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Evaluation of biological control agents for managing cucurbit powdery mildew on greenhouse-grown melon

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An evaluation was made of the ability of two mycoparasite-based products AQ10® (*Ampelomyces quisqualis*) and Mycotal® (*Lecanicillium lecanii*), as well as three strains of *Bacillus subtilis*, to manage powdery mildew disease, caused by *Podosphaera fusca* on melon seedlings maintained under different regimes of relative humidity and on plants grown under greenhouse conditions in Spain. In every case fungal and bacterial biocontrol agents (BCAs) performed better under conditions of high relative humidity (90–95% RH). In greenhouse experiments, the effectiveness of the mycoparasites to manage powdery mildew was absolutely dependent on mineral oil. The strains of *B. subtilis* provided disease control similar to that achieved with the mycoparasites or the fungicide azoxystrobin. Microscopic analysis showed the ability of these bacterial strains to efficiently colonize leaf surfaces and revealed the occurrence of antagonistic interactions between biological agents and *P. fusca* structures. These results confirmed the usefulness of these BCAs for managing powdery mildew on greenhouse-grown cucurbits either as single products or as a component of integrated control programmes.

Keywords: *Ampelomyces quisqualis*, azoxystrobin, *Bacillus subtilis*, *Cucumis melo*, *Lecanicillium lecanii*, mineral oil, *Podosphaera fusca*

Introduction

Powdery mildew fungi represent one of the most widely distributed and destructive groups of plant pathogens worldwide (Braun *et al*., 2002). Field- and greenhouse-grown cucurbit crops are often threatened by powdery mildews, which reduce yield and cause important economic losses (Jarvis *et al*., 2002). The disease is clearly distinguished by the development of white, powdery mould on both leaf surfaces, on petioles and on stems. *Golovinomyces cichoracearum* and *Podosphaera fusca* are the two most commonly recorded fungal species (Braun *et al*., 2002); however, to date, *P. fusca* has been identified as the sole cause of the disease in Spain (Torés *et al*., 1990; del Pino *et al*., 2002, Fernández-Ortuño *et al*., 2006). At present, the main management practices are the use of resistant cultivars or repeated fungicide applications. However, the limited availability of commercially acceptable resistant cultivars, lack of resistance in some cucurbit crop types and increasing public concerns about potential impact of pesticides on the environment, have necessitated alternative or complementary methods that are effective, reliable and environmentally safe (McGrath, 2001; Kiss, 2003). Biological control agents (BCAs) have received most of the attention because of their versatile modes of action to protect plants and their potential to be included in integrated management programmes (Shoda, 2000; Paulitz & Bélanger, 2001).

For biocontrol of powdery mildews, mycoparasites such as *Ampelomyces quisqualis* (Kiss, 2003; Szentivanyi & Kiss, 2003; Kiss *et al*., 2004; Sztejnberg *et al*., 2004) or *Lecanicillium lecanii* (Dik *et al*., 1998; Verhaar *et al*., 1999a) have been, by far, the most explored strategies. These micro-organisms invade and degrade fungal structures, providing adequate disease control mainly under greenhouse conditions and moderate pathogen density (Paulitz & Bélanger, 2001). Alternatively, although less studied, the use of antibiotic-producing micro-organisms represents another interesting strategy to manage powdery mildews. Yeast-like fungi belonging to the genera *Pseudozima* (Bélanger *et al*., 1994) and *Tilletiopsis* (Urquhart *et al*., 1994) and bacteria from the

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genus *Bacillus* (Romero *et al*., 2004) have been described as providing adequate control of cucurbit powdery mildew by means of the production and release to the surroundings of antifungal compounds that affect the viability of powdery mildew conidia and hyphae. *Bacillus subtilis* and other related species, ubiquitous microorganisms which are considered safe, are being widely evaluated as BCAs (Emmert & Handelsman, 1999). Their most striking features include good colonization abilities, production of endospores, which confers exceptional advantages for long-term storage and formulation of *Bacillus*-based products (Shoda, 2000; Reva *et al*., 2004), and their ability to produce numerous antimicrobials that are involved in disease suppression (Asaka & Shoda, 1996; Raaijmakers *et al*., 2002; Stein, 2005). Nevertheless, it is often assumed that the protective effect provided by *Bacillus* is the result of the combined action of multiple mechanisms (Shoda, 2000; Kloepper *et al*., 2004).

For biological control of aerial plant diseases, it has been well established that BCAs require specific environmental conditions, such as high relative humidity, for optimal suppressive activity (Verhaar *et al*., 1999b; Kiss, 2003), and it is assumed that BCAs should perform better under greenhouse conditions than under field conditions (Paulitz & Bélanger, 2001). Previous reports have demonstrated the ability of two commercial mycoparasitic fungi, *Ampelomyces quisqualis* (AQ10®) and *Lecanicillium lecanii* (Mycotal®), as well as three *B. subtilis* strains, UMAF6614, UMAF6639 and UMAF8561, to control powdery mildew disease elicited by *P. fusca* on detached melon (*Cucumis melo*) leaves (Romero *et al*., 2003; 2004). The aim of this study was to evaluate the effect of relative humidity on the biocontrol effectiveness of those agents in experiments using melon seedlings maintained in growth chambers. Additionally, the protective action of those agents was validated in greenhouse experiments where the utility of integrating BCAs with other control means was also investigated.

