Activity of carboxylic acid amide (CAA) fungicides against *Bremia lactucae*

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Abstract Four carboxylic acid amide (CAA) fungicides, mandipropamid (MPD), dimethomorph (DMM), benthiavalicarb (BENT) and iprovalicarb (IPRO) were examined for their effects on various developmental stages of *Bremia lactucae,* the causal agent of downy mildew in lettuce, in vitro and in planta. Spore germination in vitro or on leaf surfaces was inhibited by all CAA fungicides (technical or formulated). MPD was more effective in suppressing germination than DMM or BENT, whereas IPRO was least effective. CAA induced no disruption of F-actin microfilament organisation in germinating spores of *B. lactucae*. CAA applied to germinating spores in vitro prevented further extension of the germ tubes. When applied to germinated spores on the leaf surface they prevented penetration. Preventive application of CAA to intact plants inhibited infection. MPD was more effective in suppressing infection than DMM or BENT, whereas IPRO was least effective. Curative application was effective at ≤3 h post-inoculation (hpi) but not at ≥18 hpi. CAA (except IPRO) applied to upper leaf surfaces inhibited spore germination on the lower surface and hence reduced infection. CAA suppressed sporulation of *B. lactucae* on floating leaf discs and when sprayed onto infected plants two days before onset of sporulation. BENT and DMM were more effective in suppressing sporulation than MPD or IPRO. Epidemics of downy mildew in shade-house grown lettuce were suppressed by CAA. A single spray applied to five-leaf plants before transplanting controlled the disease for 50 days. The results suggest that CAA are effective inhibitors of spore germination and therefore should be used as preventive agents against downy mildew of lettuce caused by *B. lactucae*.

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Introduction

Mandipropamid (MPD) is a new mandelic acid amide fungicide (Lamberth et al. 2006) which together with dimethomorph (DMM) and flumorph (cinnamic acid amides), iprovalicarb (IPRO) and benthiavalicarb (BENT; valinamid carbamates), belongs to the carboxyl acid amide (CAA) fungicides (Anon. 2006). CAA fungicides are effective against oomycete foliar plant pathogens.

Field studies indicate that MPD is highly effective against late blight in potato and tomato, downy mildew in grapes and several downy mildews in vegetable crops (Harp et al. 2006; Huggenberger et al. 2005;

Huggenberger and Kuhn 2006). A recent report (Harp et al. 2007) has shown effective control of downy mildew in lettuce with Revus (a.i. MPD). The fungicide was shown (Hermann et al. 2005) to quickly bind to the wax layer of the leaf surface thus providing strong rain-fastness and long-lasting efficacy. A small amount of MPD is taken up by the leaf tissue, providing curative and translaminar activities against disease. All four CAA fungicides belong to one cross-resistance group, as field isolates of *Plasmopara viticola* exhibit resistance to all (Gisi et al. 2006).

A recent study (Cohen and Gisi 2007) provided a comprehensive analysis of the effects of three CAA fungicides, MPD, DMM and IPRO, on all stages in the asexual life-cycle of *Phytophthora infestans*. The most sensitive stage to CAA was shown to be germination of cystospores and sporangia (direct germination) and the most active CAA was MPD. Nano-molar doses of MPD were sufficient to block spore germination in vitro and in vivo. Mycelium growth and sporulation were less sensitive to CAA. Of the three CAA fungicides tested, MPD was most effective in suppressing late blight epidemics in shade-house grown potatoes (Cohen and Gisi 2007).

Benthiavalicarb-isopropyl, another CAA fungicide, was not studied in the previous research (Cohen and Gisi 2007). It was reported (Miyake et al. 2003) to be strongly inhibitory to germination of sporangia and cystospores, mycelial growth and sporulation of various oomycetes. The compound has not only strong preventive activity, but also curative and penetrant activity, with excellent residual effects and rainfastness.

Several attempts were made to reveal the mode of action of CAA. Morphological studies (Albert et al. 1988, 1991; Bagirova et al. 2001; Cohen et al. 1995; Cohen and Gisi 1996; Dereviagina et al. 1999; Hermann et al. 2005; Huggenberger et al. 2005; Jende et al. 1999, 2001; Matheron and Porchas 2000; Miyake et al. 2003; Reuveni 2003; Stenzel et al. 1998) indicated that DMM, IPRO and BENT, as well as the experimental CAA XR-539 (Young et al. 2005), inhibit cell wall deposition/assembly in cystospores of *P. infestans*. Biochemical studies (Griffiths et al. 2003) with the mandelamide SX 623509 in mycelium of *P. infestans* suggested alterations in phospholipid biosynthesis, with an inhibition of phosphatidylcholine (lecithin) biosynthesis as a main target. Unpublished data (Cohen and Gisi) indeed indicate that lecithin (phosphatidylcholine) compromised the inhibitory ac-

tivity of CAA on cystospore germination. Recently, Zhu et al. (2007) compared a flumorph-sensitive and a flumorph-resistant *Phytophthora melonis* and suggested that the primary site of action of flumorph is the disruption of F-actin organisation.

The aim of the present study was to examine the effects of CAA on the development of *Bremia lactucae*, the causal agent of downy mildew in lettuce, in vitro and in planta. Here, four CAA fungicides were tested, including benthiavalicarb (BENT) which was not tested with *P. infestans*.

Materials and methods

Fungicides

Four CAA (carboxylic acid amide) fungicides were used: mandipropamid (MPD; Syngenta, mw=412), dimethomorph (DMM; BASF, mw=266), iprovalicarb (IPRO; Bayer, mw=320) and benthiavalicarb (BENT; Kumiai Chemicals, mw=339). Technical grade fungicides were dissolved in DMSO (10 mg ml^{-1}) and diluted in double distilled water (DDW) to the desired concentrations. Formulated fungicides used were: mandipropamid 250SC; dimethomorph (Forum) 50WP and iprovalicarb 50WG. Benthiavalicarb (10SC) in Agsolex-8 (*N*-octylpyrrolidone) was a gift from Makhteshim, Beer Sheba, Israel. All concentrations are represented in units of active ingredient (a.i.).

Pathogen

All studies, unless stated otherwise used isolate ISR-60 of *B. lactucae* Regel (Sharaf et al. 2007) carrying 13 virulence factors (0, 1, 2, 3, 4, 5/8, 6, 7, 10, 11, 13, 15, 16, 17) obtained from A. Beharav, The Institute of Evolution, Haifa University, Israel. Some studies used isolates BL-18, BL-21, BL-24 and BL-25 (a gift from A. Lebeda, Olomouc University, Czech Republic). Isolates were maintained by repeated inoculation of lettuce cotyledons in Petri dishes in a growth chamber (15°C, 12 h light/day).

Plants

The susceptible lettuce (*Lactuca sativa*) cv. Noga (cup type; Hazera Genetics, Mivhor, Israel) was used. For

growth chamber studies, plants were grown from seeds in 175 ml pots containing 40 g peat/vermiculite mixture (1/1, *v/v*) to give 20 plants per pot. Plants grown in the greenhouse (18–32°C) were used 1 week after sowing, when two cotyledon leaves had developed. In other experiments, plants were grown either in Speedling (Hishtil, Petah-Tiqwa, Israel) trays (25 ml cells), one plant per cell and used when they had five to six true leaves, or in 0.5-l pots and used at the five to six true leaf stage.

Application of compounds

Compounds were diluted in water to a series of concentrations and applied to lettuce plants by spraying onto the upper leaf surfaces to initial run-off. Depending on the experiment, a compound was applied either before or after inoculation. In other experiments, compounds were applied as 10 µl droplets to detached cotyledon leaves, true leaves, or leaf discs. In the field, compounds were applied by spraying to initial run-off with the aid of a backpack sprayer.

