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Comparison of antagonists of *Botrytis cinerea* in greenhouse-grown cucumber and tomato under different climatic conditions

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Abstract

The efficacy of *Trichoderma harzianum* T39 and the yeasts *Aureobasidium pullulans* and *Cryptococcus albidus* against *Botrytis cinerea* in cucumber and tomato was compared with chemical control. Four experiments were conducted in cucumber grown under different climatic conditions in The Netherlands, and two experiments were done in tomato both in the Netherlands and in Israel. *T. harzianum* and *A. pullulans* showed the most consistent control of *B. cinerea*, reducing stem lesions and death of plants by 40–100% in most cases. Control of stem lesions and subsequent wilting was generally better than control of symptoms on fruits. In some cases, the biocontrol agents were more effective than the broad-spectrum fungicide tolylfluanid and the selective fungicide iprodione. The climatic conditions did not strongly influence the efficacy of the biocontrol agents, but regression analysis showed that high temperature during the day and high vapour pressure deficit during the night reduced biocontrol efficacy. From the results, prospects for biocontrol of *B. cinerea* in greenhouse vegetables appear good under a range of conditions.

Introduction

Grey mould, caused by *Botrytis cinerea* Pers.; Fr. continually threatens the productivity of greenhouse-grown tomatoes (*Lycopersicon esculentum* Mill.) and cucumbers (*Cucumis sativus* L.) in many regions of the world through effects on leaves, fruits and stems. Control of grey mould is often difficult and costly. In heated greenhouses, development of grey mould on foliage and fruits can be reduced by combined heating and ventilating to reduce relative humidity (Yunis et al., 1990; Winspear et al., 1970), but this requires a high energy input and is generally ineffective against direct infection of pruning and harvesting wounds on the stem. Lesions often girdle the stems and cause the death of the plant above the lesion, thus creating substantial yield losses. Greenhouse growers use several fungicides against grey mould, but the results have been disappointing due to the development of resistance of *B. cinerea* against benzimidazole and dicarboximide fungicides (Elad et al., 1992; Steekelenburg, 1987). The broad-spectrum fungicide tolylfluanid controls grey mould when sprayed or pasted on wounds, but export of fruits from treated plants is prohibited for several countries. For environmental reasons, there is political pressure in the Netherlands to limit the use of chemical fungicides and energy in glasshouses. The development of biological control as an alternative to the use of fungicides would greatly help the glasshouse industry in becoming more environmental-friendly and at the same time would reduce yield losses due to grey mould.

Biological control of *B. cinerea* has been researched broadly (Elad et al., 1996) and has led to the development of the commercial product Trichodex based on *Trichoderma harzianum* T39 by Makhteshim Agan Chemical Works Ltd., Be'er Sheva, Israel (Elad et al., 1993). This product has been tested mainly in non-heated greenhouses and vineyards with high

humidity or dew formation during part of most days. Its performance in heated glasshouses needs to be established. Testing a range of fungi, bacteria and yeasts in bioassays with tomato and cucumber and small greenhouse experiments has led to the selection of tw o yeasts, *Cryptococcus albidus* and *Aureobasidium pullulans*, as potential biocontrol agents (Koning and Köhl, 1995; Dik et al., 1999).

Microclimatic conditions in greenhouses can be expected to affect the performance of biocontrol agents against *B. cinerea* in cucumber and tomato. Growers employ a range of climatic regimes in these crops. Further, climatic conditions vary with season and from year to year. Because of the potential importance of climatic variables in biological control, performance of the selected biocontrol agents was tested under different climatic conditions in cucumber in the Netherlands, and in tomato in heated greenhouses in the Netherlands and non-heated greenhouses in Israel. The aim of our experiments was to assess the efficacy of Trichodex and two yeast strains against *B. cinerea*, especially their effect on stem lesions and subsequent plant death, under a range of environmental conditions in cucumber and tomato, and to gain insight into the possibilities for integration of biological control with climate control and, if necessary, chemical control.

Materials and methods

Plants. Plants of cucumber cv. Flamingo were transplanted to the greenhouse at the four to five leaf stage, in the second week of January for spring experiments and in the last week of August for autumn experiments. Plants were grown in rockwool with a standard recirculating nutrient solution. Flamingo is partially resistant to powdery mildew, so fungicides were not needed to control this disease. Insects were controlled biologically. Plants were trained according to the umbrella system (Jarvis, 1992) and fruits were harvested three times per week.

In the Netherlands, tomato plants cv. Aromata were transplanted in December of the previous year for both experiments. They were grown in rockwool in a high wire system with a standard recirculating nutrient solution and insects were controlled biologically. In Israel, tomato cv. 144 plants were grown on greenhouse tables in pots (one plant per pot) containing 10 l mixture of 1 : 2 : 1 peat : volcanic gravel : vermiculite and fertilized with 5–3–8% N–P–K every day. No pesticides were applied.

Preparation of biocontrol agents. *A. pullulans* and *C. albidus* were grown on Basal Yeast Agar (BYA, containing 20 g glucose, 0.5 g yeast extract (Difco), 10 g bactopeptone (Oxoid) and 18 g agar (Oxoid) per liter) for 5–7 days at 21°C and recovered in a sterile 0.01% Tween 80 solution. Cell concentrations of the yeasts were estimated with a haemocytometer and adjusted to $0.5–1.0 \times 10^7$ cells ml^{-1} in the final spray suspension. *T. harzianum* T39 was provided as the commercial product Trichodex 25 SP (Makhteshim Agan Chemical Works Ltd., Be'er Sheva, Israel, concentration of *T. harzianum* 10^{10} CFU g^{-1}) and was applied at the recommended rate of 4 kg ha^{-1} in the Netherlands and 2 kg ha^{-1} in Israel.

Design of cucumber experiments. Four experiments were done with cucumber in the Netherlands, one in each of four seasons: spring 1995, autumn 1995, spring 1996 and autumn 1996. The spring experiments were done in two (1995) or three (1996) greenhouses of area 189 m^2, each with a different climate regime. Spray treatments were applied to four plots of eight plants per treatment within each greenhouse. The autumn experiments were designed as split-plot experiments, with each climate regime in three (1995) or four (1996) replicate greenhouses and the spray treatments applied to one plot of 12 plants per greenhouse.