Materials and methods

Pathogen and biocontrol agents

Isolate SF26 of *P. fusca* race 1 was used (Pérez-García *et al*., 2001). The isolate was routinely grown *in planta* on cotyledons of zucchini (*Cucurbita pepo*) cv. Negro Belleza and maintained *in vitro* as described elsewhere (Álvarez & Torés, 1997). For long-term storage, the isolate was preserved at –80ºC using silica gel (Pérez-García *et al*., 2006). Three bacterial strains and two fungi were evaluated as BCAs against cucurbit powdery mildew. The mycoparasitic fungi *A. quisqualis* and *L. lecanii* were used as the formulated products AQ10® (Ecogen) and Mycotal® (Koppert Biological Systems), respectively. The *B. subtilis* strains UMAF6614, UMAF6639 and UMAF8561, previously selected for their antagonistic activity towards *P. fusca* (Romero *et al*., 2004), were also used. Bacterial strains were maintained for long-term storage at –80ºC in Luria Bertani broth (LB) with 9% DMSO as cryoprotectant.

Preparation of pathogen inoculum and control treatments

The inoculum of *P. fusca* was prepared as previously described (Romero *et al*., 2003) and the concentration of spore suspension was adjusted to 1×10^4 conidia mL^{-1} with distilled water. Spore suspensions of mycoparasites were adjusted to 5×10^5 spores mL^{-1} (Romero *et al*., 2003). For greenhouse experiments, the commercial mineral oil ADDIT (Koppert Biological Systems) was added at 2·5 mL L^{-1} (dose recommended by supplier) to spore suspensions of mycoparasites, unless otherwise specified.

For growth chamber experiments, bacterial suspensions were obtained from laboratory liquid cultures and adjusted to 10^8 cfu mL^{-1} as previously described (Romero *et al*., 2004). For greenhouse experiments, suspensions were obtained by fed-batch fermentation (Oh *et al*., 1995) in a 5 L bioreactor Biostat®-B (Sartorius AG) as follows: first, a bacterial pre-inoculum was prepared in nutrient broth (NB) after two consecutive incubations at 30ºC and agitated at 200 rpm for 24 h. The seed culture was then obtained in GCYS medium (glucose 10 g, casaminoacids 10 g, yeast extract 10 g, $MgSO_4$ 0·1 g and K_2HPO_4 1 g L^{-1}) after two consecutive incubations under the same conditions as above for 6 and 20 h, respectively. Thereafter, a 5 L bioreactor with an initial working volume of 3 L of GCYS medium was inoculated with 300 mL of seed culture to yield an initial optical density (OD) of 0·3 at a wavelength of 500 nm, and the incubation conditions were set at 30ºC, agitation at 250–1200 rpm and an aeration of 1 vvm (L air/L broth/min) by the headspace method to avoid foaming. After 17 h of incubation, the culture was fed with a peptone (200 g L^{-1}) and glucose (100 g L^{-1}) solution at a feed rate ramping from 0·8 to 2·4 mL min^{-1}. Cells were harvested at late log phase after 27–30 hours of incubation by centrifugation at 3000 *g* for 20 min at 4ºC, and the bacterial pellets were suspended in 2 L of GCYS with 9% DMSO and stored at –80ºC in aliquots of 250 mL. For each treatment, bacteria were thawed and centrifuged, and the bacterial pellet was suspended in sterile distilled water. The cell suspension was then adjusted to 10^8 cfu mL^{-1} (Ji *et al*., 2006).

A conventional fungicide treatment, (Ortiva; Syngenta), active ingredient azoxystrobin was included for comparison in the greenhouse trials. The fungicidal solution was prepared in distilled water and adjusted to 0·8 mL L^{-1} (0·2 g L^{-1} a.i. azoxystrobin), the maximum authorized dose for melon.

Growth chamber experiments

Melon seedlings of the 'universally susceptible' cv. Rochet, with the third leaf completely expanded, were used. Conidial suspensions of *P. fusca* were spread over the upper surfaces of the second and third leaves (Pérez-García *et al*., 2001). Mycoparasites were applied 3 days after inoculation of *P. fusca* (Romero *et al*., 2003). Bacterial suspensions were applied 4 h before pathogen inoculation. Inoculated plants were maintained at 25°C under a

16 h photoperiod, 3800 lux intensity and 75–80 or 90–95% relative humidity (Romero *et al*., 2004).

Greenhouse experiments

Three biocontrol experiments were conducted in an experimental greenhouse during the months of May and October of 2005 and May of 2006. For each experiment, a set of 400–500 plants of the melon cv. Rochet were raised in seedling trays in a nursery-like greenhouse under constant temperature (25ºC). After 3 weeks, plants with the first leaf completely expanded were transplanted into bigger plastic pots of 16 L and placed in the experimental greenhouse. The melon plants were routinely watered with a complete nutrient solution and pesticides were applied as required to control whiteflies and other insect pests.

At the 8-leaf stage, plants were arranged in three experimental plots consisting of six rows with 20 plants each. Distances between plants were 40 cm in the rows and 1 m between rows. All treatments were arranged within each plot in a completely randomized block design, providing at least three replicates of four plants for each treatment. The three experiments encompassed the following treatments: (i) control untreated plants, (ii) plants treated with tap water, (iii) Ortiva, (iv) AQ10 and ADDIT, (v) Mycotal and ADDIT, (vi) ADDIT, (vii) *B. subtilis* UMAF6614, (viii) *B. subtilis* UMAF6639, (ix) *B. subitlis* UMAF8561. Experiment II included an additional integrated treatment: (x) Ortiva alternated with *B. subtilis* UMAF6614 as the second treatment. Experiment III also included the following integrated treatments: (xi) ADDIT alternated with *B. subtilis* UMAF6614, (xii) *B. subtilis* UMAF6614 alternated with AQ10 and ADDIT, (xiii) *B. subtilis* UMAF6614 alternated with Mycotal and ADDIT. Additionally, in Experiment III, treatments with mycoparasite-based products without mineral oil were included: (xiv) AQ10 and (xv) Mycotal. Three or four leaves per plant were inoculated with *P. fusca* by spraying a conidial suspension over the upper surface until visible drops formed on the leaf surface. Treatments were applied twice in each experiment. The first application was done when the initial mildew colonies were observed, 3–4 days after *P. fusca* inoculation. The second application was done 10 days after the first application. Applications were carried out with a Matabi hand-sprayer (Goizper) equipped with a CHS-3AN trigger sprayer (Canyon Corporation) until drip-off and always in the evenings to ensure the longest period with the highest relative humidity.

