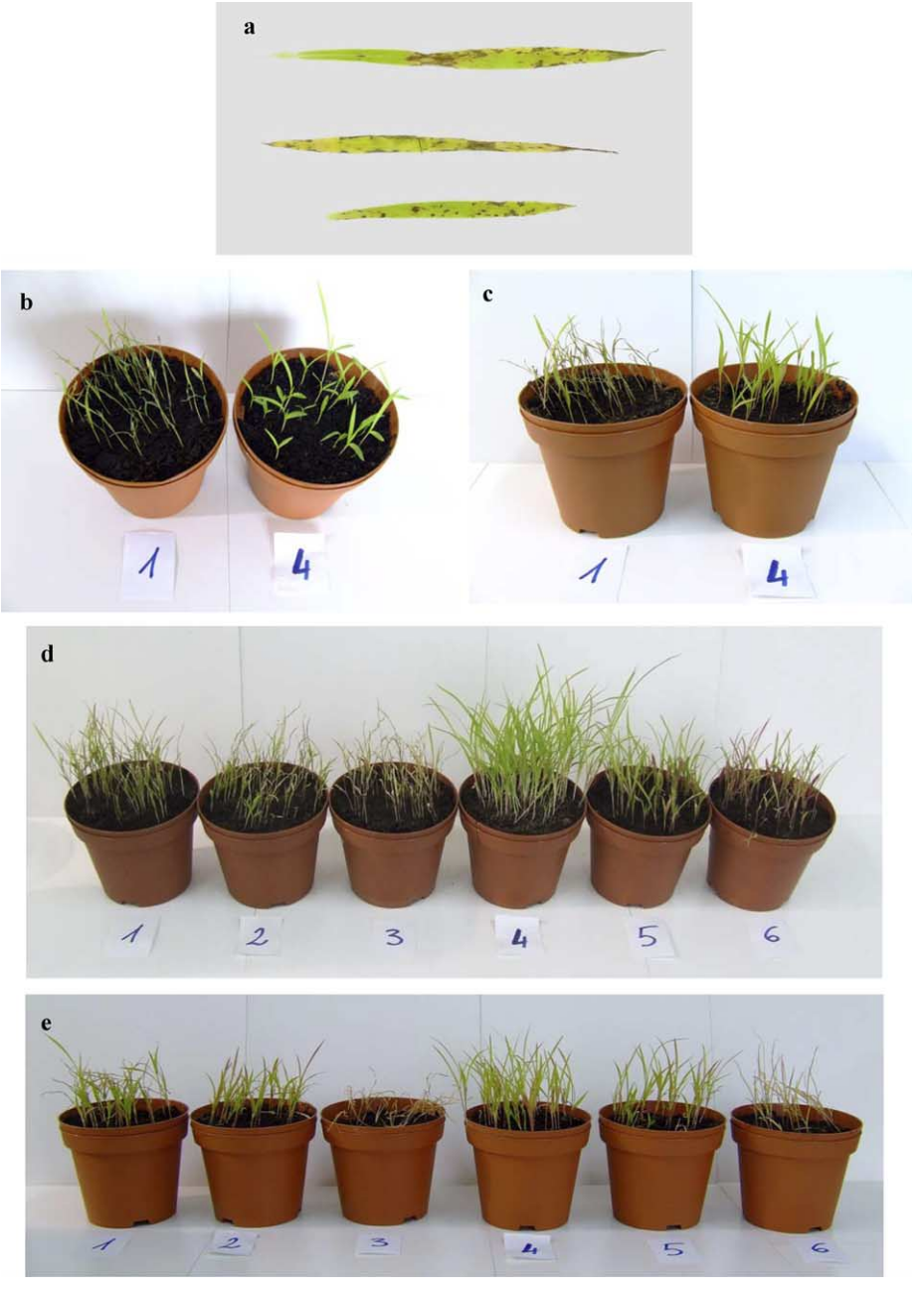


Figure 1. Symptoms caused by: (a) *E. rostratum* conidia on *S. viridis* leaves 3 DAT. The symptoms caused by the application of the other two fungi were similar to those shown here; (b) *D. gigantea* conidia (Treatment 1) on *S. viridis* seedlings, compared with the water control (Treatment 4) 1 DAT and (c) 7 DAT; (d) *E. longirostratum* and (e) *E. rostratum* on *S. viridis* 14 DAT. Treatments: 1 = conidia; 2 = conidia + wetting agent (Astrol nuovo for *E. longirostratum* and Biopower for *E. rostratum*); 3 = conidia + grass herbicide LR; 4 = water control; 5 = wetting agent control; 6 = herbicide control (Experiment 1 – see Material and methods).