Inoculation

Spores of *B. lactucae* were collected from freshly sporulating lettuce leaves into ice-cold DDW, their density adjusted to 1×10^4 spores per milliliter and then sprayed onto the upper leaf surfaces of the test plants to initial run-off with the aid of a glass atomiser. Plants were thereafter placed in a dew chamber (18°C, in the dark) for 20 h and then transferred to a growth chamber at 20°C (12 h light/day, 100 µE m^{-2} s^{-1}). At 5 days post-inoculation (dpi) plants were placed in Perspex boxes (100% relative humidity) for two days to induce sporulation of the pathogen.

Disease assessment

The number of sporulating plants was determined at the cotyledon stage with the aid of a magnifying lens (×10) at 7 dpi, unless stated otherwise.

Germination of spores in vitro

Spores were mixed (1:1) with CAA and applied to depressions in glass slides (20 µl per depression, *n*=3). Slides were kept in moist Petri dishes at 13°C for 20 h in the dark. Germination of 100 spores was recorded per depression with the aid of a dissecting microscope at ×160.

F-Actin distribution

Spores were mixed (1:1) with water (as control), 0.01 mg l^{-1} MPD or 0.1 mg l^{-1} DMM and applied to depressions in glass slides (20 µl per depression, *n*=9). Slides were kept in moist Petri dishes at 13°C for 18 h in the dark to allow for spore germination. Spores were then fixed for 10 min with formaldehyde (3.7%, 10 µl per depression), collected from the depressions, washed twice, and treated for 10 min with Alexa Fluor® 488 phalloidin (Invitrogen, Molecular Probes, Eugene, OR, USA). F-Actin distribution was visualised with a confocal microscope (Karl Zeiss, LSM-510 META), and images captured with a Zeiss AxioCam camera and analysed using Zeiss AxioVision software.

Germination of spores on leaves

Tests were performed with either cotyledon leaves or the first formed true leaf detached from 7- or 10 day-old plants, respectively. Cotyledons or leaves (*n*=4) were placed on moistened filter paper in 9 cm Petri dishes and each inoculated on the upper surface with a 20 µl droplet of spore suspension (containing 500 spores) mixed 1:1 with CAA. Dishes were incubated at 13–15°C for 20 h in the dark. A 10 µl droplet of 0.02% calcofluor (Polyscience Inc., Warrington, PA, USA) was added to each cotyledon and leaf, and germination was visualised with the aid of an UV epifluorescence microscope (Olympus AX70) equipped with an excitation filter of 390–420 nm and an emission filter of 425–450 nm. *Bremia lactucae* structures fluoresced blue; 100 spores were recorded per sample.

Temporary exposure of spores to CAA

The effect of temporary exposure of spores to CAA was assessed by incubating spores in 0, 0.5 or 1 mg l^{-1} CAA on ice for 1 h, then washing with water and allowing them to either germinate for 6 h at 13°C in the dark in vitro or to infect lettuce leaves.

Pathogen development in planta

To detect pathogen structures inside the tissue, leaves were cleared by boiling in 90% ethanol for

Fig. 1 a–j Percentage spore
germination (means and
standard deviation of the
mean, $n = 100$; germ-tube
length, $>5 \ \mu m$) and germ-
tube length of four Europe-
an (BL-18 to BL-25) and
one Israeli (ISR-60) isolates
of B. lactucae in vitro in the
presence of technical CAA
compounds (MPD mandi-
propamid, DMM dimetho-
morph, IPRO iprovalicarb,
BENT benthiavalicarb).
k Epifluorescence micro-
graphs of calcofluor-stained
B. lactucae spore germina-
tion in vitro in the presence
of 0.0005 mg l^{-1} of four
technical CAA compounds.
Scale bar=100 μm. l Germi-
nation of B. lactucae spores
in the presence of water and
CAA: Only very small
germ-tubes are produced in
the latter (arrows). Scale
bar=20 μm
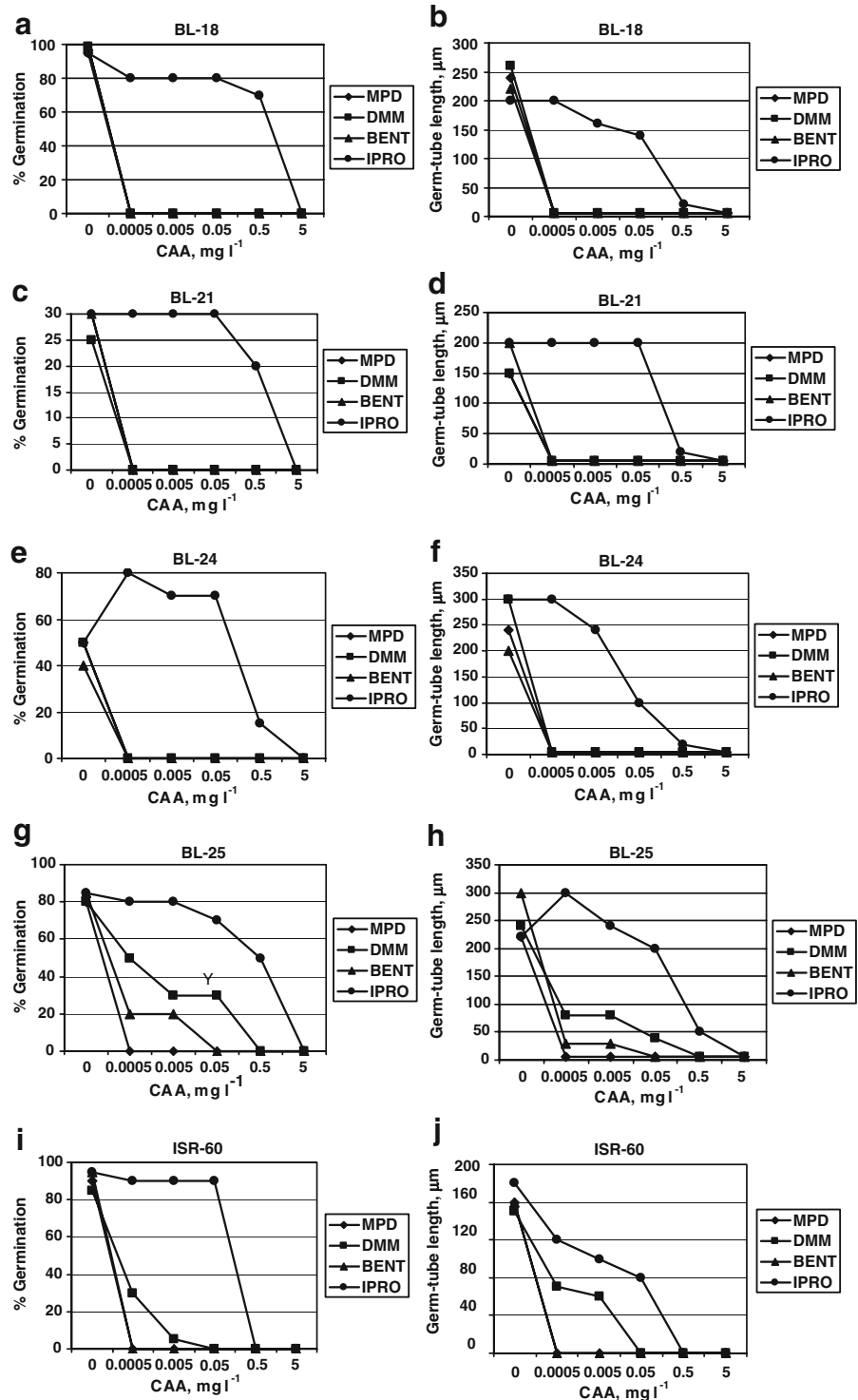
20 min, and then placed in 0.05% aniline blue in
70 mM potassium phosphate buffer (pH 8.9) at
4°C for 24 h. Stained leaves were placed on glass
slides, a drop of 0.02% calcofluor applied to the

surface and then examined by epifluorescence
microscopy, as above (Cohen et al. 1989, 1990).
Spores and germ tubes on the leaf surface fluoresced
blue; β-1–3 glucans in walls of intercellular hyphae