Disease assessment and data analysis

For the growth chamber experiments, results were recorded 16 days after inoculation. Disease severity expressed as percentage of leaf area covered by powdery mildew was estimated and disease reduction was calculated as previously described (Romero *et al*., 2004). In the greenhouse experiments, data were recorded once per week on powdery mildew severity, and the number of mildewed leaves per plant (incidence). At the end of the experiments disease reduction indexes were deduced from severity values. The effect of different treatments upon powdery mildew sporulation was estimated by conidial counts at the end of each greenhouse experiment. Five leaves per plot were sampled and leaf discs 1 cm in diameter were taken, submerged in a solution of 0·2 mL L^{-1} Tween 20 and placed on a rolling bench for 2 h. The suspended conidia were then counted on a haemocytometer and expressed as number of conidia produced per cm^2 of leaf tissue.

Data were analysed by statistics software SPSS 8·0 (SPSS). The one-way analysis of variance (ANOVA) was used and treatment means were separated by Fisher's protected least significant difference (LSD).

Bacterial colonization and microscopic analysis of interactions

The bacterial population density, as well as the proportion of endospores of each *B. subtilis* strain present on the leaves, was recorded 12 h after the first application and at the end of each greenhouse experiment as previously reported (Romero *et al*., 2004). For microscopic analysis of the interactions between *P. fusca* and BCAs, leaves of untreated plants and plants treated with bacteria or mycoparasitic fungi were randomly sampled and processed for scanning electron microscopy as described elsewhere (Pérez-García *et al*., 2001).

Results

Effect of relative humidity on biocontrol

Considering the extensively reported effect of relative humidity on the effectiveness of BCAs, experiments were carried out using melon seedlings maintained in growth chambers under different regimes of relative humidity. As shown by the percentages of leaf area covered by powdery mildew and the indexes of disease reduction (Table 1), both mycoparasites and the *B. subtilis* strains were more efficient at 90–95% relative humidity (RH), achieving disease reduction values ranging from 60 to 90%, than at 75–80% RH, with reductions up to 77%. Combining mycoparasites did not significantly improve disease reduction at 90–95% RH, although at 75–80% RH a slight synergistic effect was observed. In general terms, mycoparasites worked slightly better than *B. subtilis*; however, at 90–95% RH, differences were not statistically significant in most cases, with the only exceptions being AQ10, which achieved the best disease reduction (90%), and *B. subtilis* strain UMAF6639, which showed the lowest reduction value (60%).

Performance of BCAs under greenhouse conditions

In order to evaluate the suppressive effect of the different BCAs and integrated treatments on powdery mildew

Table 1 Effect of relative humidity (RH) on severity of powdery mildew (*Podosphaera fusca*) on melon seedlings and reduction of disease achieved by treatments with mycoparasitic fungi *Ampelomyces quisqualis* (AQ10) and *Lecanicillium lecanii* (Mycotal) and *Bacillus subtilis* strains. Disease severity expressed as percentage of leaf area covered by powdery mildew was recorded after 16 days of incubation in growth chambers

	75–80% RH		90–95% RH	
	Severity	% Reduction	Severity	% Reduction
Untreated	60 a[a]	–	63 a	–
Water	53 a	11[b]	55 a	14
AQ10	23 c	62	6 c	90
Mycotal	23 c	62	12 bc	81
AQ10 + Mycotal	14 e	77	7 bc	89
B. subtilis UMAF6614	30 bd	50	19 bc	69
B. subtilis UMAF6639	25 cd	58	25 b	60
B. subtilis UMAF8561	34 b	44	14 bc	78

[a]Values followed by different letters within a column are significantly different according to the LSD test ($P = 0{\cdot}05$).
[b]Percentage of disease reduction achieved by each treatment referred to values of leaf area covered by powdery mildew in untreated controls.