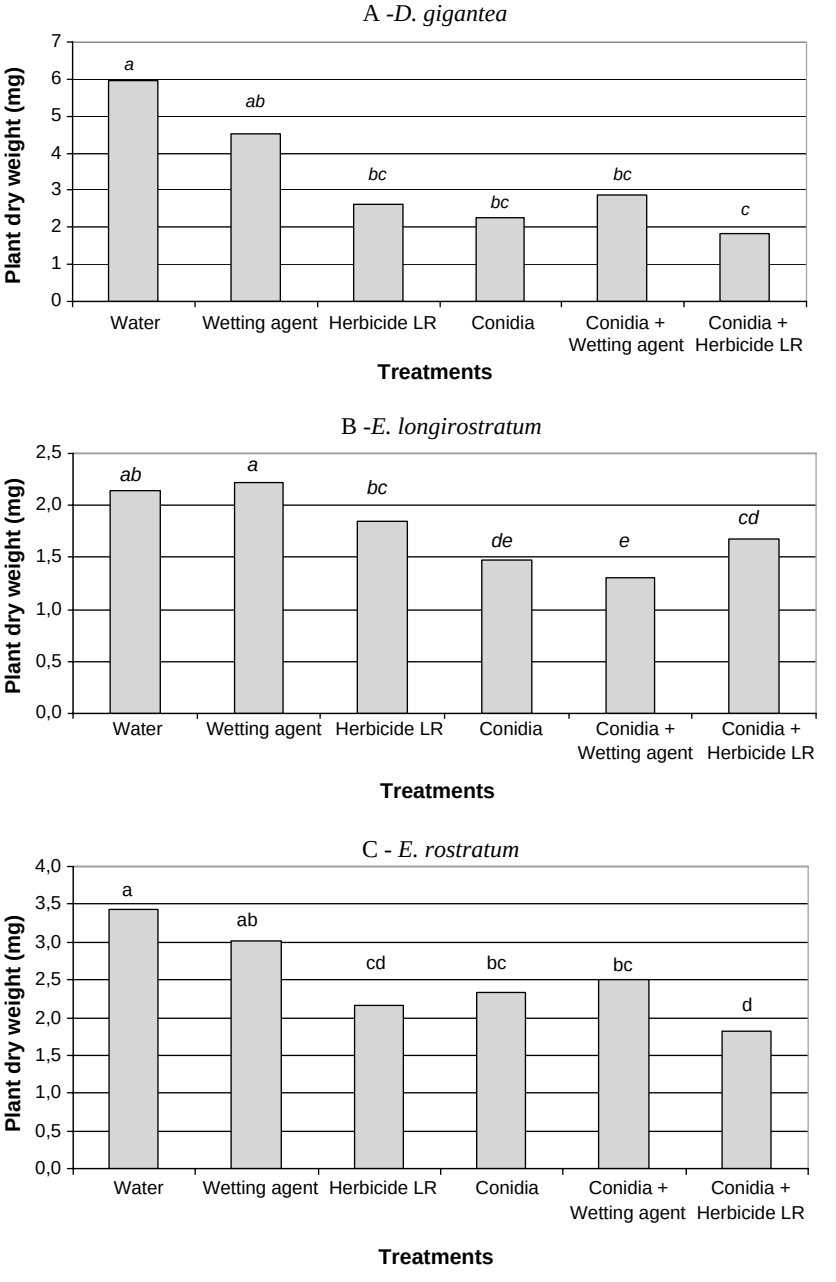


Figure 2. Effect of (a) *D. gigantea*, (b) *E. longirostratum* and, (c) *E. rostratum* treatments on dry weight of *S. viridis* plants 14 DAT. Means with the same letters are not significantly different ($\alpha = 0.05$) (Experiment 1).

necrosis became darker and well delineated in the following 2 days, especially in treatment with conidia alone (Figure 1e).

Fourteen days after treatment, the dry weight of plants sprayed with conidia alone was significantly lower (2.3 mg) than that of the water control (3.4 mg) (Figure 2c), but were not different from the treatment with the wetting agent. Plants treated with conidia alone or with conidia plus wetting agent were partially dried and reduced in size compared to the water control (Figure 2c), whereas conidia plus herbicide caused complete plant desiccation and a significant lowering of weight (Figure 2c).

Experiment 2: Compatibility with agrochemicals

D. gigantea

Seven DAI Astrol nuovo, Butisan S, Decis, Omite 57 EW and Titus had no adverse effects on mycelial growth, and the colony diameters were not significantly different compared to the control (Table 3). Significant growth reductions were observed in the presence of Biopower, Ridomil Gold MZ and Targa Flo (68, 84 and 66%, respectively) compared to the control (Table 3).

E. longirostratum

Four DAI, only Astrol nuovo and Decis had no toxic effects on mycelial growth, whereas the growth was inhibited by all other pesticides, although at different rates (Table 3): Omite 57 EW and Titus inhibited the growth of the mycelium by 25 and 29%, and Butisan S, Biopower, Targa Flo and Ridomil Gold MZ caused reductions respectively by 54, 80, 90 and 91% compared to the growth on non-amended control medium (Table 3).