Fig. 1 Continued
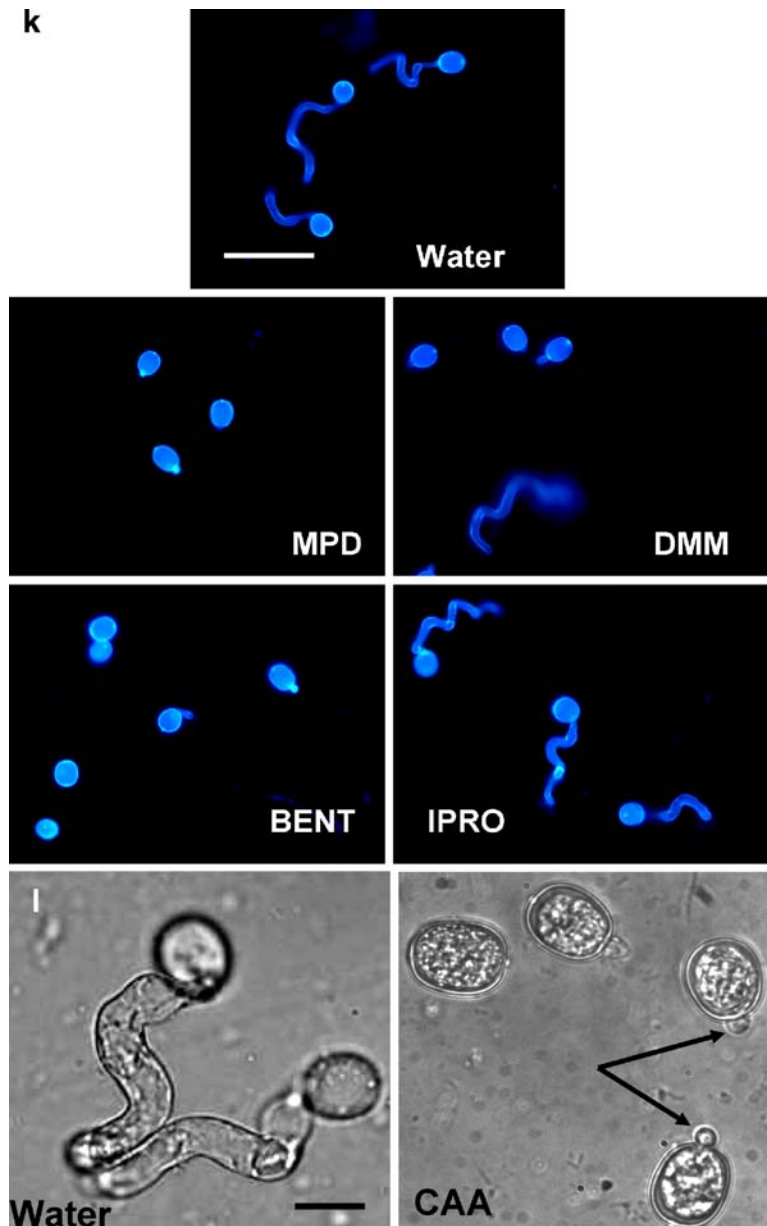
and callose deposited around haustorial necks fluoresced yellow.

Sporulation on leaves, plants and leaf discs

Leaves of plants with one true leaf were detached at 7 dpi and floated on technical CAA in 5 cm Petri dishes at 18°C for 48 h (12 h light/day). Intensity of sporulation was estimated with the aid of a magnifying lens (×10), and spore counts for leaves floating on 100 mg l^{-1} were made using a cytometer. Plants at the cotyledon stage were sprayed with technical CAA at 4 dpi, the number of sporulating plants counted at 7 dpi and spore counts measured at 11 dpi. Leaf discs (1 cm diam) from infected 10-leaf plants at 7 dpi were floated on 1 ml of either water or formulated CAA at 20°C for 20 h in the dark in six-well titer plates, and the number of sporophores per disc was determined by fluorescence microscopy after staining with calcofluor. Similar discs were incubated at 18°C with 12 h

light per day for 2 days, and the number of spores produced assessed as above.

Translaminar efficacy

Leaves formed fifth from the stem base were detached from 10-leaf plants, sprayed on upper leaf surface with 100 mg l^{-1} of four technical CAA fungicides, and placed on wet filter paper in $20\times20\times3$ cm plates. After 3 h, leaves were inverted and inoculated on their lower surface with spores of *B. lactucae*, and spore germination determined at 20 hpi as before. The number of sporulating lesions was counted at 10 dpi.

Shade-house experiments

Two experiments were conducted during 2006–2007 with whole plants of *L. sativa* cv. Noga to evaluate the efficacy of CAA in controlling *B. lactucae*. Plants were raised from seeds in Speedling