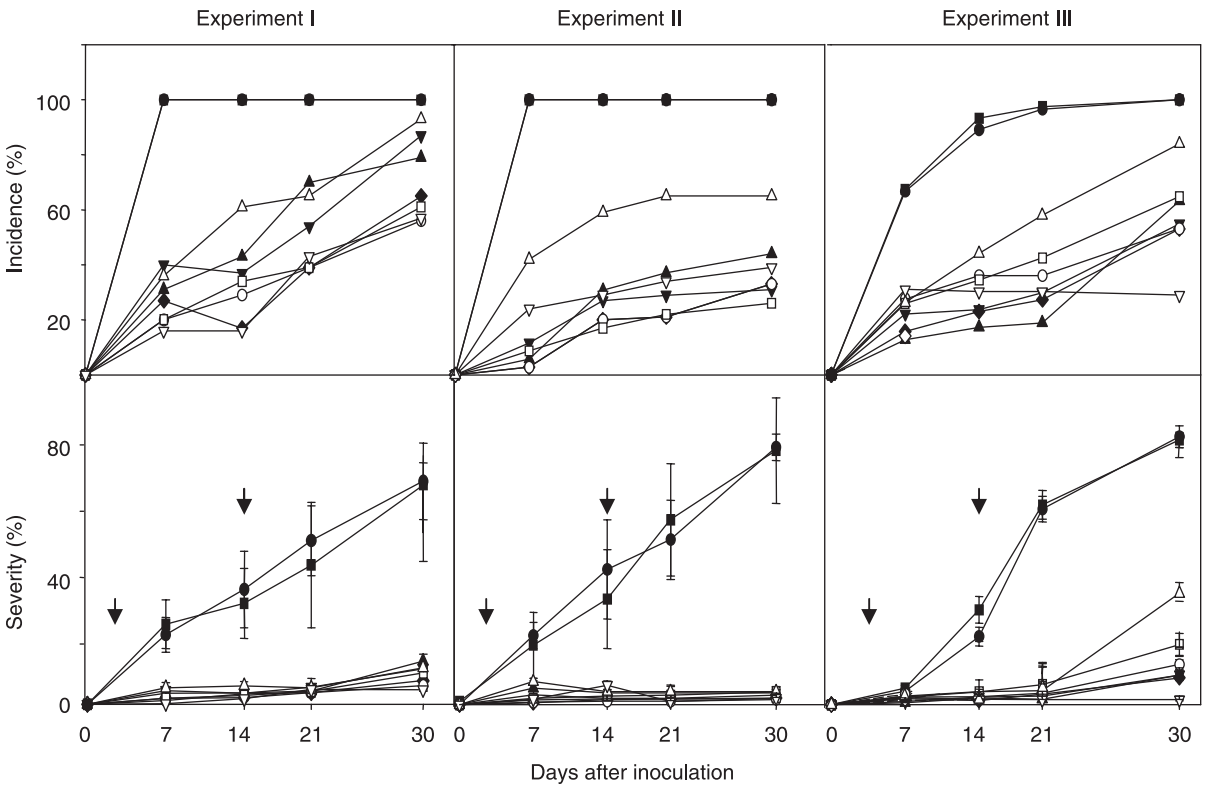

Figure 1 Disease progress of powdery mildew on melon and effect of biological control treatments in greenhouse experiments. Disease incidence (percentage of infected leaves) and disease severity (percentage of leaf area covered by powdery mildew) 7, 14, 21 and 30 days after inoculation with *Podosphaera fusca*. Applications of treatments are indicated with arrows. Means obtained in three independent experiments are shown; standard deviations are only given for severity values. Treatment included: untreated (●), water (■), Ortiva (▽), AQ10+ADDIT (□), Mycotal+ADDIT (○), *Bacillus subtilis* UMAF6614 (◆), *B. subtilis* UMAF6639 (▼), *B. subtilis* UMAF8561 (▲), ADDIT (△).

disease progress in greenhouse experiments, the disease incidence and severity were recorded (Fig. 1). Inoculation of plants with *P. fusca* conidial suspensions adjusted to 10^4 conidia per mL resulted in severe disease symptoms, causing incidence values of 100%, as observed for untreated plants 7 or 14 days after inoculation, and severity records ranging from 60 to 80% of leaf area covered by powdery mildew after 30 days (Fig. 1). The experiments were terminated 30 days after pathogen inoculation, when disease symptoms became extremely severe with a predominance of necrotic and senescent leaves (Fig. 3a).

In general all the biological treatments delayed the progress of powdery mildew disease compared with the untreated controls, but the biological treatments performed differentially depending on the season in which the experiments were carried out. The best results obtained for all the biological treatments were achieved in experiment II conducted in October 2005, when the lower oscillations in RH were recorded (Fig. 4) and where the

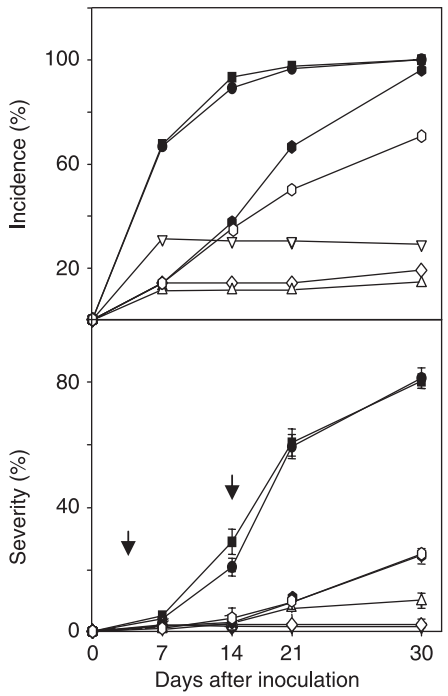

Figure 2 Disease progress of powdery mildew on melon and effect of integrated treatments in greenhouse experiments. Disease incidence (percentage of infected leaves) and disease severity (percentage of leaf area covered by powdery mildew) 7, 14, 21 and 30 days after inoculation with *Podosphaera fusca*. Applications of treatments are indicated by arrows. Treatments included: untreated (●), water (■), Ortiva (▽), 1st Ortiva, 2nd *Bacillus subtilis* UMAF6614 (◇), 1st ADDIT, 2nd *B. subtilis* UMAF6614 (△), 1st *B. subtilis* UMAF6614, 2nd AQ10 (⬮), 1st *B. subtilis* UMAF6614, 2nd Mycotal(0).

incidence in experiment II was generally lower than in the other experiments (Fig. 1). Azoxystrobin (Ortiva) was the most effective control treatment, maintaining powdery mildew disease at especially low levels during the different experiments, independently of the season (Table 2).

Only in combination with the mineral oil ADDIT were the mycoparasite-based products AQ10 or Mycotal most effective, showing disease reductions of 80–95%. In the absence of mineral oil, however, severity values obtained for plants treated with mycoparasites were not statistically different from untreated or water controls (Table 2). Furthermore, applications of ADDIT alone also provided very good protection levels with the exception of experiment III. The *B. subtilis* strains without any complementary additive were also very effective in controlling disease, achieving a disease reduction similar to mycoparasites with mineral oil. Integrated treatments in which the strain UMAF6614 was applied, alternately with the other control methods (fungicide, mineral oil or mycoparasites) did not significantly improve the protective effect of the bacteria alone (Fig. 2). When *B. subtilis* alternated with mycoparasites, disease control was less effective (Table 2).