In the spring experiment in 1995 (exp. C1), differences in VPD were created between the two greenhouses by continually closing a horizontal polyethylene screen in the top of one greenhouse and maintaining a 15% opening in the screen in the other greenhouse at similar temperature (Table 1). The difference in irradiation between 100% and 0% closed is only 7% (Graaf, 1985), so it can be assumed to be negligible in our settings. In the spring experiment in 1996 (exp. C2), the aim was to create differences in temperature between climates 1 and 2 and in VPD between climates 1 and 3, by the use of screens, and differential ventilation as in exp. C1, (Table 1).

In the autumn experiment in 1995 (exp. C3), two climate regimes were used. In climate 1, the set point for ventilation was 4 °C above that for heating, and in climate 2 ventilation started at 10 °C outside temperature, increasing by 1% per degree increase in outside temperature. In the autumn experiment in 1996 (exp. C4), the same regimes for climates 1 and 2 were used and an extra one was added with the set point for heating at the actual temperature reached in climate 1 and for ventilation at 0.5 °C above this (climate 3). Each climate

Table 1. Description of climate regimes and resulting climatic conditions in all experiments

Exp.	Season	Climate	Description	Heating set point (°C)	Ventilation set point (°C)	Horizontal screen in top	Greenhouses per climate	T_{av}[1] (°C)	VPD_{av}[2] (kPa)	T_d[3] (°C)	VPD_d[4] (kPa)	T_n[5] (°C)	VPD_n[6] (kPa)
C1	spring 1995	1	normal T + humid	21.5	22.0	100%	1	22.5	0.293	24.4	0.342	20.4	0.224
		2	normal T + dry	21.5	22.0	85%	1	22.7	0.642	24.7	0.692	20.5	0.562
C2	spring 1996	1	warm + humid	21.5	26.0	100%	1	22.7	0.300	25.3	0.356	19.8	0.247
		2	normal T + dry	21.5	22.0	85%	1	20.7	0.396	22.3	0.443	19.0	0.363
		3	normal T + humid	21.5	22.0	100%	1	21.9	0.271	24.1	0.327	19.5	0.212
C3	autumn 1995	1	warm + humid	22.0	26.0	0%	4	22.1	0.408	23.1	0.499	20.3	0.371
		2	normal T + dry	22.0	10.0 outside[7]	0%	4	21.5	0.596	22.3	0.687	19.7	0.499
C4	autumn 1996	1	warm + humid	22.0	26.0	0%	3	21.8	0.467	23.0	0.520	20.3	0.456
		2	normal T + dry	22.0	10.0 outside[7]	0%	3	21.1	0.613	22.1	0.624	20.0	0.571
		3	warm + dry	T measured in climate 1	0.5 above heating	0%	3	21.9	0.580	23.1	0.676	20.4	0.618
T1	spring 1996	1	humid nights	day: 19.5 night: 17.0	day: 21.5 night: 20.0	100% during 3 h before sunrise	1	18.7	0.447	19.8	0.572	17.6	0.327
T2	autumn 1996	1	humid nights	16.5	17.5	100% during 3 h before sunrise	1	17.2	0.349	18.5	0.494	15.8	0.207
		2	normal	16.5	17.5	0%	1	17.2	0.388	19.0	0.500	15.8	0.279
T3	spring 1996	1	fluctuating	no heating	manual	0%	1	18.3	0.311	23.1	0.563	14.6	0.165
T4	spring 1997	1	fluctuating	no heating	manual	0%	1	16.1	0.250	20.3	0.469	11.9	0.071

[1]Average temperature from first treatment until end; [2]Average VPD from first treatment until end; [3]Average daytime temperature from first treatment until end; [4]Average daytime VPD from first treatment until end; [5]Average nighttime temperature from first treatment until end; [6]Average nighttime VPD from first treatment until end; [7]Ventilation starting at 10°C outside temperature and increasing with 1% per degree increase in outside temperature.

regime was used in four greenhouses of area 156 m^2 in exp. C3 and three greenhouses in exp. C4 (Table 1).

Design of the tomato experiments. In the Netherlands, tomato plants were grown in greenhouses of area 453 m^2. One greenhouse was used for the spring experiment in 1996 (exp. T1), and two were used for the autumn experiment in 1996 (exp. T2). In the spring and in one greenhouse in autumn, a horizontal thermal screen was closed from 3 h before sunrise until sunrise. In the other greenhouse in autumn, no screen was used. Treatments were applied to four plots of ten plants per greenhouse.

In Israel, one experiment was carried out in spring 1996 (exp. T3) and one in spring 1997 (exp. T4). In the greenhouse, a polyethylene cover was put above each of the greenhouse tables so greenhouses of area 4 m^2 were formed to create conditions conducive for grey mould. Each treatment was applied to five groups of ten plants.

Treatments. All experiments shared five common treatments, applied weekly: 1. control sprayed with Tween 80 (0.01%); 2. chemical control: in the Netherlands tolylfluanid (Eupareen-M, Bayer, 1.5 g l^{-1}), in Israel iprodione (Rovral 50 WP, Rhône-Poulenc Agro BV., 0.5 g l^{-1}); 3. *A. pullulans*; 4. *C. albidus*; 5. *T. harzianum* T39 (Trichodex 25 WP, Makhteshim Agan Chemical Works Ltd.).

In exp. C3, two controls were added: 6. untreated; 7. only water, to compare the effect of Tween 80 and water to untreated. In exp. C4, five treatments were added: 6. *A. pullulans* applied biweekly; 7. *C. albidus* applied biweekly; 8. *T. harzianum* T39 applied biweekly; 9. iprodione applied biweekly; 10. *T. harzianum* T39 and iprodione applied alternately. In treatment 10, *T. harzianum* T39 was applied in the same week as in treatment 8, whereas iprodione was applied in the alternate week in both treatments 9 and 10. In exp. T2, three treatments were added: 6. *A. pullulans* applied biweekly; 7. *C. albidus* applied biweekly; 8. *T. harzianum* T39 applied biweekly. The extra treatments in exps. C4 and T2 aimed at assessing the effect of the time interval between applications on efficacy of the antagonists. In exp. T3, the control was sprayed with water instead of Tween since all the biocontrol agents were applied in water. In exp. T4, three treatments were added: 6. control with water; 7. *T. harzianum* T39 and *A. pullulans* in combination; 8. iprodione, in order to asses the combined effect of two antagonists and of a selective fungicide.