trays in the greenhouse, and transplanted when they had five true leaves into polystyrene containers ($1.2\times0.6\times0.2$ m) filled with peat and vermiculite (1:1, v/v), to give 10 plants per container. Containers were located in shade-houses in the field at Bar-Ilan University Farm. Shade-houses were covered with white plastic nets (50 holes per square inch, mesh) to avoid aphid and viral infections. In 2006, plants were sprayed twice with three concentrations of formulated MPD, with the first spray at 4 weeks after planting, when plants had reached the 10–12 leaf stage. The second spray was applied after a further 8 days. In 2007, plants were sprayed once with 500 mg l^{-1} of each of three formulated CAA fungicides, before transplanting into the shade-house. BENT was not tested outdoors because no formulated solo product was available. In both experiments plants were inoculated with a spore suspension of *B. lactucae* (1×10^3 ml^{-1}) in the evening on the same day as the treatment. After inoculation, plants were covered with plastic sheets
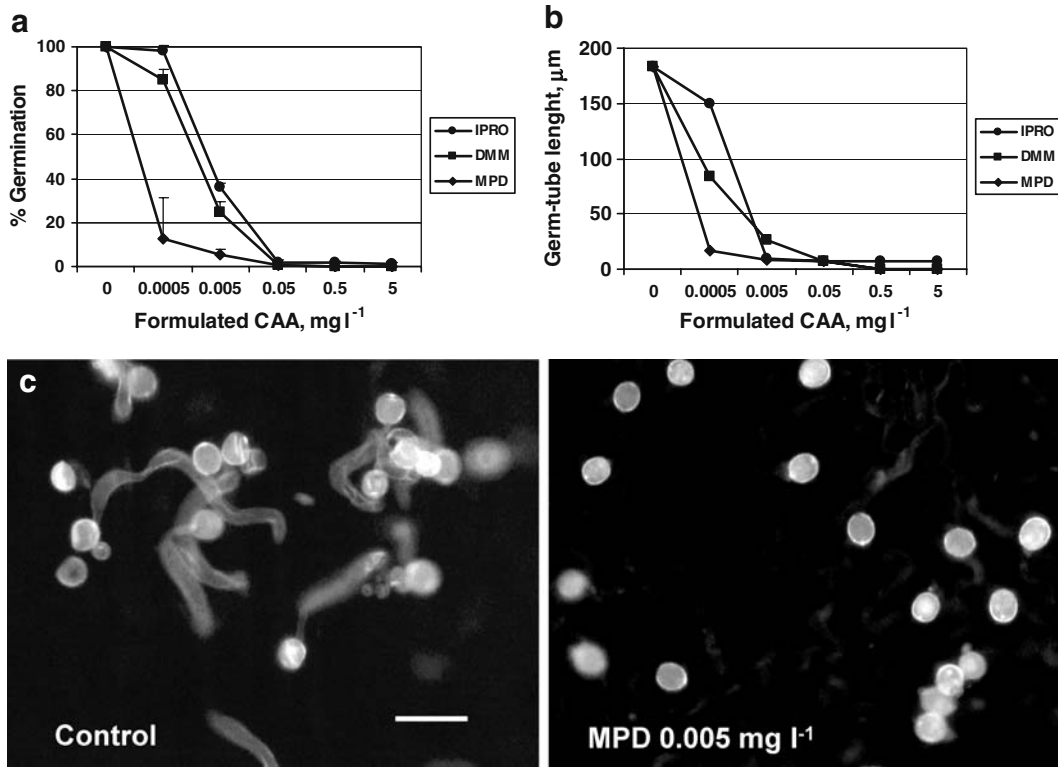
Fig. 2 Percent spore germination (means and standard deviation of the mean, $n=100$) (**a**) and germ-tube length (**b**) of *B. lactucae* isolate ISR-60 on leaf surfaces treated with three formulated CAA compounds (see Fig. 1 for abbreviations).
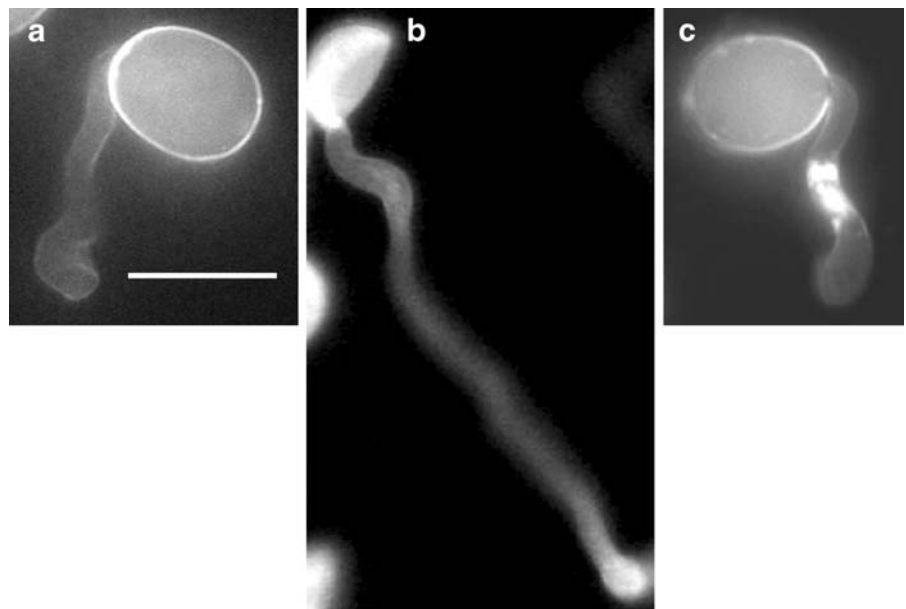
c Epifluorescence micrographs of calcofluor-stained *B. lactucae* spore germination on leaf surfaces treated with water (control) and technical MPD. Scale bar=50 μm
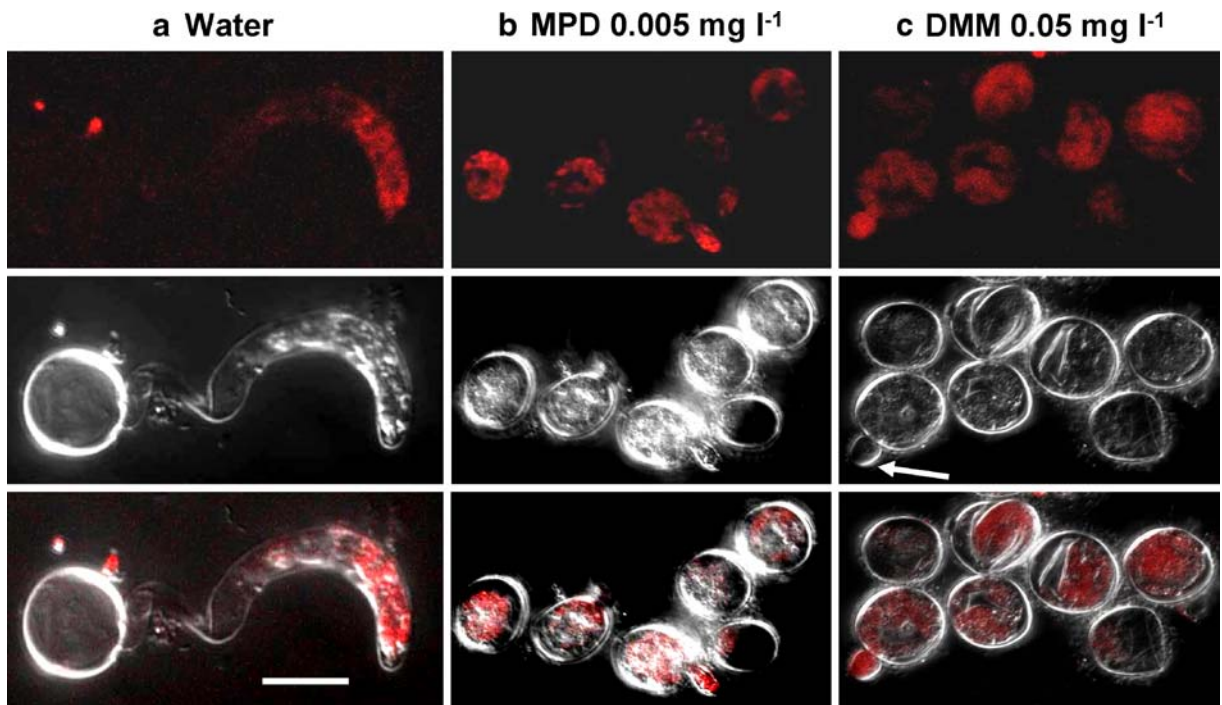
Fig. 3 Laser scanning confocal micrographs of phalloidin-stained *B. lactucae* spore germination in vitro showing no effect of CAA on F-actin organization in the germinating spores (*arrow* small germ-tube). Scale bar=20 μm

for the night to ensure infection. In the first experiments, CAA was applied with a hand sprayer, or a manual backpack sprayer, at a rate of about 20–30 ml per plant. Disease severity was recorded at 42 or 50 dpi by counting the number of downy mildew lesions developing on 10 plants in a container.

Results

Spore germination

Four European and one Israeli isolate of *B. lactucae* were tested for sensitivity to technical CAA fungi-

Fig. 4 Epifluorescence micrographs of calcofuor-stained germinating spores of *B. lactucae* after adding 0.005 mg l^{-1} MPD 3 h after start of germination in vitro. **a** Germ-tube at 3 h after start of germination (time of treatment). Control (**b**) and treated (**c**) germ-tubes at 48 h after start of germination. Scale bar=20 μm

cides in vitro. CAA strongly suppressed spore germination, but enabled spores to produce very small germ-tubes (Fig. 1l). True germination was considered to have occurred only when a spore produced a germ-tube of ≥ 5 µm (spore diam ~20 µm).

Figure 1a–f shows that the European isolates BL-18, BL-21 and BL-24 were totally inhibited by 0.0005 mg l^{-1} (lowest concentration tested) of MPD, DMM and BENT, and by 5 mg l^{-1} of IPRO. Strangely, low concentrations of IPRO stimulated germination of BL-24 (Fig. 1e). BL-25 (Fig. 1g, h) was similarly sensitive to MPD and IPRO, but required ×100 and ×1,000 more BENT and DMM, respectively, to be suppressed fully. The Israeli isolate ISR-60 (Fig. 1i, j) was more sensitive to IPRO, and controlled fully with 0.5 mg l^{-1}, compared with the European isolates. It was highly sensitive to MPD and

BENT (totally inhibited with 0.0005 mg l^{-1}) and ×100 less sensitive to DMM.

Figure 1k shows the in vitro germination of *B. lactucae* spores in water and in four technical CAA fungicides at 0.0005 mg l^{-1} (for numerical data see Fig. 1a–j). At inhibitory concentrations, all fungicides allowed the formation of very short germ-tubes (about one fifth of the spore size, Fig. 1l) in about 20% of the spore population.