Table 3. Effect of agrochemicals on fungal mycelial growth (% variation of colony diameter compared to the control) and on conidial germination (% of variation of germination compared to the control.

Product	Growth[1]			Germination[2]		
	DG	EL	ER	DG	EL	ER
CONTROL	0 ab	0 ab	0 a	0 a	0 c	0 b
ASTROL NUOVO	−18 bc	−4 b	−15 b	−1 a	+28 ab	−7 bc
BIOPOWER	−68 cd	−80 f	−83 e	−2 a	+15 b	+3 b
BUTISAN S	+18 ab	−54 e	−65 d	−58 d	−20 d	−40 d
DECIS	+26 ab	+1 a	−14 ab	−2 a	+33 a	−13 c
OMITE 57 EW	+47 a	−25 c	−17 b	−30 c	+34 a	+17 a
RIDOMIL GOLD M.Z.	−84 d	−91 g	−91 e	−52 d	−100 e	−100 e
TARGA FLO	−66 cd	−90 g	−86 e	−15 b	−1 c	+20 a
TITUS	+21 ab	−29 d	−40 c	−1 a	+27 ab	−1 bc

[1]After 7 days of exposure for DG (*Drechslera gigantean*), and 4 days of exposure for EL (*Exserohilum longirostratum*) and ER (*E. rostratum*).
[2]Germination rate determined after 24 h of exposure.
Along each column, means with the same letters are not significantly different ($\alpha = 0.05$).

E. rostratum

The mycelial growth of this isolate in presence of the insecticide Decis did not differ at 4 DAI from the non-amended PDA (Table 3). With Astrol nuovo and Omit EW 57, there was a slight negative effect on growth (15 and 17%, respectively), whereas with Titus, the reduction was more severe (40%) and much higher for Butisan S, Biopower, Targa Flo and Ridomil Gold MZ, with reductions of 65, 83, 86 and 91%, respectively (Table 3).

Experiment 3: Effect of selected agrochemicals on conidial germination

D. gigantea

Conidial germination was not affected by exposure to Astrol nuovo, Biopower, Decis and Titus (Table 3). With Butisan S, Omit 57 EW, Ridomil Gold MZ and Targa Flo, the reduction of conidial germination was moderate with values ranging between 15 and 58%. The inhibition in the case of treatment with the fungicide Ridomil Gold MZ was 52%.

E. longirostratum

Targa Flo did not inhibit germination compared to the water control (Table 3), whereas the exposure to Astrol nuovo, Biopower, Decis, Omite 57 E.W and Titus resulted in a higher germination percentage (between 15 and 34%). Butisan S reduced germination by 20%, whereas Ridomil Gold MZ completely inhibited germination.

E. rostratum

The exposure to Astrol nuovo, Biopower and Titus did not have an adverse effect on conidia germination, whereas conidia exposed to Omite 57 EW and Targa Flo had higher germination (20%) compared to the control (Table 3). Decis and Butisan S reduced germination by 13 and 40%, respectively, whereas with Ridomil Gold MZ conidial germination was completely suppressed.

Experiment 4: Effects of media and lights on fungal growth and conidia production

Colony growth

All the factors considered in the experiment (fungal nourishment, source of light and growth time) had significant influence on the growth of fungi as well as on the conidia yield.

D. gigantea. Under continuous fluorescent light (Figure 3a), 4 DAI, colonies had grown on PDA (73 mm diameter) and TA (68 mm) were statistically higher than on MA (56 mm) and Czapek (40 mm). Under continuous darkness (Figure 3b), the colony growth was much faster on MA and TA (final diameters 84 and 86 mm, respectively), compared to PDA (53 mm) and Czapek (28 mm). Almost the same results were obtained when the strain was grown under Wood's lamps (Figure 3d). Under conditions of alternate 12 h D:12 h L (Figure 3c), the growth was not different

compared to that observed under Wood's lamps, with the difference of a good growth also on Czapek (79 mm).

E. longirostratum. On 12 DAI, no statistical differences were found among the four substrates under the four lighting conditions (Figure 4). In all cases, the growth covered the entire plate (86 mm). Significant differences were found during the early growth phase under the four lighting conditions: TA often shows an average diameter significantly higher than those obtained on the other media, allowing, in the case of continuous light (Figure 4a), the colonization of the entire plate even by 4 DAI.

E. rostratum. On 12 DAI under continuous fluorescent light, Wood's lamps and alternate dark/light conditions (Figure 5a,c,d, respectively), *E. rostratum* behaved similar to *E. longirostratum*; only under continuous darkness (Figure 5b) was the average diameter value of the colony grown on MA (65 mm) statistically lower than those on PDA and TA. Significant differences were found in the intermediate stages of growth, in particular on 4 DAI where the colonies had grown more on TA under all the four conditions.