In cucumber, treatments started six weeks after planting in the spring experiments and four weeks after planting in the autumn experiments. In tomato, treatments started 15 and 38 weeks after planting in exps. T1 and T2, respectively. In Israel, treatments were applied only once in exp. T3 and subsequently the plants were inoculated with a *B. cinerea* suspension (10^5 conidia ml^{-1}). In exp. T4, plants were inoculated with *B. cinerea* (10^5 conidia ml^{-1}) three days before the first application of the treatments, which were applied weekly.

In the Netherlands, treatments were applied with a 10 l backpack sprayer (Gloria 172 RT, Gloria-Werke, Wadersich, Germany) at a rate of 1500 l ha^{-1} in cucumber and 1000 l ha^{-1} in tomato with pressure of 2–4 atm. In Israel, treatments were applied with pressurized hand sprayers at a rate of 1000 l ha^{-1}.

Climate registration. In the experiments in the Netherlands, dry and wet bulb temperatures were measured at 1 min intervals with an aspirated psychrometer at a height of 1.5 m in the canopy. Relative humidity (RH) and vapour pressure deficit (VPD) were calculated. Mean values for each hour were stored in a VAX mainframe computer (Digital, Utrecht, The Netherlands). In Israel, temperature was measured with copper–constantan thermocouples and RH with PCRC-II electro-humidity sensors. Data were recorded hourly with C21 X data loggers (Campbell Scientific Inc., Logan, UT, USA).

Temperature and VPD were averaged per 24 h (T_{av} and VPD_{av}), for daytime (T_d and VPD_d, sunrise to sunset) and for nighttime (T_n and VPD_n, sunset to sunrise). Averages of these values were calculated from the first application date of treatments until the end of the experiments.

Disease assessments. In cucumber, diseased fruits were counted and subsequently removed except in exp. C1. Incidence, length and position of lesions on stems and incidence of dead plants were recorded. In tomato, incidence and length of stem lesions and incidence of dead plants were recorded in the Netherlands. No disease was found on fruits or leaves. In Israel, diseased fruits, stem lesions and dead plants were counted. Leaf symptoms were recorded as the estimated percentage leaf area with symptoms of grey mould.

Disease assessments were done at 6–15 day intervals. The average length of lesions was calculated for all assessment dates in exp. T2 and for the first three assessment dates in exps. C2–C4, because death of plants interfered with subsequent assessments.

In exp. C4, powdery mildew severity was assessed 59 days after planting on two plants per plot by estimating the percentage diseased leaf area for all leaves. The average diseased leaf area was calculated per plant and averaged per plot.

Population densities of biocontrol agents on stems. In exps. C1–C4, T1 and T2, rectangular peelings of the stem of one plant per plot were sampled with a potato peeler at four different heights on several sampling dates. Samples were always taken at the same time in the morning to reduce variability between sampling dates. The samples were shaken in 50 ml sterile Tween 80 (0.01%) for 1 h in a Griffin Flask Shaker (Stuart Scientific Co Ltd., Redhill, UK) in exps. C1 and C2 and in 10 ml sterile Tween 80 (0.01%) for 30 min in a tube shaker (IKA-Vibrax-VXR, IKA Labortechnik, Staufen, Germany) in the other experiments. The washings were deposited on two plates each of BYA and PDA per sample with a Spiral Plating System (Spiral Biotech, Bethesda, MD, USA). Afterwards, the length and width of the peelings were measured and area was calculated. The plates were incubated at 22 °C for 2–7 days. Colonies of the biocontrol agents were counted and the CFU per cm^2 stem was calculated.

Assessment of visible residue. In exp. C3, the presence of visible residue of the treatments on the fruits was recorded after three applications of the treatments on 47 days after planting.

Yield. The fresh weight of the harvested fruits was assessed in exps. C2, C3 and C4 on 8, 6 and 6 plants per plot, respectively. Fruits were classified as first or second class quality, depending on size, shape and colour according to the criteria of the Dutch auction. The total yield per plant and the percentage of first class quality fruits were calculated.

Statistical analysis. The Area Under the Curve (AUC) was calculated for stem lesions per plant (AUCles) and for the percentage of dead plants per plot (AUCdpl). The differences between treatments in cumulative number of diseased fruits, AUCles, AUCdpl, average lesion length (exps. C2–C4, T2), yield per plant and percentage of first class quality fruits were examined by analysis of variance and means were compared by Fisher's protected LSD test at $P = 0.05$. If necessary to stabilize variance, the data were transformed before analysis with square-root transformation for number of diseased fruits and according to Fry (1978) for AUCles and AUCdpl. In exps. C1, C2 and T2, the separate greenhouses were treated as separate experiments. Experiments C3 and C4 were analysed as split-plot experiments with climate as main factor and treatments as subfactor. Overall difference between climates and between treatments, and the differences between treatments within the same climate were tested with Fisher's protected LSD test. For AUCles and AUCdpl in the cucumber experiments, the percentage inhibition compared to the control treatment was calculated. Stepwise multiple linear regression was done on the percentage inhibition of AUCles and of AUCdpl against T_{av}, VPD_{av}, T_d, VPD_d, T_n and VPD_n. Acceptance of fitted equations was based on significance of the estimates at $P \leq 0.05$, chi-square values, distribution of residuals and $R^2_{adj.}$ with a minimum number of parameters (Campbell and Madden, 1990). All statistical analyses were done with Genstat (Genstat 5 Committee, 1992).

Results

Climatic conditions. In exp. C1, RH was above 85% in climate 1 with the closed screens and ranged between 40% and 80% in climate 2 with very similar temperatures (Table 1). In exp. C2, temperatures differed between all three climates while VPD was similar in climates 1 and 3 and lower in climate 2 (Table 1). In both autumn experiments, climate 1 with the ventilation set point at 26 °C resulted in a warmer and more humid climate than the regime with continuous ventilation. The third climate in exp. C4 had similar temperatures as climate 1 and similar VPDs as climate 2, thus resulting in a warm and dry climate (Table 1).

In the Netherlands, the average temperature in the tomato experiments was lower than in the cucumber experiments. In exp. T2, the main difference between the two greenhouses was the RH at night: with screens, between 85% and 95%; and without screens, between 80% and 85% (Table 1). In Israel, temperatures were more variable between day and night than in the heated greenhouses in the Netherlands. RH ranged from 80% during the day to 95% during the night (Table 1).