The impact of treatments on the sporulation of *P. fusca* was evaluated at the end of the greenhouse experiments 30 days after pathogen inoculation (Table 3). Untreated leaves and leaves treated with water produced large numbers of conidia (1200–1800 conidia cm^{-2}), quantities consistent with the previously observed disease severities of around 65–80% of leaf area covered by powdery mildew (Table 2). In contrast, in most of the biological treatments, conidial production was practically eliminated (Table 3), resulting in sporulation reduction indexes around 90% which were not significantly different from those observed

Table 2 Severity and percentage disease reduction of powdery mildew (*Podosphaera fusca*) in greenhouse-grown melon plants. Disease severity as percentage of leaf area covered by powdery mildew was recorded 30 days after inoculation

	Experiment I		Experiment II		Experiment III	
Treatments	Severity	% Reduction	Severity	% Reduction	Severity	% Reduction
Untreated	65 a[a]	–	78 a	–	81 a	–
Water	63 a	3[b]	77 a	1	80 a	1
Ortiva (0·8 mL L^{-1})	4 d	94	2 b	97	2 d	97
AQ10 + ADDIT	9 bcd	86	4 b	95	18 c	78
Mycotal + ADDIT	6 cd	91	2 b	97	12 dc	85
ADDIT (2·5 mL L^{-1})	11 bc	83	3 b	96	34 b	58
Bs UMAF6614[c]	7 bcd	89	2 b	97	8 d	90
Bs UMAF6639	10 bcd	84	2 b	97	11 d	86
Bs UMAF8561	12 b	80	3 b	96	9 d	89
1st Ortiva, 2nd *Bs* UMAF6614[d]	–	–	2 b	97	2 d	97
1st ADDIT, 2nd *Bs* UMAF6614	–	–	–	–	9 dc	89
1st *Bs* UMAF6614, 2nd AQ10 + Addit	–	–	–	–	24 cb	70
1st *Bs* UMAF6614, 2nd Mycotal + Addit	–	–	–	–	25 cb	69
AQ10	–	–	–	–	73 a	9
Mycotal	–	–	–	–	65 a	19

[a]Mean values followed by different letters within a column were significantly different accordingly to the LSD test ($P = 0{\cdot}05$).
[b]Percentage of disease reduction achieved by each treatment referred to values of leaf area covered by powdery mildew in untreated controls.
[c]*Bs* = *Bacillus subtilis*.
[d]First and second applications consisted of different treatments.