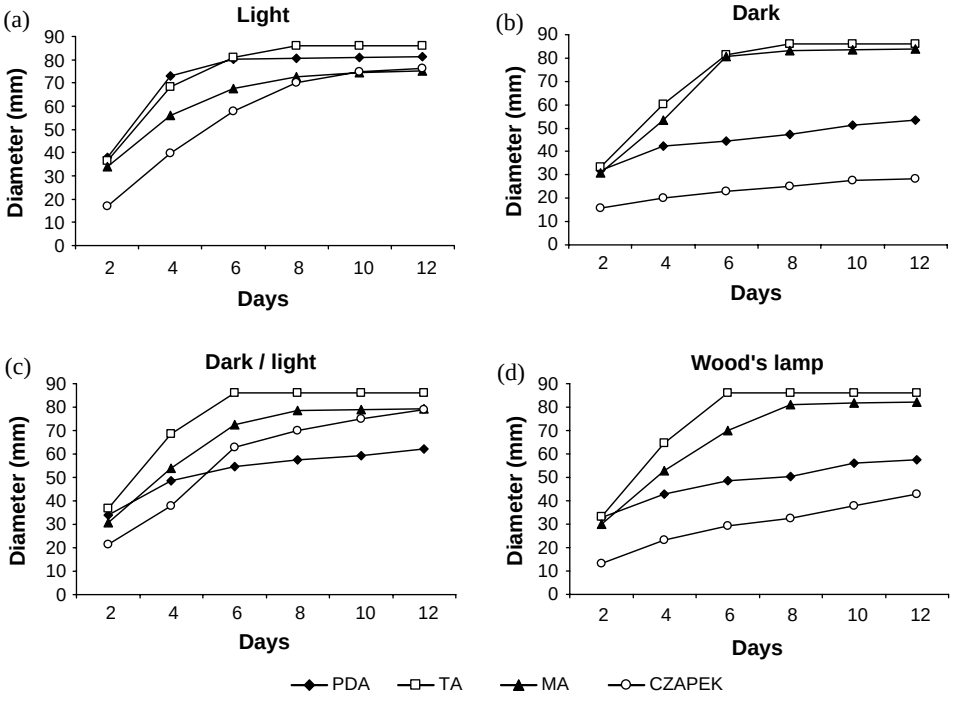


Figure 3. *D. gigantea* growth over time (colony diameter) under different lighting and media conditions: (a) Continuous fluorescent light; (b) continuous dark; (c) alternate dark/light; (d) Wood's lamp (near UV light). LSD = 15.2 ($\alpha = 0.01$) (Experiment 4).

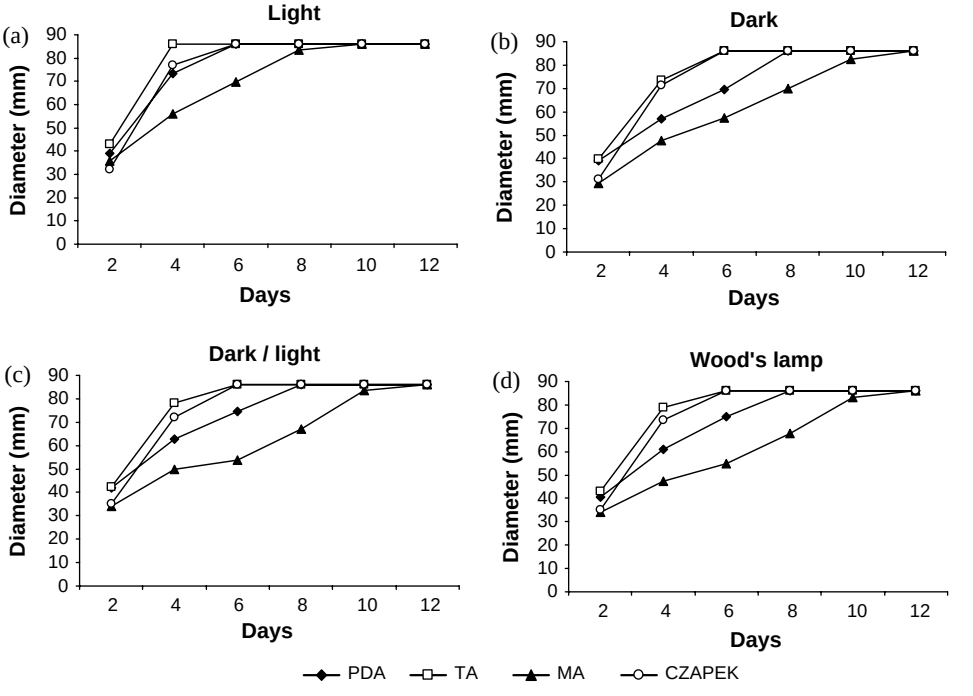


Figure 4. *E. longirostratum* growth over time (colony diameter) under different lighting and media conditions: (a) Continuous fluorescent light; (b) continuous dark; (c) alternate dark/light; (d) Wood's lamp (near UV light). LSD = 2.8 ($\alpha = 0.01$) (Experiment 4).

