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EMS and UV irradiation induce unstable resistance against CAA fungicides in *Bremia lactucae*

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Abstract Wild type (WT) field isolates of *Bremia lactucae* failed to germinate in vitro or infect lettuce leaves in the presence of CAA (carboxylic acid amide) fungicides. Minimal inhibitory concentrations (MIC) for mandipropamid, dimethomorph, benthiavalicarb and iprovalicarb were 0.005, 0.5, 0.5 and 5 $\mu g ml^{-1}$, respectively. Mutagenesis experiments showed that spores exposed to EMS (ethyl methane sulphonate) or UV irradiation (254 nm) could infect lettuce leaves in the presence of up to 100 $\mu g ml^{-1}$ CAA. The proportion of infected leaves relative to the number of spores inoculated (infection frequency) was inversely related to the concentration of CAA used, ranging between 0 and 160 per 1×10^6 spores. Resistant mutants (RM) lost their resistance within 1–14 reproduction cycles on CAA-treated plants. Crosses were made between RMxWT isolates and RMxRM isolates with an attempt to obtain stable homozygous resistant off-springs. Such crosses yielded few resistant but unstable progeny isolates. Mutagenic treatments given to hybrid isolates also failed to produce stable resistance. Previous gene sequencing data showed that stable resistance to CAAs is based on a single SNP in the *cellulose synthase 3* (*CesA3*) gene of *Plasmopara viticola*. Therefore, we sequenced a 582 bp DNA fragment of *Ces3A* of WT, RM and hybrid isolates of *B.lactucae*. No mutation in this gene fragment was found. We conclude that mutagenic agents like EMS or UV may induce resistance to CAA in *Bremia lactucae* but this resistance is not stable and not linked to mutations in *CesA3* gene.

Keywords Chemical control · Lettuce downy mildew · Oomycetes

Abbreviations

BENT	Bentiavalicarb
BC	Back-cross
CAA	Carboxylic acid amides
Ces	Cellulose synthase
Dpi	Days post inoculation
DMM	Dimethomorph
EMS	Ethyl methanesulfonate
IPRO	Iprovalicarb
MIC	Minimal inhibitory concentration
MPD	Mandipropamide
RM	Resistant mutant
PCR	Polymerase chain reaction
UV	Ultra violet
WT	Wild type

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Introduction

The Carboxylic Acid Amides (CAA), mandipropamide (MPD), dimethomorph (DMM), bentiavalicarb (BENT) and iprovalicarb (IPRO) are effective fungicides against oomycete plant pathogens including *Phytophthora infestans* (Cohen and Gisi 2007), *Plasmopara viticola* (Reuveni 2003; Gisi et al. 2007), *Pseudoperonospora cubensis* (Cohen et al. 1995) and *Bremia lactucae* (Cohen et al. 2008). Resistance against CAAs occurs in field populations of *P. viticola* (Gisi et al. 2007) and *P. cubensis* (Y.Cohen, unpublished data, CAA FRAC working group, www.frac.info) but not in *P. infestans* or *B. lactucae*. The reasons for the differential occurrence of field resistance in some but not other oomycetes are not clear. Major reasons may be that resistance is hardly fixed in the population because of its recessive nature controlled by one or two nuclear genes (Gisi et al. 2007) and that pathogen species differ in basic biological processes such as frequency of sexual reproduction and migration. With *P. infestans*, we were able to artificially mutate sporangia for stable resistance against the phenylamide fungicide mefenoxam but failed to select mutants with stable resistance against CAAs (Rubin et al. 2008), suggesting a low risk of resistance developing in this pathogen in field populations against CAAs.

The aim of the present study was to estimate the likelihood of *B. lactucae* to develop resistance against CAA fungicides. This was done by exposing spores to UV light or to the mutagenic agent EMS and examining their capability to infect lettuce leaves after being mixed with CAA, or to infect CAA-treated leaves. The information provided here is important for formulating resistance management strategies to be employed in the field. Preliminary reports were published recently (Cohen et al. 2009; Werdiger and Cohen 2008).

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 to the desired concentrations. Formulated fungicides used were: mandipropamid 250SC, dimethomorph (Forum) 50WP and Benthiavalicarb (5EC), a gift from Syngenta. All concentrations are presented in units of active ingredient (a.i.).