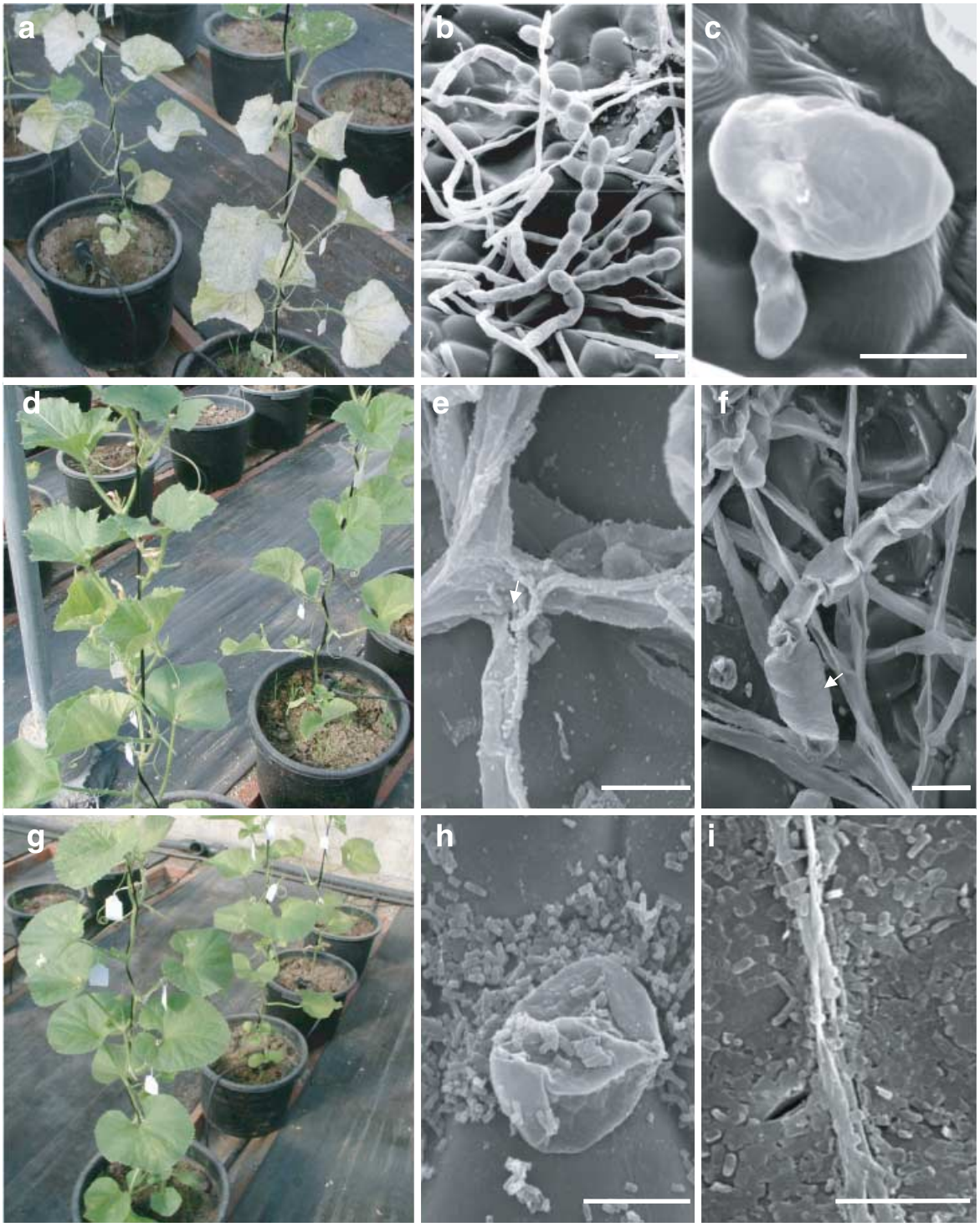

Figure 3 Effect of biocontrol agents on cucurbit powdery mildew and scanning electron microscopy (SEM) analysis of the interactions between *Podosphaera fusca* and biocontrol agents. Pictures and samples for SEM analysis were taken 30 days after pathogen inoculation. (a) Untreated plants showing abundant and typical powdery mildew symptoms. (b) SEM micrograph of a *P. fusca* colony from an untreated plant showing typical conidiophores. (c) Detail of a germinating conidium of *P. fusca*. (d) Plants treated with AQ10 and ADDIT and showing only few powdery mildew colonies. (e) The arrow indicates hyphae of *Lecanicillium lecanii* surrounding and penetrating hyphae of *P. fusca*. (f) Interaction between *Ampelomyces quisqualis* and *P. fusca*; note an altered conidiophore of *P. fusca*. The arrow shows a typical swelling at the base of a conidiophore produced by internal development of a pycnidium of *A. quisqualis*. (g) Plants treated with *Bacillus subtilis* UMAF6614 and showing only few powdery mildew symptoms. (h) Attachment of cells of *B. subtilis* UMAF6614 to a *P. fusca* conidium and bacterial aggregates on melon leaf surface. (i) Cells of *B. subtilis* UMAF6639 colonizing a hypha of *P. fusca*. Bars = 10 μm.

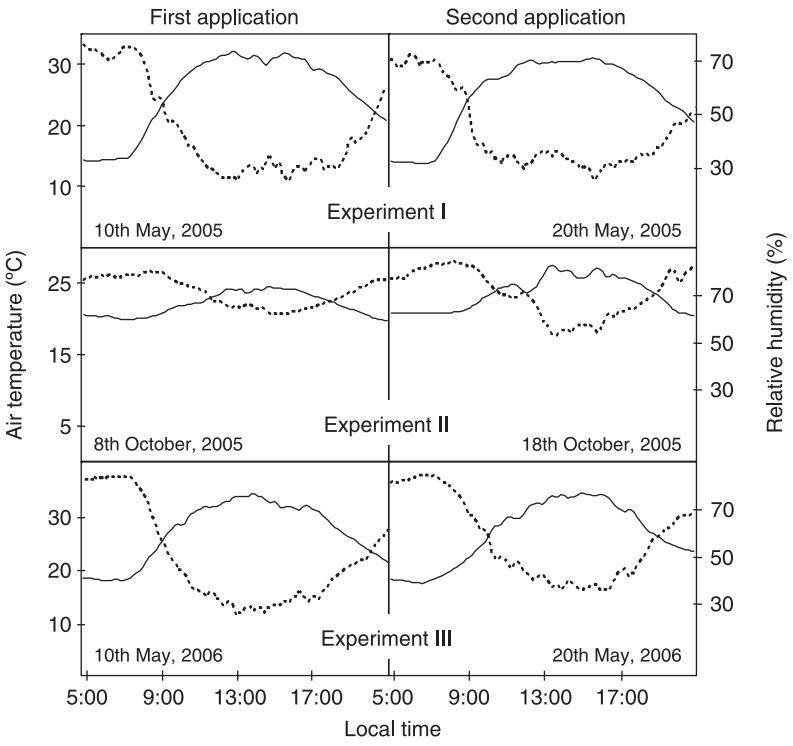

Figure 4 Environmental conditions inside the greenhouse recorded for the first day after each application of biological treatments against cucurbit powdery mildew. Data for air temperature (continuous line) and relative humidity (dashed line) are given. Two applications per experiment were performed during the evenings before the dates indicated.

Table 3 Effect of different biological control treatments on sporulation of *Podosphaera fusca* in greenhouse-grown melon recorded 30 days after pathogen inoculation and expressed as conidia cm^{-2}. Percentage of reduction of sporulation achieved by treatments is also indicated

	Experiment I		Experiment II		Experiment III	
Treatments	Conidia	Reduction	Conidia	Reduction	Conidia	Reduction
Untreated	1333 a[a]	–	1595 a	–	1812 a	–
Water	1285 a	4[b]	1173 a	26	1778 a	2
Ortiva (0·8 mL L^{-1})	81 b	90	66 b	96	83 c	95
AQ10 + ADDIT	138 b	89	80 b	94	388 cd	79
Mycotal + ADDIT	49 b	96	32 b	97	272 cd	85
ADDIT (2·5 mL L^{-1})	170 b	87	53 b	96	634 b	65
Bs UMAF6614[c]	123 b	91	56 b	96	133 c	93
Bs UMAF6639	192 b	85	132 b	92	192 c	89
Bs UMAF8561	137 b	89	90 b	94	313 cd	83
1st Ortiva, 2nd *Bs* UMAF6614[d]	–	–	81 b	95	116 c	93
1st ADDIT, 2nd *Bs* UMAF6614	–	–	–	–	119 c	93
1st *Bs* UMAF6614, 2nd AQ10 + Addit	–	–	–	–	554 bd	69
1st *Bs* UMAF6614, 2nd Mycotal + Addit	–	–	–	–	390 bcd	78
AQ10	–	–	–	–	1470 a	19
Mycotal	–	–	–	–	1700 a	6