Grey mould epidemics and control efficacy in cucumber. In all treatments in the spring experiments (exps. C1 and C2), grey mould was observed chiefly on aborting fruits and only late in the season did stem lesions develop, resulting mainly from invasion of adhering fruits by the pathogen. The number of dead plants was very small. In all treatments in the autumn experiments (exps. C3 and C4), symptoms were observed on fruits and stems. Disease incidence was high in all climates. Stem lesions were found mainly on lower parts of the stem in association with pruning wounds and incidence of stem lesions and subsequent death of plants were higher than in spring.

In exps. C3 and C4, split-plot analysis showed a significant effect of climate on the number of diseased fruits (higher in climate 1 than in climate 2, and climate 3 in exp. C4) and AUCdpl (higher in climate 2 (and climate 3 in exp. C4) than in climate 1). AUCles was not significantly affected by climate in these experiments. Similar trends were observed in exps. C1 and C2.

When applied weekly, the biocontrol agents in most instances reduced the number of diseased fruits on cucumber plants as effectively as or better than tolylfluanid (Tables 2–5). In cucumber stems, the biocontrol agents and tolylfluanid both delayed the onset of the epidemic and decreased its rate of development (Figure 1). AUCles and AUCdpl values were similar in cucumbers treated with biocontrol agents or with tolylfluanid (Tables 2–5). Against stem lesions, tolylfluanid was in one case more effective than *A. pullulans* applied weekly (exp. C3) and in one case less effective than *T. harzianum* T39 (exp. C1). In exp. C3, an unexpected control of *B. cinerea* by the application of water and Tween 80 was found (Table 4). For logistic reasons, in this experiment the treatments with biocontrol agents were all sprayed suspended in rain water and the treatments with Tween 80 and water were sprayed with tap water, which was later shown to slightly reduce *B. cinerea*. In the other experiments, all treatments were sprayed with the same water.

No significant interaction between treatments and climates was found in the split-plot analysis for exps. C3 and C4 for diseased fruits, AUCles and AUCdpl. The antagonists were effective in most climates with a few exceptions. *A. pullulans* reduced AUCles in climates 1 and 2 but not in climate 3 in exp. C4. *C. albidus* controlled AUCles in exp. C4 in climates 1 and 3 but not in climate 2. *T. harzianum* was more effective in climates 1 and 2 but also gave some control in climate 3 in exp. C4. Stepwise multiple regression of efficacy (Table 6) of *A. pullulans* against climatic parameters yielded a significant negative effect of T_d and VPD_n on inhibition of AUCles and AUCdpl (percentage inhibition in AUCles $= 1048 - 38.3T_d - 273.9VPD_n$, $R^2_{adj.} = 89.7$; Percentage inhibition in AUCdpl $= 641 - 19.6T_d - 312.7VPD_n$; $R^2_{adj.} = 96.7$). For *C. albidus* and *T. harzianum* T39, no significant effect on inhibition of AUCles was found. Inhibition of AUCdpl was best described by VPD_{av} for *C. albidus* (percentage inhibition in AUCdpl $= 187 - 264.1VPD_{av}$; $R^2_{adj.} = 71.6$) and by T_d, T_n and VPD_n for *T. harzianum* (percentage inhibition of AUCdpl $= 51.7 - 21.7T_d + 32.9T_n - 325.4VPD_n$; $R^2_{adj.} = 98.1$).

When applied once every two weeks, all three biocontrol agents lost part or all of their efficacy against stem lesions by *B. cinerea* and death of plants (Figure 1,

Table 2. The effect of a fungicide and biocontrol agents on number of diseased fruits per plant and on stem lesions produced by *B. cinerea* in cucumbers grown in two greenhouse climates in spring (exp. C1)

	Climate 1 (normal T and humid)		Climate 2 (normal T and dry)	
Treatment	Diseased fruits (no. plant^{-1})	AUCles[1]	Diseased fruits (no. plant^{-1})	AUCles
Tween 80	13.0 a[2]	11.2 a	3.9 a	0.0 a
tolylfluanid	10.0 b	8.3 ab	2.4 b	1.0 a
A. pullulans in Tween	5.3 c	3.7 bc	0.7 c	1.4 a
C. albidus in Tween	6.9 c	4.5 abc	1.5 bc	1.8 a
T. harzianum T39 in water	5.9 c	1.1 c	0.6 c	0.0 a

[1]AUC for stem lesions per plant (lesion days); [2]values within one column followed by the same letter are not significantly different at $P = 0.05$.

Table 3. The effect of a fungicide and biocontrol agents on number of diseased fruits per plant, on stem lesions produced by *B. cinerea* and on death of plants due to *B. cinerea* in cucumbers grown in three greenhouse climates in spring (exp. C2)

Treatment	Climate 1 (warm and humid)			Climate 2 (normal T and dry)			Climate 3 (normal T and humid)		
	Diseased fruits (no. plant^{-1})	AUCles[1]	AUCdpl[2]	Diseased fruits (no. plant^{-1})	AUCles	AUCdpl	Diseased fruits (no. plant^{-1})	AUCles	AUCdpl
Tween 80	15.2 a[3]	1.27 a	0 a	0.4 a	4.27 a	164 a	9.8 ab	5.89 a	65.6 a
tolylfluanid	15.6 a	2.47 a	0 a	0.6 a	0.44 b	0 b	13.6 a	2.62 a	0 b
A. pullulans in Tween	8.4 b	2.44 a	109 a	0.4 a	0.33 b	0 b	8.0 b	1.86 a	0 b
C. albidus in Tween	10.7 ab	1.50 a	0 a	1.0 a	0.33 b	0 b	13.6 a	4.12 a	0 b
T. harzianum T39 in water	12.0 ab	1.41 a	109 a	1.5 a	0.33 b	33 b	8.8 b	6.94 a	0 b

[1]AUC for stem lesions per plant (lesion days); [2]AUC for percentage of dead plants per plot (percentage days); [3]values within one column followed by the same letter are not significantly different at $P = 0.05$.