Conidia production

D. gigantea. For *D. gigantea* (Figure 6a), the best conidia yield was obtained on PDA under continuous fluorescent light (1.2×10^6 conidia mL^{-1}). Lower yield was obtained by using alternate dark/light (0.93×10^6 conidia mL^{-1}) or on MA under Wood's lamp (0.84×10^6 conidia mL^{-1}) and constant light (0.98×10^6 conidia mL^{-1}), but significantly higher than those relative to all other conditions. Conidia production was even significantly lower on TA and Czapek, regardless of the lighting conditions used.

E. longirostratum. As shown in Figure 6b, the best yield was obtained on TA under alternate dark/light (2.5×10^5 conidia mL^{-1}). A slightly lower production was obtained on the same medium, under constant fluorescent light (1.3×10^5 conidia mL^{-1}). All the other growth conditions negatively affected conidia production.

E. rostratum. On PDA under continuous darkness, the conidia production (7.9×10^5 conidia mL^{-1}) was significantly higher than that obtained on TA (3.7×10^5) and MA (5.1×10^5). Under the other tested conditions, conidia production was very low (Figure 6c).

The best cultural conditions for conidia production are summarized in Table 4.

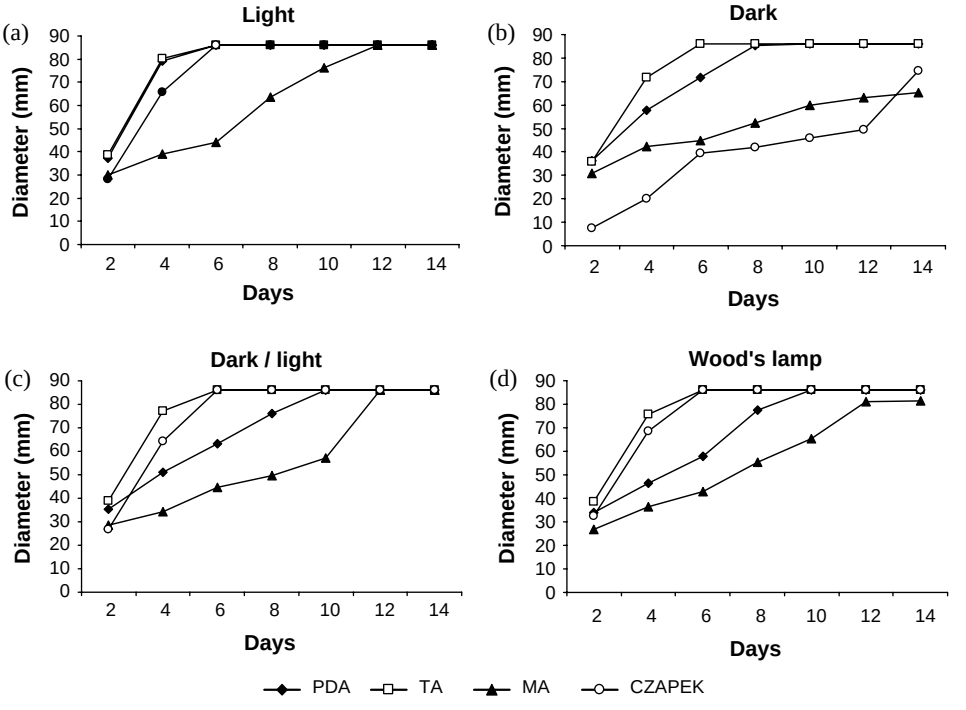


Figure 5. *E. rostratum* growth over time (diameter colony) under different lighting and media conditions: (a) Continuous fluorescent light; (b) continuous dark; (c) alternate dark/light; (d) Wood's lamp (near UV light). LSD = 15.4 ($\alpha = 0.01$) (Experiment 4).

Experiment 5: Effects of solid media on conidia production

The conidia yield obtained after 35 days of growth are shown in Figure 7 ($\alpha = 0.05$). For *D. gigantea*, the highest number of conidia per gram of substrate was obtained on broken corn grains and bran (1.43×10^6 and 1.02×10^6 conidia g^{-1}, respectively). For *E. longirostratum*, conidia production was most abundant on broken wheat grains and rice (9.37×10^6 and 8.15×10^6 conidia g^{-1}, respectively). *E. rostratum* gave best yields on broken wheat grains, broken corn grains and rice (4.13×10^6, 2.08×10^6 and 2.54×10^6 conidia g^{-1}, respectively).