Figure 2 shows the germination of *B. lactucae* spores in the presence of three formulated CAA fungicides (0.0005–5 mg l^{-1}) on plant leaf surfaces. At 0.0005 mg l^{-1}, MPD (1.2 nM) was significantly more effective than DMM or IPRO, causing 90% inhibition. Formulated IPRO in planta was much more effective than technical IPRO in vitro (compare with Fig. 1). All fungicides strongly affected not only the number of germinating spores (Fig. 2a, c) but also
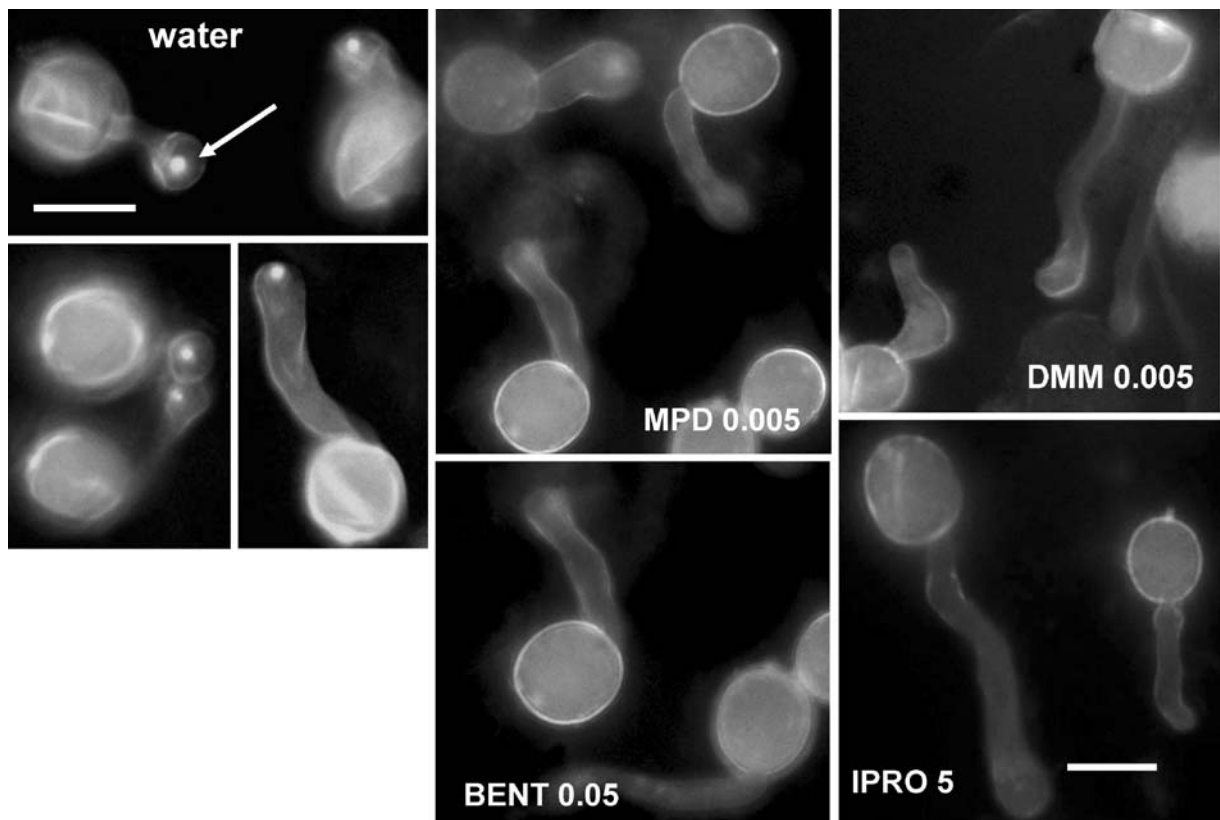
Fig. 5 Epifluorescence micrographs of germinating spores of *B. lactucae* treated with technical CAA (0.005–5 mg l^{-1}; see Fig. 1 for abbreviations) and water at 3 h post-inoculation. Penetration of *B. lactucae* into lettuce leaf tissue occurs in water-treated leaf tissue but not in CAA-treated tissue. Arrow indicates point of penetration (yellow fluorescence). Aniline blue staining followed by calcofluor staining; scale bar=20 µm
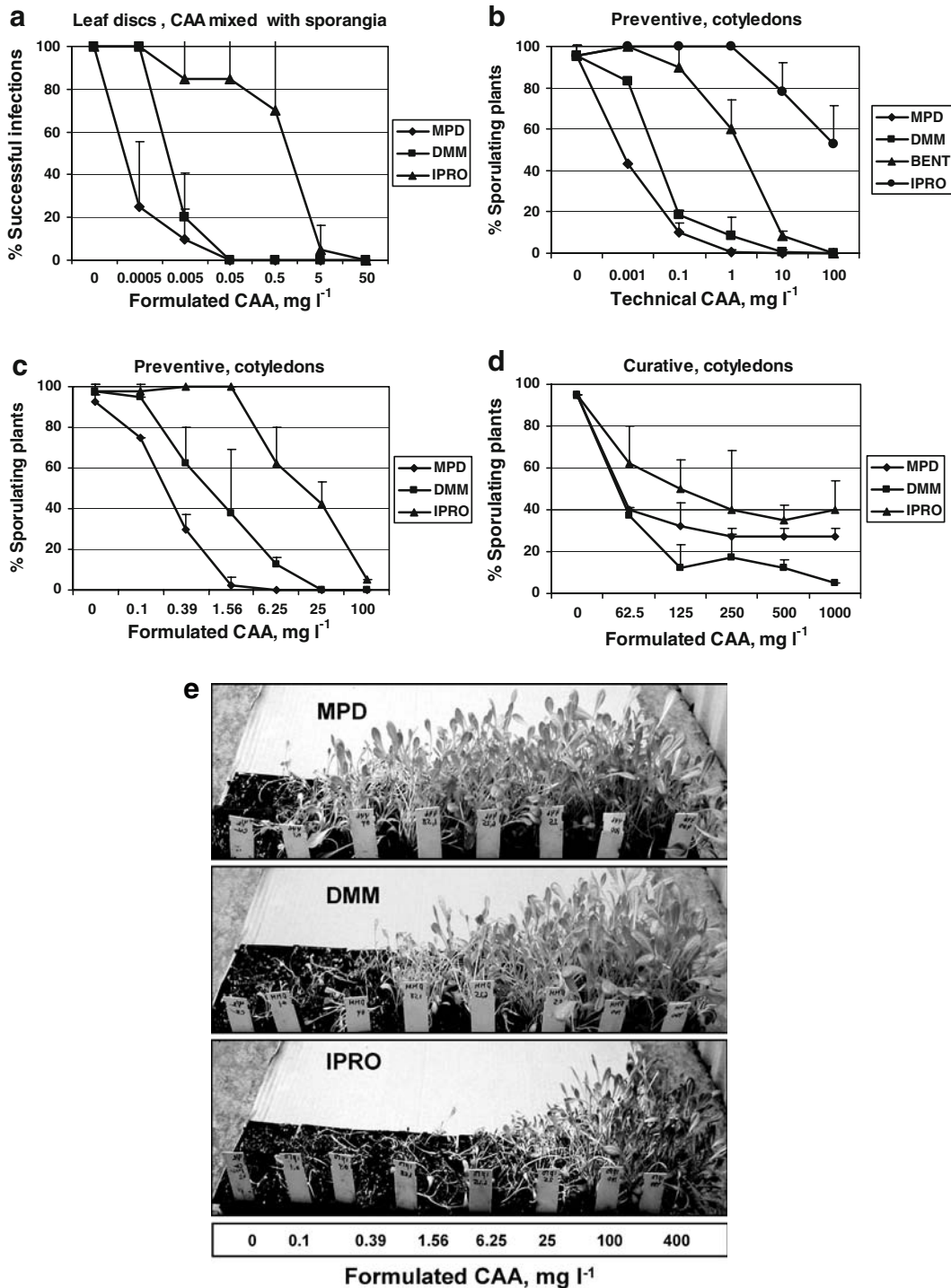
Fig. 6 Downy mildew development in lettuce plants treated with CAA (see Fig. 1 for abbreviations). **a** CAA mixed with spores of *B. lactucae* before inoculation (means and standard deviation of the mean, *n*=40). **b**, **c** CAA applied preventively 4 h before inoculation (*n*=80). **d** CAA applied curatively 1 dpi (*n*=80). Disease symptoms recorded at 7 dpi. **e** The appearance of the treated and inoculated plants shown in **c** at 21 dpi

the size of the germ-tubes (Fig. 2b, c). BENT was not available as a formulated solo product; a formulation (10% a.i.) was therefore prepared in Agsolex-8 and used for testing germination in vitro. Results confirm that BENT at 0.005 mg l^{-1} (lowest concentration tested) allowed 11% germination (88% inhibition relative to water control). The germinated spores produced a germ-tube of 11 μm compared to 170 μm in the water control.