Table 4. The effect of a fungicide and biocontrol agents on number of diseased fruits per plant, on stem lesions produced by *B. cinerea* and on death of plants due to *B. cinerea* in cucumbers grown in two greenhouse climates in autumn (exp. C3)

Treatment	Climate 1 (warm and humid)			Climate 2 (normal *T* and dry)		
	Diseased fruits (no. plant^{-1})	AUCles[1]	AUCdpl[2]	Diseased fruits (no. plant^{-1})	AUCles	AUCdpl
Tween 80	3.1 bc[3]	20.2 bcd	154 b	0.6 a	39.5 b	1546 b
tolylfluanid	1.8 c	9.5 d	269 ab	0.3 a	16.4 d	928 d
A. pullulans	3.1 bc	23.5 bc	153 b	0.2 a	22.4 cd	1174 cd
C. albidus	4.2 ab	22.1 bcd	155 b	0.5 a	24.9 cd	1185 cd
T. harzianum T39	5.2 a	12.4 cd	0 b	0.2 a	20.0 cd	1042 d
untreated control	4.8 a	53.0 a	513 a	0.6 a	67.2 a	2067 a
water	3.7 ab	32.8 b	267 ab	0.1 a	32.2 bc	1507 bc

[1]AUC for stem lesions per plant (lesion days); [2]AUC for percentage of dead plants per plot (percentage days); [3]values within one column followed by the same letter are not significantly different at $P = 0.05$.

Table 5). The trends for the three climates were the same as for the weekly treatments.

In exp. C4, iprodione was not effective against stem lesions when applied biweekly, but in alternation with *T. harzianum* T39 efficacy improved from 37.5% to 85.3% in climate 1, from 12.9% to 70.0% in climate 2 and from 0% to 57.4% in climate 3. The alternation treatment was also more effective than biweekly application of *T. harzianum* T39, but not when compared to weekly application of *T. harzianum* T39.

The average length of lesions in the autumn experiments was only occasionally influenced by the treatments. In exp. C3, lesion length was significantly larger only in the tolylfluanid treatment 49 days after planting and significantly reduced by *T. harzianum* T39 and *C. albidus* 55 days after planting compared to the control. In climate 2, lesion length was significantly larger than in climate 1, 55 days after planting. In exp. C4, no effect of climate on average lesion length was found for any of the assessment dates. Only once, lesion length was reduced in climate 1 by all treatments except biweekly application of *C. albidus*.

Powdery mildew was not significantly influenced by any of the treatments in exp. C4.

Grey mould epidemics and biological control efficacy in tomato. No disease was observed in exp. T1. In exp. T2, symptoms were observed only on stems, starting in September. In the control, AUCles was similar in both greenhouses, but the effect of the biocontrol agents was larger in the greenhouse with the screens closed during part of the night. *T. harzianum* T39 applied weekly and *A. pullulans* applied every two weeks significantly reduced AUCles in climate 1 (Table 7). In climate 2, no effect of any of the treatments including tolylfluanid on AUCles was found. AUCdpl and average lesion length were not influenced by any of the treatments in either greenhouse.

In Israel, only leaf symptoms were observed in exp. T3. It reached a level of 34% in the control and was significantly reduced by 40–53% by all five biocontrol agents with no differences among antagonists.

In exp. T4, symptoms were found on all plant parts (Figure 2, Table 8). The number of fruits with ghost spots was reduced significantly by *T. harzianum* T39. Leaf symptoms were significantly reduced by *T. harzianum* T39 compared to water but not by *A. pullulans* and *C. albidus* compared to Tween 80. Iprodione was significantly more effective against leaf symptoms than *T. harzianum* T39. AUCles was reduced significantly by all three biocontrol agents compared to the relevant control. The efficacy of *T. harzianum* T39 was greater than that of the yeasts and similar to that of iprodione. The combination of *T. harzianum* T39 and *A. pullulans* significantly increased control efficacy against stem lesions compared to either agent alone. Plant mortality at the end of the experiment was reduced significantly by *A. pullulans*, *T. harzianum* T39 and by iprodione. The combination of *A. pullulans* and *T. harzianum* T39 did not increase control efficacy against death of plants (Table 8).

Populations of biocontrol agents. In cucumber, the population densities of the yeasts on stem peelings ranged from 5×10^3 to 1×10^6 CFU cm^{-2}. The population density of *T. harzianum* T39 generally ranged

Table 5. The effect of a fungicide and biocontrol agents on number of diseased fruits per plant, on stem lesions produced by *B. cinerea* and on death of plants due to *B. cinerea* in cucumbers grown in three greenhouse climates in autumn (exp. C4)

Treatment	Interval of application (days)	Climate 1 (warm and humid)			Climate 2 (normal T and dry)			Climate 3 (warm and dry)		
		Diseased fruits (no. plant^{-1})	AUCles[1]	AUCdpl[2]	Diseased fruits (no. plant^{-1})	AUCles	AUCdpl	Diseased fruits (no. plant^{-1})	AUCles	AUCdpl
Tween 80	7	1.6 abcd[3]	46.9 ab	272 ab	0.3 a	33.3 a	729 ab	0.2 a	18.8 abc	340 ab
tolylfluanid	7	1.1 cd	12.2 ef	29 b	0.1 a	17.0 bcd	632 abc	0.2 a	16.3 abc	243 b
A .pullulans in Tween	7	1.8 abc	26.2 cde	146 ab	0.3 a	21.8 abcd	564 bc	0.3 a	21.6 abc	418 ab
C. albidus in Tween	7	2.2 a	18.5 def	39 b	0.2 a	32.1 ab	758 ab	0.1 a	10.0 c	243 b
T. harzianum T39 in water	7	2.1 ab	17.8 def	78 ab	0.3 a	15.2 cd	418 cd	0.3 a	12.2 bc	263 ab
A. pullulans in Tween	14	1.6 abcd	36.9 bc	282 ab	0.2 a	26.1 abc	690 abc	0.1 a	27.5 ab	574 a
C. albidus in Tween	14	2.1 a	53.3 a	389 a	0.3 a	31.6 ab	904 a	0.4 a	22.5 abc	408 ab
T. harzianum T39 in water	14	1.4 bcd	25.5 cde	165 ab	0.1 a	19.3 abcd	603 abc	0.1 a	16.9 abc	311 ab
T. harzianum T39/iprodione	7	1.9 ab	6.9 f	0 b	0.2 a	10.0 d	243 d	0.3 a	8.0 c	185 b
iprodione	14	1.0 d	29.3 cd	263 ab	0.1 a	29.0 abc	797 ab	0.3 a	29.3 a	457 ab

[1]AUC for stem lesions per plant (lesion days); [2]AUC for percentage of dead plants per plot (percentage days); [3]values within one column followed by the same letter are not significantly different at $P = 0.05$.