Experiment 6: Speed and rate of conidial germination

Conidial germination rate of the three isolates (Figure 8), based on readings taken every 2 h for the first 10 h and then after 24 h indicates that the percentage of germination was high for all three isolates. In particular, *D. gigantea* conidia germinated at 68% after 2 h and reached 94% at 10 h of incubation. At 24 h, germination rate increased to 99%. For *E. longirostratum*, the germination rate was 52% after 2 h of incubation and 86% at 10 h. In the case of *E. rostratum*, conidia germination occurred only more slowly, being just 3% after 2 h and reaching 75% at 24 h.

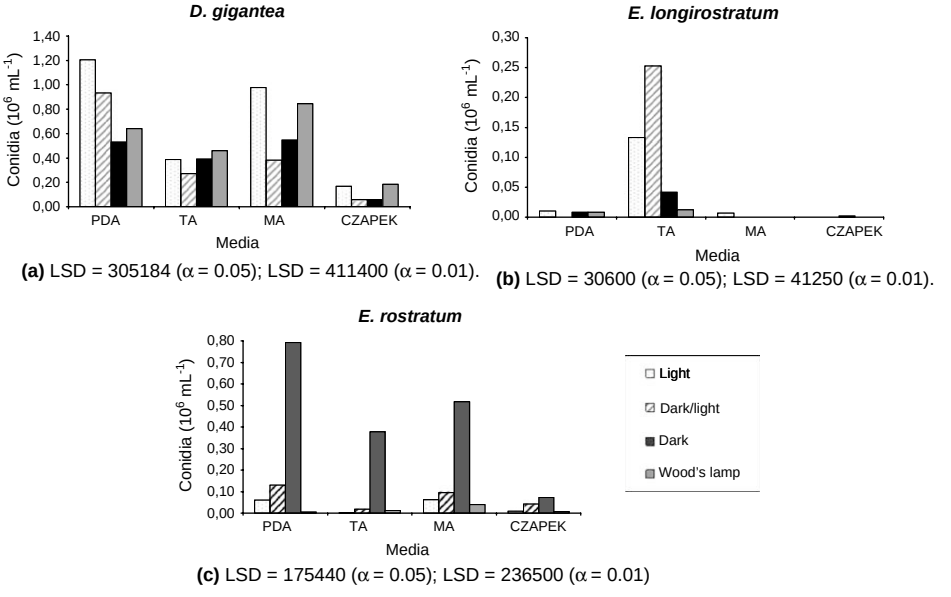


Figure 6. Conidia yield after 15 days growth on different lighting conditions and media. (a) *D. gigantea*; (b) *E. longirostratum*; (c) *E. rostratum* (c). Interaction media × light (Experiment 4).

Experiment 7: Specificity of the potential bioherbicide

In this test, no disease symptoms due to the tested isolates of *D. gigantea*, *E. longirostratum* and *E. rostratum* developed on any of the horticultural crop species screened. The plants grew normally, confirming their nonsusceptibility to the fungal strains and hence the safety in the use of the strains as bioherbicide agents.

Discussion

A very important aspect to consider when evaluating a potential bioherbicide is the speed in colonizing host tissues and in developing disease symptoms. From this point of view, *D. gigantea* and *E. longirostratum* appear to be particularly interesting (Experiment 1). Even as early as 1 DAT, these pathogens caused clear symptoms, consisting in necrotic spots widely distributed on the leaf surface, which became more severe with time. This property is particularly important in the case of grass

Table 4. Best cultural conditions for conidia production.

Isolate	Media	Lighting
D. gigantea	PDA	Light
	MA	Light
	PDA	Dark/light
	MA	Wood's lamp
E. longirostartum	TA	Dark/light
E. rostratum	PDA	Dark