Phalloidin staining indicated no disruption by CAA in *B. lactucae* spores (Fig. 3). Spores germinating in water (18 h, 13°C, darkness) produced a germ-tube of about 80 μm. The F-actin (red) was concentrated in the distal, growing part of the germ-tube, moving with the cytoplasm towards the tip. In CAA-treated spores, most produced no germ-tube, while some produced a germ-tube of about 3–5 μm. In non-germinating spores the red stain remained within the spore. In those that produced a small germ-tube, the red stain representing the F-actin microfilaments moved into the germ-tube (Fig. 3).

Effect of CAA on germ-tube growth

Microscopic observations (Fig. 4) made in vitro (13°C, darkness) showed that CAA (technical, 0.005–50 mg l^{-1}) added to spores at 3 h after the start of germination inhibited further extension of germ-tubes. At 3 h, when CAA was added, 50% of the spores produced a germ-tube of 40 μm (±standard error of 10 μm); at 48 h (45 h after adding CAA) mean germ-tube length in control, water-treated spores was 250±50 μm. In contrast, spores treated with ≥0.005 mg l^{-1} of technical MPD, DMM or BENT showed no extension of the germ-tubes. Spores treated with IPRO at 0.005, 0.05, 0.5, 5 and 50 mg l^{-1} produced germ-tubes of 150±30, 100±20, 80±20, 60±10, and 40±10 μm, respectively.

Effect of CAA on penetration

Spores were allowed to germinate on lettuce leaf surfaces at 13°C, and CAA added at 3 hpi when 50% of the spores had germinated with a germ-tube of 40±10 μm. Leaves were incubated for an additional 17 h at 13°C to allow penetration of the host. At 20 hpi, epifluorescence microscopy showed that penetration occurred in water-treated control leaves (Fig. 5, arrow) but not in leaves treated with CAA at 0.005–50 mg l^{-1}, except IPRO which stopped penetration at ≥5 mg l^{-1} (Fig. 5).

Temporary exposure

Spores incubated in water showed 80–90% germination (germ-tube=30–50 μm). Spores exposed to 0.5 or 1 mg l^{-1} MPD or DMM for 1 h on ice failed to germinate or infect leaves when the CAA was removed by washing with water. In contrast, spores exposed to 0.5 or 1 mg l^{-1} BENT or IPRO for 1 h germinated in vitro and produced lesions when inoculated onto detached leaves equivalent to control spores.

Effect on infection

Spores were mixed with formulated CAA and inoculated onto lettuce leaf discs laid lower surface uppermost on moist filter paper in 9 cm Petri dishes.
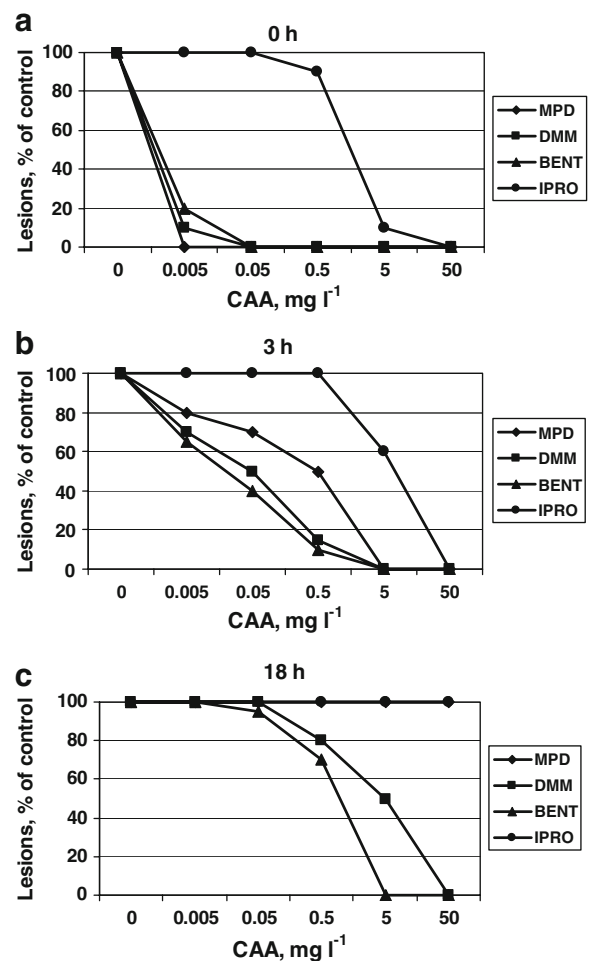

Fig. 7 Control of downy mildew lesion development in lettuce by CAA (see Fig. 1 for abbreviations) applied in **a** at 0 hpi, **b** at 3 hpi, and **c** at 18 hpi

The number of successful infections (sporulating lesions) at 10 dpi is shown in Fig. 6a. MPD was most effective while IPRO was least effective. The inhibitory concentrations of the three CAA fungicides resemble those required to inhibit spore germination in vitro (Fig. 1). Microscopic observations made at 1 dpi revealed that the failure to infect (for example in the presence of MPD) was a consequence of inhibited spore germination. In control inoculated plants, primary and secondary vesicles were seen in epidermal cells from which hyphae were emerging.