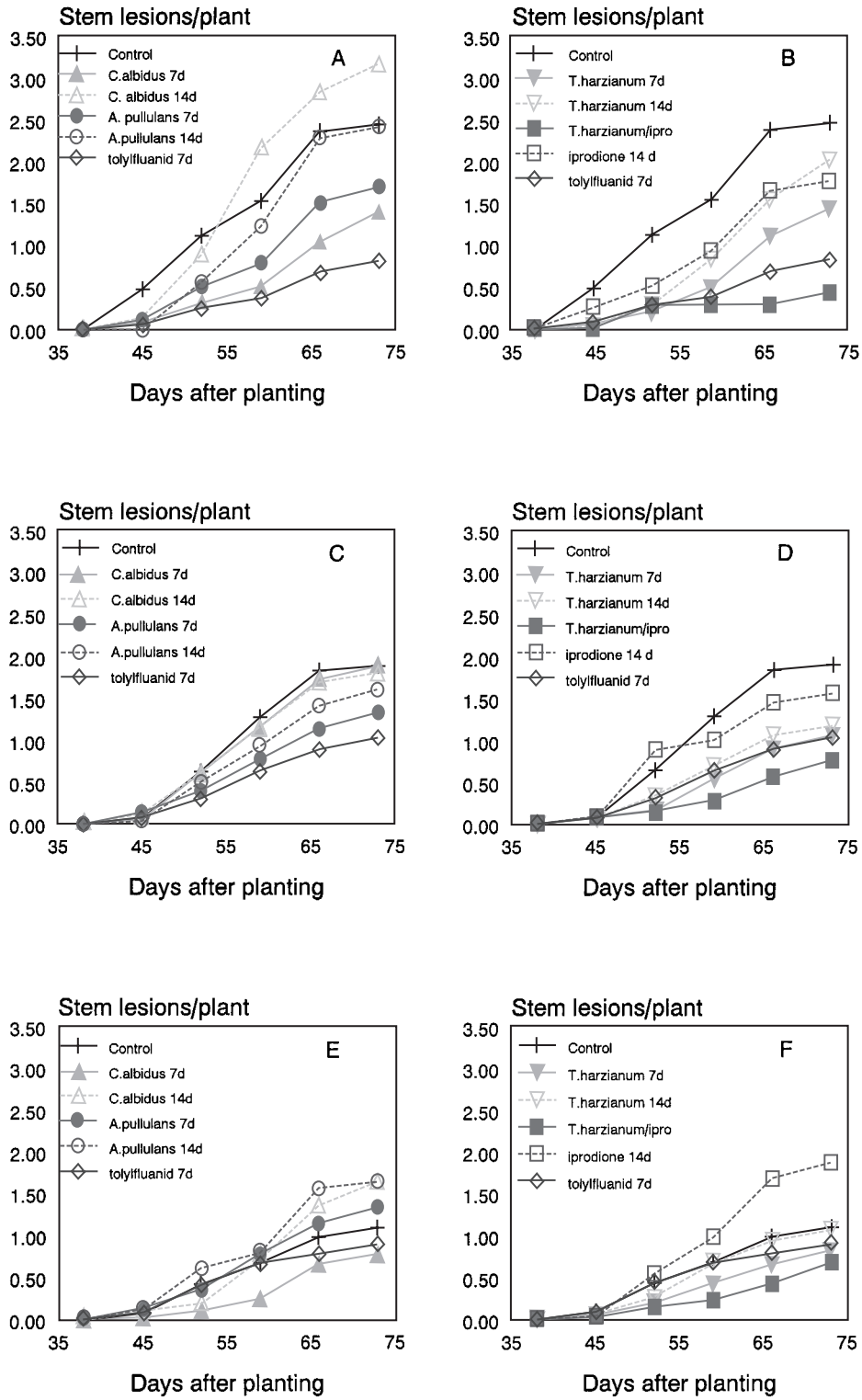

Figure 1. The effect of chemical and biological control agents on stem lesions produced by *B. cinerea* in cucumbers grown in three greenhouse climates (exp. C4). A + B: climate 1; C + D: climate 2; E + F: climate 3.

Table 6. Percentage reduction in AUC for stem lesions per plant (AUCles) and in AUC for percentage of dead plants per plot (AUCdpl) exerted by the biological and chemical control agents compared to the control treatment in four experiments with cucumber grown in different greenhouse climates

Exp.	Climate	Description of climate	Inhibition in AUCles (%)				Inhibition in AUCdpl (%)			
			tolylfluanid	*A. pullulans*	*C. albidus*	*T. harzianum* T39	tolylfluanid	*A. pullulans*	*C. albidus*	*T. harzianum* T39
C1	1	normal T + humid	25.9	67.0	59.8	90.2	*	*	*	*
C1	2	normal T + dry	*[1]	*	*	*	*	*	*	*
C2	1	warm + humid	0.0	0.0	0.0	0.0	*	*	*	*
C2	2	normal T + dry	89.7	92.3	92.3	92.3	100	100	100	79.9
C2	3	normal T + humid	55.5	68.4	30.1	0.0	100	100	100	100
C3	1	warm + humid	82.1	55.7	58.3	76.6	47.6	70.2	69.8	100
C3	2	normal T + dry	75.6	66.7	62.9	70.2	55.1	43.2	42.7	49.6
C4	1	warm + humid	74.0	44.1	60.6	62.0	89.3	46.3	85.7	71.3
C4	2	normal T + dry	48.9	34.5	3.6	54.4	13.3	22.6	0.0	42.7
C4	3	warm + dry	13.3	0.0	46.8	35.1	28.5	0.0	28.5	22.6

[1] * = not calculated because value of 0.0 in the control treatment.

Table 7. The effect of a fungicide and biocontrol agents on stem lesions produced by *B. cinerea* and on death of plants due to *B. cinerea* in tomatoes grown in two greenhouse climates in autumn (exp. T2)

Treatment	Interval of application	Climate 1 (humid nights)		Climate 2 (normal)	
		AUCles[1]	AUCdpl[2]	AUCles	AUCdpl
Tween 80	7	16.2 a[3]	0 a	15.6 a	44 a
tolylfluanid	7	11.7 ab	114 a	20.3 a	329 a
A. pullulans	7	11.4 ab	0 a	22.9 a	176 a
C. albidus	7	18.3 a	0 a	24.7 a	264 a
T. harzianum T39	7	7.6 b	44 a	15.4 a	166 a
A. pullulans	14	9.4 b	80 a	16.0 a	114 a
C. albidus	14	13.4 ab	114 a	36.3 a	150 a
T. harzianum T39	14	18.0 a	44 a	21.5 a	80 a

[1]AUC for stem lesions per plant (lesion days); [2]AUC for percentage of dead plants per plot (percentage days); [3]values within one column followed by the same letter are not significantly different at $P = 0.05$.