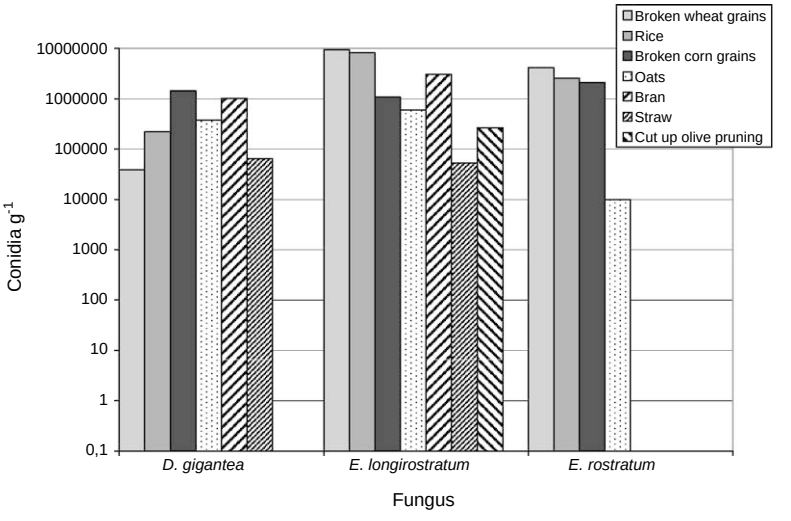
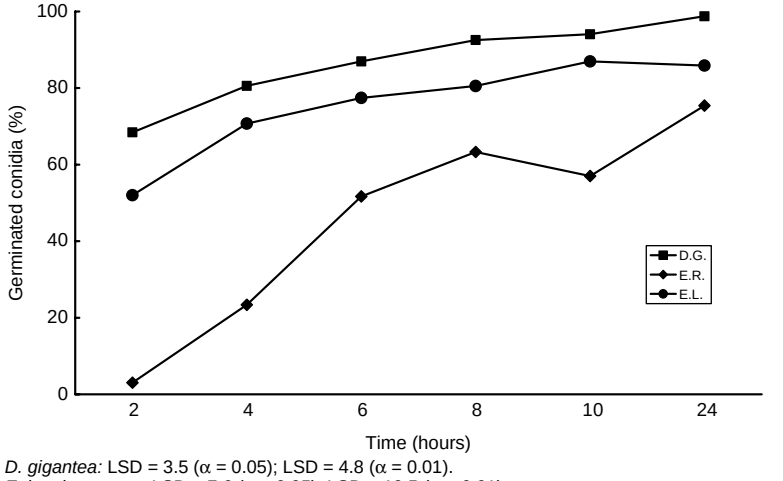


Figure 7. Conidia yield (number per dried gram) on solid organic substrates, 35 DAI. Means with the same letters are not significantly different ($\alpha = 0.05$) (Experiment 5).

weeds that are fast growing and can recover from pathogen attack, especially if the attack is not sufficiently fast in producing the disease. Moreover, the rapid symptom appearance is presumably correlated with rapid germination of conidia and penetration in the host tissues, making the pathogen less dependant on, or influenced by, the environmental conditions.

With regard to compatibility with agrochemicals (Experiment 2 and 3), it is necessary to find substances that help disperse the conidia in the suspension, prevent clumping, and favour its attachment to the host plant surface. This is



D. gigantea: LSD = 3.5 ($\alpha = 0.05$); LSD = 4.8 ($\alpha = 0.01$).
E. longirostratum: LSD = 7.6 ($\alpha = 0.05$); LSD = 10.5 ($\alpha = 0.01$).
E. rostratum: LSD = 8.5 ($\alpha = 0.05$); LSD = 11.7 ($\alpha = 0.01$).

Figure 8. Conidial germination of *D. gigantea*, *E. longirostratum* and *E. rostratum* (Experiment 6).

particularly important in the case of grass weeds, whose leaves are not very suitable for spore adhesion due to their physical structure and chemical composition. Another need for compatibility is with herbicides for synergic purposes. One approach could be the joint application of the bioherbicide with a grass herbicide at reduced rate, in order to improve the efficacy of the treatment. Another option is to use the bioherbicide with dicot herbicides, in order to control a larger spectrum of weeds. Moreover, because other pesticides might be used in integrated agriculture, it is necessary to prevent any risk of treatment failure in the field due to incompatibilities between chemical pesticides and the bioherbicide.

The agrochemicals could have two different detrimental effects (Experiments 2 and 3). The first is a direct toxic effect on conidia, affecting their viability or germination and consequently on pathogenicity and disease development. The second is an effect on the growth of the mycelium, affecting its virulence. To test these possibilities, two different experiments were carried out. The two wetting agents used behaved quite similarly, even at different concentrations, being able to negatively affect mycelia development and in general to favour conidia germination. The herbicides (Butisan, Targa Flo and Titus) generally negatively affected mycelium growth or conidia germination, or both. Butisan had quite a strong effect, being able to partially suppress (between 20 and 65%) both germination and growth. Targa Flo was much more potent against mycelial growth (up to 90% inhibition) and had lower effect on germination. Titus proved to be the less active. In the case of herbicides, the problem of toxicity could be overcome when using them at reduced rates (data not shown). As suspected, the fungicide was strongly active and almost completely inhibited the growth and germination. The other two tested agrochemicals, an insecticide and a miticide, proved to have different levels of compatibility with the different fungi. This is a further confirmation that the effects of pesticides can only be predicted in general, depending on their class, but a case-by-case test should be done to prove the actual compatibility.

With regard to the effects of different media and light conditions (Experiment 4), *D. gigantea* was more influenced by the type of media than by light conditions. The best media proved to be TA followed by MA. However, the growth in dark or under near UV light increased the differences between the media, with the growth being slower on PDA and Czapek. In the case of the two species of *Exserohilum*, their behaviour was quite similar; on average both grew faster than *D. gigantea*. They grew slowly on MA regardless of the light conditions, whereas on Czapek the growth was particularly influenced by the presence of light, with very modest growth at dark and very fast growth under all the other conditions. By contrast, for PDA, only the continuous fluorescent light positively influenced the growth.