Preventive application of four technical or three formulated CAA fungicides to 7 day-old lettuce plants efficiently protected against downy mildew. Plants were sprayed with CAA at various concentrations and inoculated 4 h later. The proportion of
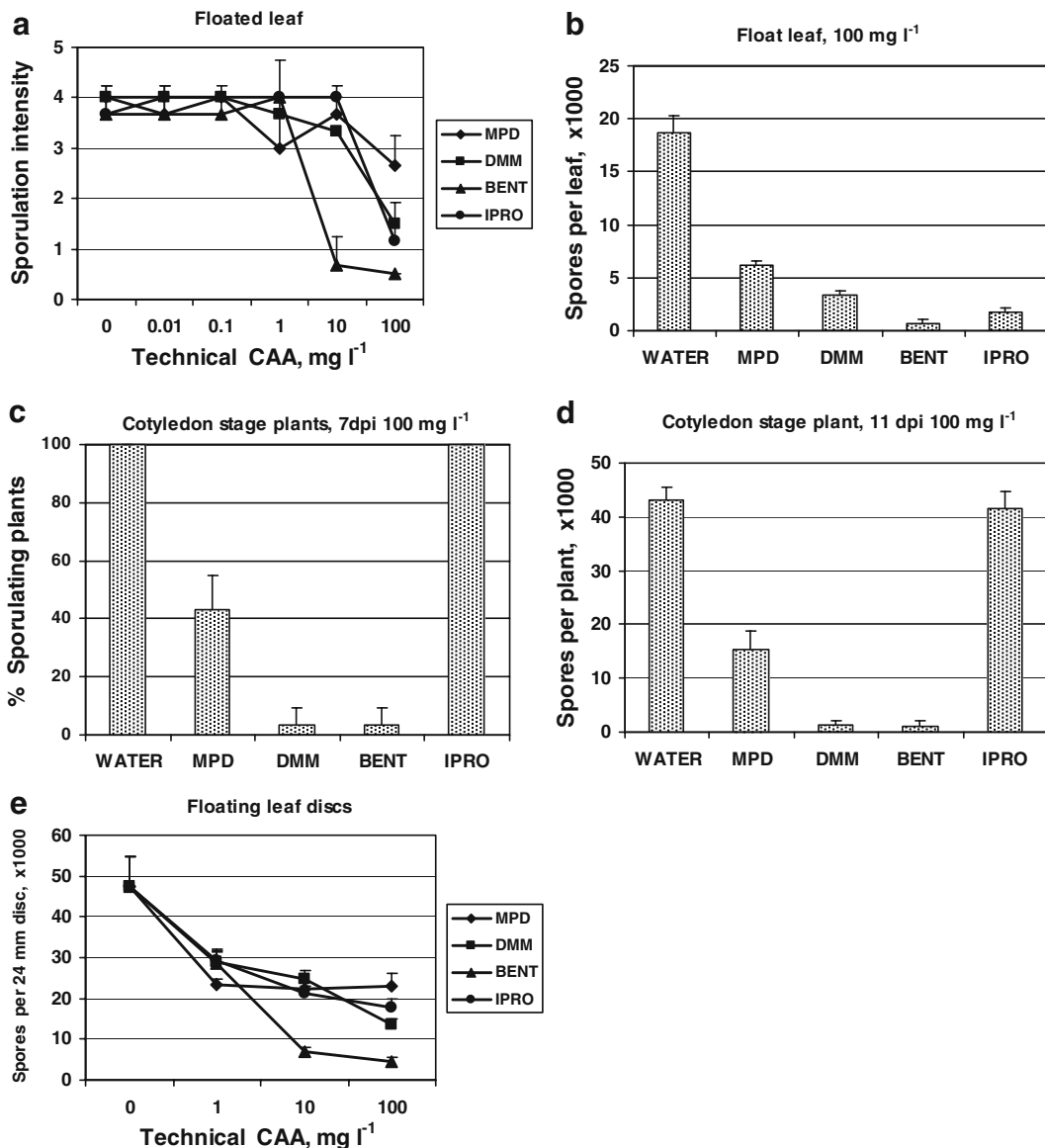
Fig. 8 Inhibition of sporulation of *B. lactucae* by CAA (see Fig. 1 for abbreviations), shown as sporulation intensity (visual assessment, means and standard deviation of the mean, $n=20$) (**a**), spores per leaf ($n=10$) (**b**), percent sporulating plants ($n=20$) (**c**), spores per plant ($n=20$) (**d**) and spores per leaf disc ($n=10$) (**e**). **a** and **b** First-formed leaves floating on CAA. **c** and **d** Whole plants at cotyledon stage, 7 and 11 dpi respectively, sprayed with CAA. **e** Leaf discs (24 mm diam) floating on CAA

plants showing sporulation of *B. lactucae* at 8 dpi is shown in Fig. 6b and c, and the appearance of the plants treated with formulated CAA at 21 dpi is shown in Fig. 6e. The fungicides differed in their efficacy in the order of MPD > DMM > BENT > IPRO.

Post-infection effects

Formulated CAA fungicides exhibited reduced control efficacy when applied at 1 dpi, when penetration of the pathogen into the leaf had already taken place (Fig. 6d). Much higher concentrations were required for inhibition of disease compared to preventive application suggesting that mycelium growth in planta is less sensitive to CAA than spore germination.

Post-infection efficacy was also tested with technical CAA in detached leaves. Leaves were inoculated and treated with the compounds at 0, 3, 18 or 45 hpi, and lesion production was assessed at 8 dpi (Fig. 7). The efficacy of the fungicides was strongly dependent on the time of their application post-inoculation. When applied at 0 h after inoculation (Fig. 7a) they were highly efficient (except IPRO) in inhibiting disease, probably due to their strong inhibitory effect on spore germination. When applied at 3 hpi efficacy decreased and the order of efficacy was BENT ≥ DMM > MPD > IPRO (Fig. 7b). At 18 hpi (Fig. 7c), MPD and IPRO lost efficacy whereas BENT and DMM showed full suppression of the disease at 5 and 50 mg l^{-1}, respectively. This suggests that BENT and DMM might affect pathogen development after penetration. Application of CAA at

45 hpi did not affect symptom production relative to untreated inoculated controls.

Effect on sporulation

BENT was most suppressive to sporulation: at 10 mg l^{-1} it caused 82% inhibition, while the other fungicides showed 0–17% inhibition at this concentration (Fig. 8a). At 100 mg l^{-1}, BENT was most suppressive and MPD least suppressive to sporulation (Fig. 8b). For whole plants at the cotyledon stage, both the number of sporulating plants (Fig. 8c) and the number of spores produced per plant (Fig. 8d) were reduced strongly by DMM and BENT, and moderately by MPD. In contrast, IPRO had no effect. The number of spores produced on leaf discs at 7 dpi and treated with technical CAA is shown in Fig. 8e. BENT was most inhibitory to sporulation, reducing the number of spores by 85% at 10 mg l^{-1} compared to 48–55% with the other fungicides. Other experiments revealed that BENT formulated with Agsolex-8 prevented sporophore emergence from stoma. BENT at 6.25 or 25 mg l^{-1} reduced the number of sporophores by 75% and 100%, respectively, relative to water controls (=130 sporophores per disc). The other fungicides had no effect on sporophore formation.

Translaminar efficacy

Data on germination of spores and sporulating lesions on detached leaves inoculated on the undersurface following treatment with CAA on the upper surface are given in Fig. 9a and b. Percentage inhibition of
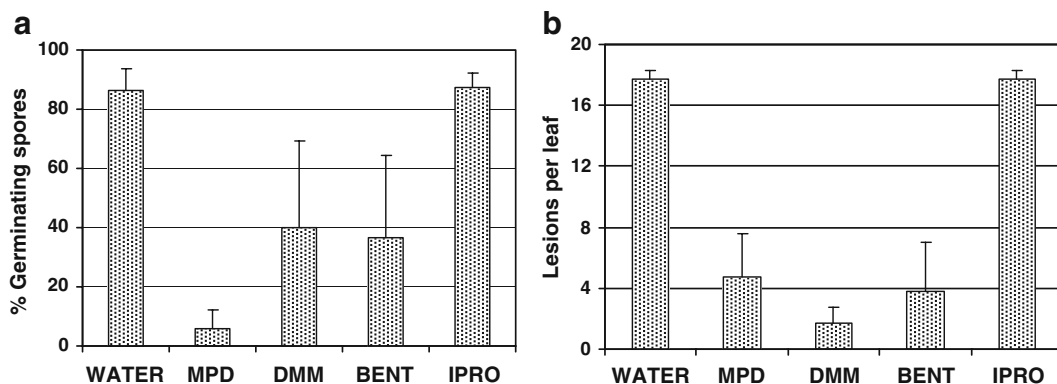
Fig. 9 Germination of *B. lactucae* spores on lower leaf surface (means and standard deviation of the mean, *n*=100) (**a**), and lesion development (*n*=10) (**b**), in detached leaves treated with CAA (see Fig. 1 for abbreviations) on upper leaf surface and inoculated on lower leaf surface

spore germination at 20 hpi was 94, 54, 58 and 0 (Fig. 9a), and percentage inhibition of lesion at 10 dpi formation was 73, 90, 79 and 0 for MPD, DMM, BENT and IPRO, respectively (Fig. 9b). The data show that while MPD was superior to the other fungicides in translocation to the opposite surface of the leaf in assessments of spore germination, DMM was superior in inhibiting lesion formation. This suggests that DMM might have post-germination effects on *B. lactucae*.

Shade-house experiments

Data from the experiments to assess the efficiency of CAA for controlling epidemics in shade-house grown plants are presented in Fig. 10. In 2006, all three concentrations were effective similarly, providing about 95% and 90% protection at 27 and 42 days after the second (last) spray, respectively (Fig. 10a and b). In 2007, a single spray of CAA provided about 90% protection at 50 days after treatment (Fig. 10c).