[a]Mean values followed by different letters within a column were significantly different accordingly to the LSD test ($P = 0{\cdot}05$).
[b]Percentage of sporulation reduction achieved by each treatment referred to values of conidia production in untreated controls.
[c]*Bs* = *Bacillus subtilis*.
[d]First and second applications consisted of different treatments.

for control with azoxystrobin. As previously shown for disease progress, the largest conidial reductions (92–97%) were achieved in October 2005. The effect of integrated treatments on sporulation was similar to the effect previously observed for severity; alternations of fungicide or mineral oil with *B. subtilis* UMAF6614 were very effective, whereas alternation of UMAF6614 with mycoparasites had a limited impact on conidial production. In contrast, as expected, the application of mycoparasites without mineral oil proved ineffective at reducing sporulation, which remained at statistically similar levels for this treatment group and the untreated controls.

Table 4 Population of *Bacillus subtilis* strains on melon phylloplane of greenhouse-grown plants after treatment against powdery mildew (*Podosphaera fusca*). Total bacterial populations were estimated after the first application (initial) and 30 days after inoculation of *P. fusca* (final) and expressed as log cfu cm^{-2}. Percentages of dormant spores at the end of the experiments are also indicated

	Experiment I			Experiment II			Experiment III		
Treatments	Initial	Final[a]	Spores	Initial	Final	Spores	Initial	Final	Spores
Untreated	1·6 ± 0·2	1·6 ± 0·1	100	1·6 ± 0·1	1·6 ± 0·1	100	3 ± 0·2	3·0 ± 0·1	29
Bs UMAF6614	4·0 ± 0·2	4·0 ± 0·6	11	4·0 ± 0·6	5·0 ± 0·2	< 1	5·5 ± 0·3	6·0 ± 0·2	3
Bs UMAF6639	4·5 ± 0·5	3·4 ± 0·1	15	4·5 ± 0·5	5·0 ± 0·5	3·5	5 ± 0·1	6·0 ± 0·3	< 1
Bs UMAF8561	4·6 ± 0·5	5·0 ± 0·2	25	5 ± 0·2	4·5 ± 0·4	8·6	6 ± 0·1	6·0 ± 0·1	10

Bs = *Bacillus subtilis*.

[a]Each value represents mean ± standard error from three replicates (three plants per replicate).

Survival of *B. subtilis* on melon phylloplane

To determine the colonization characteristics of the *B. subtilis* strains on melon phylloplane under greenhouse conditions, population studies were carried out (Table 4). The suspensions of *B. subtilis* were composed of a mixture of vegetative cells (main fraction) and endospores that resulted in initial population levels of 10^4–10^5 cfu cm^{-2} of leaf surface after the first application. The indigenous bacterial microbiota, represented by an analysis of untreated and non-infected leaves, hardly varied from 10^2–10^3 cfu cm^{-2}, and many of these bacteria did not show the colony morphology typical of the antagonistic strains applied, although they also were spore-forming bacteria. Total bacterial populations and the spore fractions of untreated and treated leaves were recorded 30 days after pathogen inoculation. In general terms, *B. subtilis*-populations remained unchanged on the leaves (10^4–10^5 cfu cm^{-2}). In most cases only 1–10% of the bacterial population remained as dormant spores, and only in experiment I was the spore fraction relatively higher than the others (11–25%).

Visualization of interactions between *P. fusca* and biocontrol agents

The bacterial survival results indicated that the *B. subtilis* cells applied upon the leaves were able to remain in this habitat, probably as stable cellular aggregates. This hypothesis was supported by scanning electron micrographs taken of samples obtained at the end of the greenhouse experiments 30 days after inoculation of the pathogen. The antagonistic bacteria were not randomly distributed upon the leaf surface but rather colonized the leaves by forming orderly microcolonies following epidermal cell junctions, in which a sort of fibrillar material connected bacterial cells to each other and to the leaf surface. Furthermore, bacteria were observed closely attached to the surfaces of *P. fusca* conidia (Fig. 3h) and hyphae (Fig. 3i) which showed collapsed shapes in contrast to the typical features of *P. fusca* hyphae, conidophores (Fig. 3b) and conidia (Fig. 3c) observed on untreated plants. SEM analysis of leaves treated with mycoparasites confirmed their presence on the leaves as well as the occurrence of an extensive parasitism of *P. fusca* structures. *Lecanicillium lecanii* interacted ectoparasitically with *P. fusca*, penetrating the host hyphae (Fig. 3e). In the interaction between *A. quisqualis* and *P. fusca* typical alterations of the host revealed parasitism as illustrated by the typical swelling at the base of *P. fusca* conidiophores corresponding to the internal formation of *A. quisqualis* pycnidia and the abnormal deformation of conidia (Fig. 3f).

Discussion

Previous experiments on detached melon leaves suggested that *P. fusca* could be controlled by the mycoparasite-based products AQ10® and Mycotal® or by the antagonistic *B. subtilis* strains UMAF6614, UMAF6639 and UMAF8561 (Romero *et al*., 2003, 2004). It has been extensively reported that biocontrol efficiency depends on a combination of factors: the characteristics of the BCAs, the epidemiology of the target pathogen and the environmental conditions in which the relationship is taking place (Paulitz & Bélanger, 2001). Mycoparasites used here were found to be more effective when relative humidity values were above 80% and most likely influenced by a combination of different factors, but mainly, the higher germination rate of mycoparasitic spores in high humidity and secondly, the lower growth rate of *P. fusca* under these conditions (Verhaar *et al*., 1999b). Similarly, bacterial strains performed better at high relative humidity, probably because these environmental conditions favour a more efficient colonization of leaves, which could support the effective production and secretion of antifungal compounds.