From a technological point of view, the need for light for growth and production of conidia is certainly one of the most important limiting factors for the microbial mass production. Indeed, they are usually grown in large solid fermentation systems where light sources cannot be applied. Thus, strains that grow in the dark are of interest for further industrial development.

In this regard, conidial yield of *D. gigantea* (Figure 6) was influenced by both light and media, although in general, higher yields were obtained in the presence of light. However, on MA there was less difference between continuous light and near UV light. With regard to *E. longirostratum*, the situation was different, because the

main limiting factor was clearly the source of nourishment. This fungus yielded ample conidia (Figure 6) only on TA, and only in the presence of sufficient amount of light (better with continuous light). On the other hand, conidia production by *E. rostratum* was negatively affected by light, regardless of the type of media, with PDA at dark providing the best conditions for sporulation. Therefore, from a technological point, this latter strain would be more interesting for further studies.

With regard to conidial yield on solid media (Experiment 5), ours was an attempt to use some common and low-cost sources of nutrients (Figure 7), such as grains or food industry wastes (e.g., tomato skins). On average, broken corn or wheat kernels and rice supported better conidia production by the three fungi. This was not surprising, as kernels are a good source of nutrients, and maintain a good physical structure (hardiness and texture) which is suitable for the growth of filamentous fungi. Oat and bran also supported good conidia production. No differences were observed in percentage and speed of germination for conidia obtained from the different sources (data not shown). Only *E. longirostratum* was capable of growing on cut olive pruning. In this case, besides being a less favourable source of nutrients, oleuropein present in the olive tissue could have had a different antifungal effect against the tested strains (Bisignano et al. 1999). No growth was observed on tomato skins, winery dregs and crushed olive kernels. This was not surprising, because they all are wastes and exhausted material from industries. In particular, tomato skins were probably not suitable due to their physical structure (very tiny and crispy layers), presumably not supporting the fungal colonization. The crushed wastes of olive stones are obtained after pressure-extraction of the oil (extra virgin oil), and then a further extraction by solvents (oil of sansa). So, it is a poor source of nutrients and, as it contains mostly lignin, it is frequently used as a natural fuel. This medium, although not favourable to fungal growth, could be used as a physical support, for example by mixing it with other cheap suitable sources of nutrients. Indeed, from a physical point of view, it could resemble other polymeric inert supports that have been used in fermentation processes (Ooijkaas, Weber, Buitelaar, Tramper and Rinzema 2000). The winery dregs are obtained after yeast fermentation, and could be not suitable for further microbial growth because of the high content in potassium and sodium salts of tartaric acid, or to the presence of tannins. Also in this case, they could be mixed with other nutrients, obtaining cheap recipes suitable for fungal sporulation. The use of agro-industrial wastes would also add an environmental value to the use of biopesticides, because it would contribute to the recycling of organic materials. This is the reason why, besides some very common and cheap kernels, we tried some other wastes easily and cheaply available in Mediterranean agriculture.

From an industrial perspective, besides efficacy, the most important factors affecting the attractiveness of a bioherbicide are the total conidia yield (or, better, the cfu), the ease of production, and a fairly low effective dose to be applied to the fields (conidia ha^{-1}). Obviously, a conidia yield that is 10-fold higher or lower can completely change the efficiency of a biofactory producing the bioherbicide product and its competitiveness in the market.

The speed of colonizing the host tissue seems to be confirmed by the prompt germination of conidia observed in the *in vitro* test (Experiment 6). In that experiment, the conidia of all strains were highly capable, with a percentage of germination at nearly 100%, 24 h after treatment. The germination was already

high 8 h after treatment, but particularly noteworthy is that, after 2 h, around 50–70% of conidia of *E. longirostratum* and *D. gigantea*, respectively, had already germinated. This finding is in agreement with the results of the greenhouse experiments, in which these two fungi produced symptoms the fastest. In particular, *D. gigantea* conidia, when applied alone, acted much faster than the herbicide, and its joint application with the surfactant appeared to be partially hampered by this latter (see above). *E. longirostratum* was very active on *S. viridis* and even on *Poa annua* (data not presented) causing clear symptoms 3 DAT. In this case, the efficacy of the fungus seems to be improved by adding both the herbicide at reduced rate and the surfactant. *E. rostratum* was faster than *E. longirostratum* in producing disease, and it seems likely that its efficacy can be helped by the joint application of the grass herbicide at reduced rate. Thus, all three fungi were effective in controlling 2-week-old green foxtail plants, when applied at 10^6 conidia mL^{-1}. Treatment of older plants or application of lower doses resulted in a lowering of efficacy (data not shown).

The results obtained further validate the potential of the three pathogens as biological control agents against *S. viridis*.

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