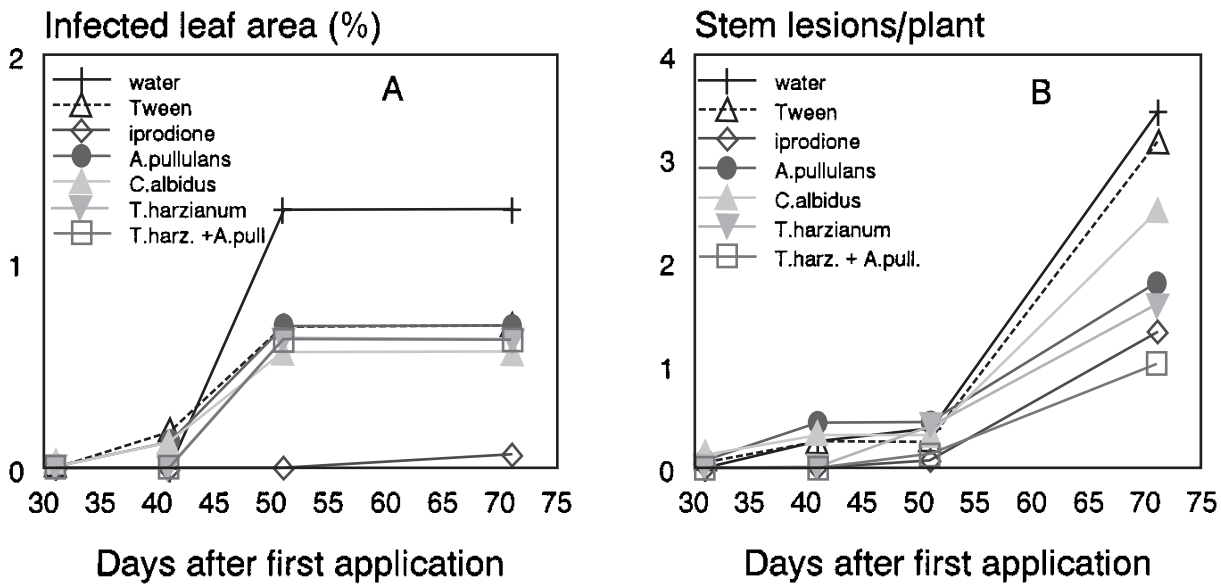

Figure 2. The effect of chemical and biological control agents on diseased leaf area and stem lesions by *B. cinerea* in tomatoes (exp. T4).

from 100 to 3000 CFU cm^{-2}. The climatic conditions in the greenhouses did not affect population densities of any of the biocontrol agents. Application once per two weeks resulted in lower population densities of the three biocontrol agents than did weekly application.

Yeasts and *T. harzianum* T39 persisted for several weeks after the last application in exp. T1 (Figure 3). In exp. T2, population densities of *A. pullulans* were between 3 and 57 $\times$ 10^3 CFU cm^{-2}. The population density of *C. albidus* was between 9 and 120 $\times$ 10^3 CFU cm^{-2} and no differences between the two greenhouses were observed. *T. harzianum* T39 population densities ranged from 1.5 to 15 $\times$ 10^3 CFU cm^{-2} in both greenhouses. In the biweekly applied treatments with *A. pullulans* and *T. harzianum* T39, the populations declined faster in the greenhouse without screens.

Visible residue. In exp. C3, the treatments with *T. harzianum* T39 and tolylfluanid gave visible residue on fruits and leaves. The amount of residue was similar for those two treatments.

Table 8. The effect of a fungicide and biocontrol agents on number of diseased fruits per plant, on diseased leaf area, on stem lesions produced by *B. cinerea* and on death of plants due to *B. cinerea* in tomatoes in spring (exp. T4)

Treatment	Fruits with ghost spots per plant (%)	AUCleaf[1]	AUCles[2]	Dead plants at the end of the experiment[3] (%)
Water	26.3 a[4]	18.8 a	42.4 a	50 a
Tween 80	24.0 a	12.2 b	38.1 b	46 a
A. pullulans	21.0 a	11.6 b	28.9 d	20 b
C. albidus	27.6 a	9.6 c	33.3 c	50 a
T. harzianum T39	11.3 b	9.3 c	16.3 e	21 b
T. harzianum T39 + *A. pullulans*	5.8 b	9.3 c	11.8 f	27 b
iprodione	19.9 a	0.3 d	14.0 ef	21 b

[1]AUC for percentage of diseased leaf area (percentage days); [2]AUC for stem lesions per plant (lesion days); [3]Percentage of dead plants per plot at the end of the experiment; [4] values within one column followed by the same letter are not significantly different at $P = 0.05$.

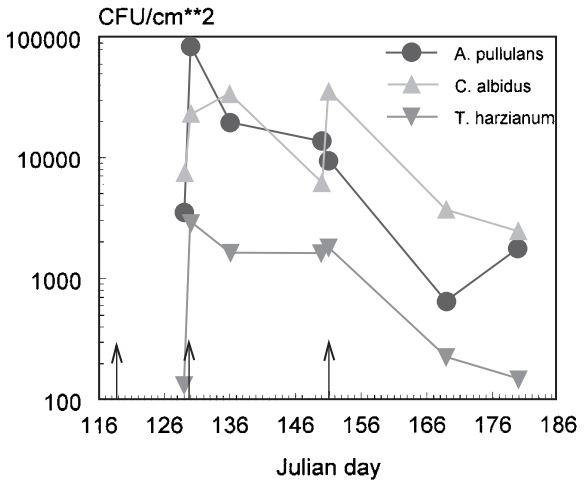

Figure 3. Population densities of the biocontrol agents in tomatoes (exp. T1). Arrows indicate application of treatments.

Yield. In exp. C2, no differences were found in yield per plant or percentage of first class quality fruits between treatments in any of the climates. The yield ranged from 4.8 to 6.2 kg plant^{-1} with 85–90% first class quality fruit. In exp. C3, it ranged from 3.7 to 4.9 kg plant^{-1} with percentage of first class quality fruit between 85% and 90%. Averaged over all treatments, the yield was significantly higher in climate 1 than in climate 2 (4.5 and 4.0 kg plant^{-1}, respectively). In climate 1, *T. harzianum* T39 increased the yield significantly. The other treatments had no effect on yield or percentage of first class quality fruit. In exp. C4, no differences in yield per plant or percentage of first class quality fruit were found between treatments or climates. The yield ranged from 4.5 to 5.3 kg plant^{-1} with 50–60% first class quality fruits.