Discussion

Carboxylic acid amide (CAA) fungicides are shown here to control effectively (with intrinsic differences) downy mildew in lettuce in growth chamber and shade-house experiments. The toxicity of four CAA compounds towards *B. lactucae* has been compared quantitatively in vitro and in planta, by exposing the pathogen to CAA at various stages of its life-cycle: spore germination, germ-tube extension, penetration, colonisation, sporophore formation, spore production and epidemics in the field.

It appeared that spore germination was the stage in the life-cycle most sensitive to CAA. Germination in vitro and on leaf surfaces was inhibited with nano-molar concentrations of MPD (0.0005 μg ml^{-1}) and micromolar concentrations of DMM and BENT (0.005 μg ml^{-1}) or IPRO (0.5 μg ml^{-1}). Similar results were reported recently for germination in vitro of sporangia and cystospores of *P. infestans* (Cohen and Gisi 2007). Unlike cystospores or sporangia of *P. infestans* which produce no germ-tubes in CAA (Cohen and Gisi 2007), spores of *B. lactucae* produce a small germ-tube (3–5 μm) in the presence of CAA. The formation of these germ-tubes suggests that the
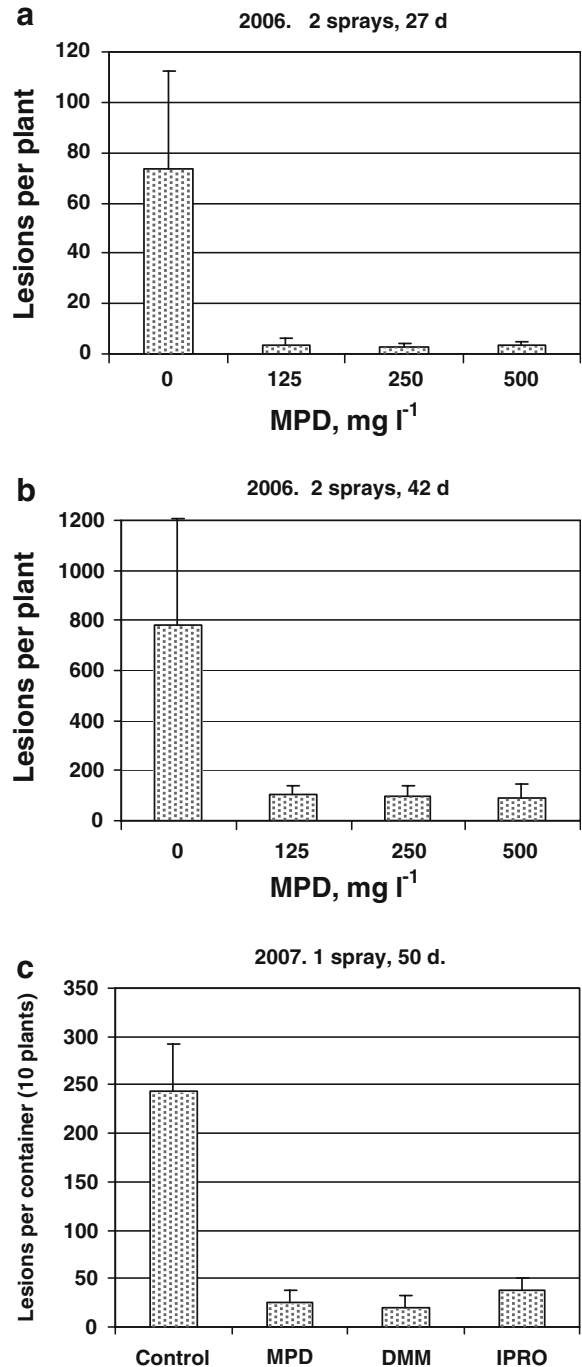
Fig. 10 Downy mildew development in shade-house grown lettuce plants treated with 125, 250 or 500 mg l^{-1} MPD (formulated) at 27 (**a**) and 42 (**b**) days after the second (last) spray. **c** Downy mildew development in shade-house grown lettuce plants treated with three CAA compounds (formulated; see Fig. 1) applied once at a dose of 500 mg l^{-1}. Lesions per ten plants in a box recorded at 50 days after spraying. $n=10$ boxes per dose treatment per fungicide

polarity of the spores of *B. lactucae*, required for germ-tube formation, is not disturbed by CAA.

Phalloidin is a specific stain for F-actin microfilaments and was applied recently by Zhu et al. (2007) to flumorph-treated cystospores and mycelium of *P. melonis* in vitro. They concluded that flumorph disrupts microfilament organisation. In the present study, no such disruption in F-actin organization was seen in spores of *B. lactucae* treated with MPD or DMM. More work is needed to elucidate the mode of action of CAA and the target site in oomycetes.

When germ-tubes were allowed to be formed during 3 h in water (reaching ~40 μm long), CAA stopped their further extension in vitro. Equivalent experiments on leaf surfaces showed that CAA prevented them from producing appressoria and penetrating the host. Experiments also determined that post-penetration stages of *B. lactucae* infection are less sensitive to CAA and require much higher doses to be controlled. As disease control by CAA is a consequence of inhibition of spore germination and germ-tube extension, only preventive application might reduce disease development effectively.

CAA compounds applied to upper lettuce leaf surfaces were shown here to inhibit spore germination of *B. lactucae* on the lower leaf surface. This suggests that CAA travels across the leaf (translaminar movement) to reach the lower surface of the opposite, untreated side of the leaf. CAA compounds greatly differ in their translaminar movement, with MPD being superior to others. Similar results were obtained for MPD compared to DMM and IPRO in potato and tomato (Cohen and Gisi 2007). Studies with C^{14}-MPD showed that within 1.5 h after application, 4.9% and 1.3% of the applied compound reached the mesophyll of potato and grape leaves, respectively (Hermann et al. 2005). Three days after application, 54.9%, 36.4% and 8.7% of the applied radioactive MPD were on the potato leaf surface (water wash), bound on the surface and in wax (organic solvent wash), and in the leaf extract, respectively. Seven days after application, 49% of the C^{14} MPD applied to potato leaves could be recovered from leaf surface and wax (Hermann et al. 2005). This prolonged binding of MPD to the leaf cuticle, its good rain-fastness (Hermann et al. 2005) and its pronounced translaminar mobility may ensure its prolonged availability to lower leaf surfaces. No data, however, are available for how much C^{14}-MPD is present on the opposite, lower, surface of the leaf.

Miyake et al. (2003) reported that BENT has not only strong preventive activity, but also curative and penetrant activity, with excellent residual effects and rainfastness.

The biotrophic nature of *B. lactucae* does not allow testing the effects of CAA on mycelium growth or sporulation in vitro. Therefore, the interpretation of in planta experiments should consider also the incorporation of the CAA compounds into the leaf tissue. Indeed, application of CAA to detached leaves at 18 h post-inoculation, when primary and secondary vesicles have already developed in the epidermal cells, did not prevent the pathogen from completing its life-cycle by producing new spores. Nonetheless, two compounds, BENT and DMM, did partially inhibit sporulation, and BENT also suppressed sporophore development.

CAA fungicides were found to be highly effective in controlling downy mildew in lettuce in the shade-house experiments. Unfortunately, doses applied were too high to distinguish differences in efficacy among the compounds. Previous data with late blight control in potato (Cohen and Gisi 2007) showed superior activity of MPD over DMM, and of DMM over IPRO. More studies are required to evaluate the performance of CAA, including BENT, in the field.

In conclusion, CAA fungicides were found here to be extremely potent inhibitors of spore germination of *B. lactucae*, as they were in *P. infestans* (Cohen and Gisi 2007). CAA inhibited germ-tube extension when applied to germinating spores. The compounds had poor curative efficacy when applied at 1 dpi against *B. lactucae*, as was determined previously for *P. infestans* (Cohen and Gisi 2007). All the compounds, except IPRO, showed translaminar movement across lettuce leaves, with MPD superior to DMM and BENT. When applied to mature lesions, BENT was superior to the other compounds in suppressing sporulation of *B. lactucae*. CAA fungicides, therefore, should be used preventively to achieve the best control of downy mildew in lettuce.

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