The preliminary results of the growth chamber experiments enabled the proper conditions for application of biocontrol treatments in the subsequent greenhouse experiments, which were set up near sunset in order to ensure the longest period of time with the highest relative humidity values (above 80%). Moreover, the greenhouse fluctuations in relative humidity (approximately between 50% and 90%) through the day–night cycle necessitated applying the mycoparasites with mineral oil to generate a favourable microclimate upon the leaves that, combined with the high humidity required for adequate germination of mycoparasitic spores, ensured effective management of

powdery mildew as previously reported (Verhaar *et al*., 1999b; Kiss, 2003). Mycoparasites applied without mineral oil proved to be ineffective compared with the treatments with the mineral oil ADDIT. These results and the fact that oil itself provided reasonable disease reduction agree with the suggestion that mineral oil not only keeps the leaf surface wet longer, thus providing a microclimate optimal for mycoparasites, but also exerts a direct deleterious effect upon *P. fusca* conidia, probably by inducing alterations in the membrane that could collapse the spore, causing it to become unviable (Northover & Scheider, 1996; McGrath & Shishkoff, 1999; Fernández *et al*., 2006).

As previously reported (Romero *et al*., 2004), the bacterial suspensions mainly composed of vegetative cells also produced efficient disease control in greenhouse experiments. Furthermore, bacterial populations recovered 30 days after treatment were also composed mainly of vegetative cells that remained stable on melon phylloplane or even increased, as in experiment III. These findings, in close agreement with those reported by other authors, provide evidence that vegetative cells, rather than endospores, of *B. subtilis* are responsible for its ability to protect against disease (Sonoda *et al*., 1996; Collins & Jacobsen, 2003; Collins *et al*., 2003). Discussions about bacterial colonization and its relationship with biocontrol performance have encouraged several studies that have not only established a direct relationship between the two variables, but have also shown that colonization is a requirement to ensure the permanence of the BCA in the habitat where it is supposed to perform (Beattie & Lindow, 1999; Shoda, 2000; Raaijmakers *et al*., 2002). This study has established that bacterial populations remained at 10^4–10^6 cfu per cm^2 for 16 days after the second application which yielded disease reductions of 80–97%, therefore suggesting that this number of bacteria is suitable for effective control of powdery mildew disease. The good colonization capabilities of diverse bacteria seem to be related to the ability to form stable biofilms upon the leaf surface, protecting the cells against the harshness of environmental flux (Raaijmakers *et al*., 2002; Demoz & Korsten, 2006). SEM micrographs showed the antagonistic bacteria occurred preferentially in the plant epidermal cell junctions, where availability of nutrients seems to be ensured, supporting the permanence of bacterial microcolonies as vegetative cells rather than dormant endospores (Collins & Jacobsen, 2003; Demoz & Korsten, 2006). This ability to colonize and to form microcolonies provides protection to the bacteria but also has ecological implications involving the exclusion of other microorganisms from the occupied niche or the increased production of antimicrobials (Stein, 2005).

Several *B. subtilis* strains are receiving great attention because of their versatility in conferring protection on plants. The antagonistic mechanisms encompass antibiosis, competition or induction of systemic plant responses (Emmert & Handelsman, 1999; Shoda, 2000; Ongena *et al*., 2005). Considering the epidemiology of powdery mildews, it has been assumed that the production of antifungals able to repress spore germination should successfully control the disease (Bélanger *et al*., 1998; Avis & Bélanger, 2001). Early studies pointed out antibiosis could be involved in the disease protection provided by these *B. subtilis* strains (Romero *et al*., 2004). It has been demonstrated that antifungal compounds from the iturin and fengycin families of lipopeptides are produced by these strains which play a major role in the antagonism towards *P. fusca* determined to a large extent by the suppression of conidia germination (Romero *et al*., 2007). The presence of bacterial cells in close relation to visibly collapsed *P. fusca* conidia and hyphae suggests the local secretion of antifungal substances at sufficient concentrations to induce structural damage, resulting in the concomitant inhibition of spore germination and vegetative growth (Asaka & Shoda, 1996; Raaijmakers *et al*., 2002). Moreover, the same study has shown the occurrence of iturin and fengycin upon leaves treated with *B. subtilis*, providing clear evidence for production *in situ* and secretion of these antifungal compounds to the surroundings (Romero *et al*., 2007). Taken together, the correlation between bacterial density and disease reduction, the production *in situ* of antifungals, and the microscopic alterations which the *B. subtilis* strains induced in *P. fusca* strongly highlight the relevant role of antibiosis as the main mechanism used by these strains to protect melon plants against powdery mildew.

BCAs are increasingly becoming attractive alternatives to chemicals but the current investigations demonstrated they did not perform as expected in all crop conditions, suggesting that combining control strategies may be required in the form of integrated management programmes (IMP) (Ji *et al*., 2006; Omar *et al*., 2006; Nofal & Haggag, 2006). In order to establish an efficient IMP, it is important to know the epidemiology of pathogens, as well the mechanisms used by the different control methods to be pooled for managing the disease (Dik *et al*., 2002). In this study, the *B. subtilis* strain UMAF6614 – as a paradigm for antibiosis – was combined with the fungicide azoxystrobin, a mineral oil ADDIT or mycoparasitic fungi in independent applications. All of these combinations provided good disease protection, with the exception of the alternations of *B. subtilis* and mycoparasites, a result that may indicate incompatibility when these latter two agents are used together. These results demonstrate that these *B. subtilis* strains may be suitably arranged in different treatment combinations determined by environmental conditions, disease state and growth stage of the pathogen.

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