Discussion

The antagonists tested were moderately to strongly effective against *B. cinerea* in tomato and cucumber, in most climate regimes controlling the disease at least as well as tolylfluanid. Differences between climates may seem small, but were large enough in cucumber to influence grey mould epidemics, with increased disease on fruits under humid conditions and increased plant death under dry conditions.

In cucumber, *A. pullulans* and *T. harzianum* T39 performed slightly better than *C. albidus* and both inhibited *B. cinerea* effectively under high temperature/low VPD conditions and normal temperature/high VPD conditions in climates 1 and 2, respectively, but were less effective in the combination of high temperature and high VPD in climate 3 in exp. C4. Regression analysis indicated a detrimental effect of high daytime temperature and of high nighttime VPD on the efficacy of these two antagonists. Apparently, in a warm and humid climate and in normal temperature and dry conditions, only T_d or VPD_n, respectively, play a role, thus resulting, overall, in the same efficacy of the antagonists against grey mould in these climates. But in a warm and dry climate, both high T_d and high VPD_n reduce the efficacy of the two antagonists. Since climates 1

and 2 are used in practice and climate 3 was added for scientific reasons, it can be concluded that in the commonly used climatic regimes in cucumber biological control will be effective. However, in the autumn experiments, the percentage inhibition of AUCdpl was generally larger than the percentage inhibition for AUCles under humid conditions (climate 1) and smaller under dry conditions (climates 2 and 3), which makes climate 1 the preferred climate.

Population densities of the biocontrol agents were similar in all climates in cucumber, even when efficacy was reduced. Maybe more frequent sampling during the day and night would have shown larger differences due to desiccation or high temperatures. Bio-assays have shown the antagonists to remain effective up to at least 30 °C (Dik et al., 1999), but the exact maximum VPD has not been established yet. The population densities of *T. harzianum* T39 were less affected by the conditions in our experiments than by the continuous high VPD used by Elad and Kirshner (1993), probably because sufficiently long periods of favourable conditions occurred and prevented harmful effects.

In tomato, *T. harzianum* T39 and *A. pullulans* were more effective than *C. albidus* in both countries. The use of thermal screens in one experiment in the Netherlands increased the survival and the performance of the biocontrol agents, probably due to the higher humidity during part of the night.

The alternation of *T. harzianum* T39 with iprodione, tested in exp. C4, was more effective against grey mould than iprodione alone, but not, as effective as *T. harzianum* T39 applied weekly as a stand-alone treatment. Thus, purely biological control was as good as integrated control in all climates in this experiment. The decision-support system (BOTMAN) for integration of Trichodex with chemical fungicides advises the application of a fungicide when temperatures are low during the night (Shtienberg and Elad, 1997). Prevention of these conditions in heated greenhouses in the Netherlands resulted apparently in a more consistent performance of Trichodex than in unheated greenhouses in the Mediterranean countries and, therefore, in heated greenhouses integration with chemical fungicides may not be necessary. However, if necessary because of adverse climatic conditions, the yeasts and *T. harzianum* T39 can be used in integrated control schedules with chemical fungicides, since the isolates are compatible with a number of fungicides (Dik and Elad, unpublished results). The performance of iprodione was good in exp. T4 in Israel but not in exp. C4, underscoring inconsistencies in chemical control similar to those reported earlier (Elad et al., 1992; Steekelenburg, 1987).

The biocontrol effect of the yeasts is generally believed to occur as a result of competition for nutrients. Schrattenholz and Flesch (1993) have reported the presence of toxins in culture medium extracts of *A. pullulans* but the inhibitory metabolites seemed to play no role in antagonism against *Alternaria solani* on tomato leaves (Flood and Rees, 1986). No inhibition zone was found when *A. pullulans* and *B. cinerea* were grown together on PDA plates, indicating that no antibiotics were produced (Dik, unpublished results). From observations of De Meyer et al. (1998) and Zimand et al. (1996), *T. harzianum* T39 suppresses *B. cinerea* by a combination of nutrient competition, interference with pathogenicity enzymes of the pathogen and induced resistance. To be effective, the yeasts and *T. harzianum* T39 have to be used preventively. Once disease has been established, the biocontrol agents have no effect on the development rate of the lesions, as reflected in the average lesion length. This is consistent with results in bio-assays, in which germination of the pathogen and disease incidence were reduced but not disease severity (Dik et al., 1999). Also, *T. harzianum* T39 failed to control *B. cinerea* already established in tomato stems (O'Neill et al., 1996).

The effects of the rate and method of application of the microbial agents have to a certain extent been established. *T. harzianum* T39 is effective in a dose of 2 kg ha^{-1}, but at high disease pressures a dose of 4 kg ha^{-1} is preferred. The effect of application of *A. pullulans* and *C. albidus* at the concentration used in the experiments described here and in a ten- and hundredfold dilution in cucumber showed that *C. albidus* lost its efficacy when diluted, whereas *A. pullulans* was equally effective at those three concentrations (Dik, unpublished results). This indicated that the concentration of *A. pullulans* in the spray suspension can be reduced without loss of efficacy, which increases commercial applicability. *T. harzianum* T39 was equally effective in tomato when applied as a spray suspension and pasted to pruning wounds in a ten-fold higher concentration (Dik and Buitelaar, 1995).

Climate control is one of the possible methods to be used in an integrated control programme for grey mould. Cucumber growers generally prefer a climate regime with a high ventilation set point to elevate temperature and humidity and thereby improve fruit quality (Bakker, 1991). This climate regime also has the advan-

tages that (i) death of plants caused by *B. cinerea* is reduced; (ii) the biocontrol agents are highly effective under these conditions; (iii) the effect of the biocontrol agents on prevention of death of plants is larger than that under drier conditions. Overall, in an integrated approach, this climate, together with good crop maintenance to remove diseased fruits, provides excellent opportunities for non-chemical control of grey mould. In tomato, the use of thermal screens during part of the night reduces heat loss and thus energy input. The use of thermal screens also provides a better climate for the biocontrol agents. Our general conclusion is that both *T. harzianum* T39 and *A. pullulans* show appropriate characteristics as biological control agents of grey mould in tomato and cucumber. Integration of biological control with climate control will lead to a reduction in the input of energy and chemical fungicides in both crops.

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