Fig. 1 **a**. The effect of CAA fungicides on germination of six wild type isolates of *Bremia lactucae* in vitro. Spores (about 200 in 20 µl droplets) were mixed with technical grade CAAs and placed in glass slide depressions. The proportion of germinating spores (producing a germ-tube of≥10 µm) was determined microscopically after incubation at 15°C in the dark for 20 h. Vertical bars represent standard deviation of the mean (n=3). **b**. One-way Anova analysis of the germination data. Anova unit=square root of arc-sin% germination. Horizontal diagonal=mean; vertical diagonal=standard error. In each panel, diagonals sharing the same letter are not significantly different (Tukey-Kramer test, α=0.05)

Pathogen

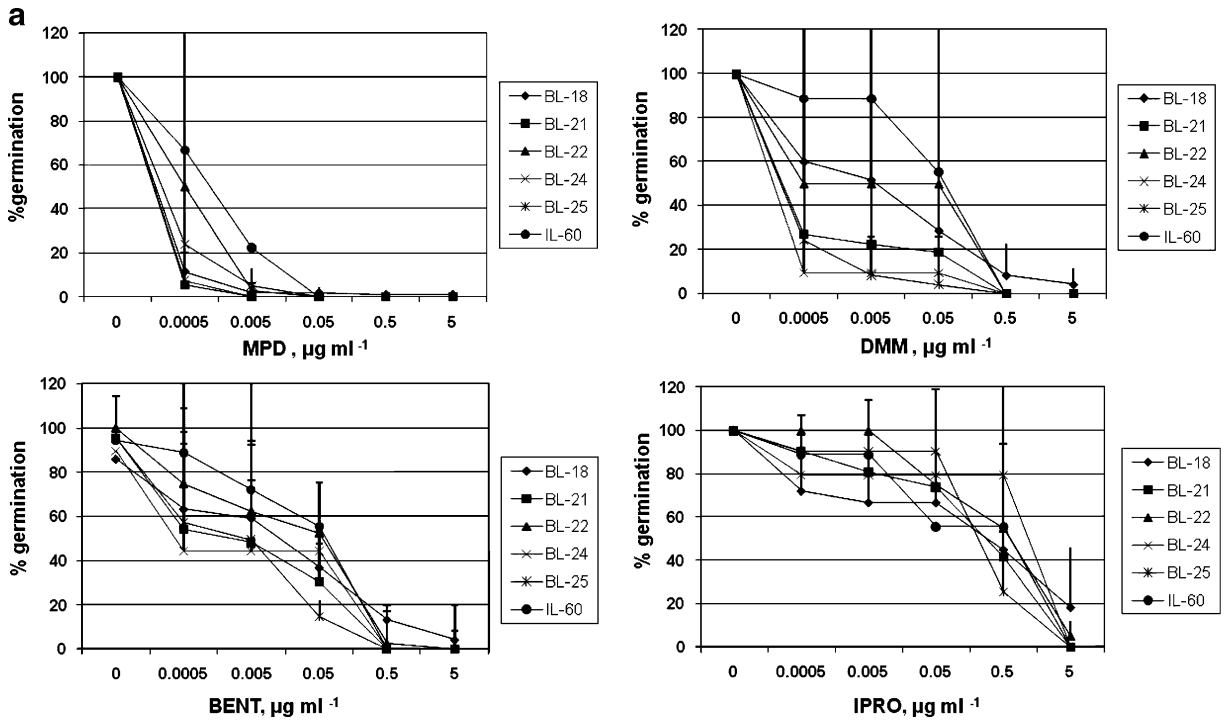
The following wild type field isolates of *B. lactucae* Regel were used: IL60 and BS (both from Israel); BL-18, BL-21, BL-24 and BL-25 (from A. Lebeda, Olomouc University, Czech Republic); and isolate CH (from Syngenta, Stein, Switzerland). Isolates were maintained by repeated inoculation on detached 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. Plants were grown from seeds in 250 ml pots containing peat/vermiculite mixture (1/1, v/v) to give 30–40 plants per pot. Plants were grown in a growth cabinet (20–22°C) and used 1 week after sowing, when two cotyledon leaves had developed. Also detached cotyledons laying on a wet filter paper in Petri dishes were used. In some experiments, plants were grown in 0.5-L pots and used at the 10th leaf stage.

Mutagenesis

Two mutagenic agents were used: EMS and UV irradiation. EMS (ethyl methane sulphonate) was purchased from Sigma. It was dissolved in water and supplied to spores of *B. lactucae* at the concentrations


a
%germination
MPD , µg ml -1
% germination
DMM , µg ml -1
BENT, µg ml -1
IPRO, µg ml -1
0
0.0005
0.005
0.05
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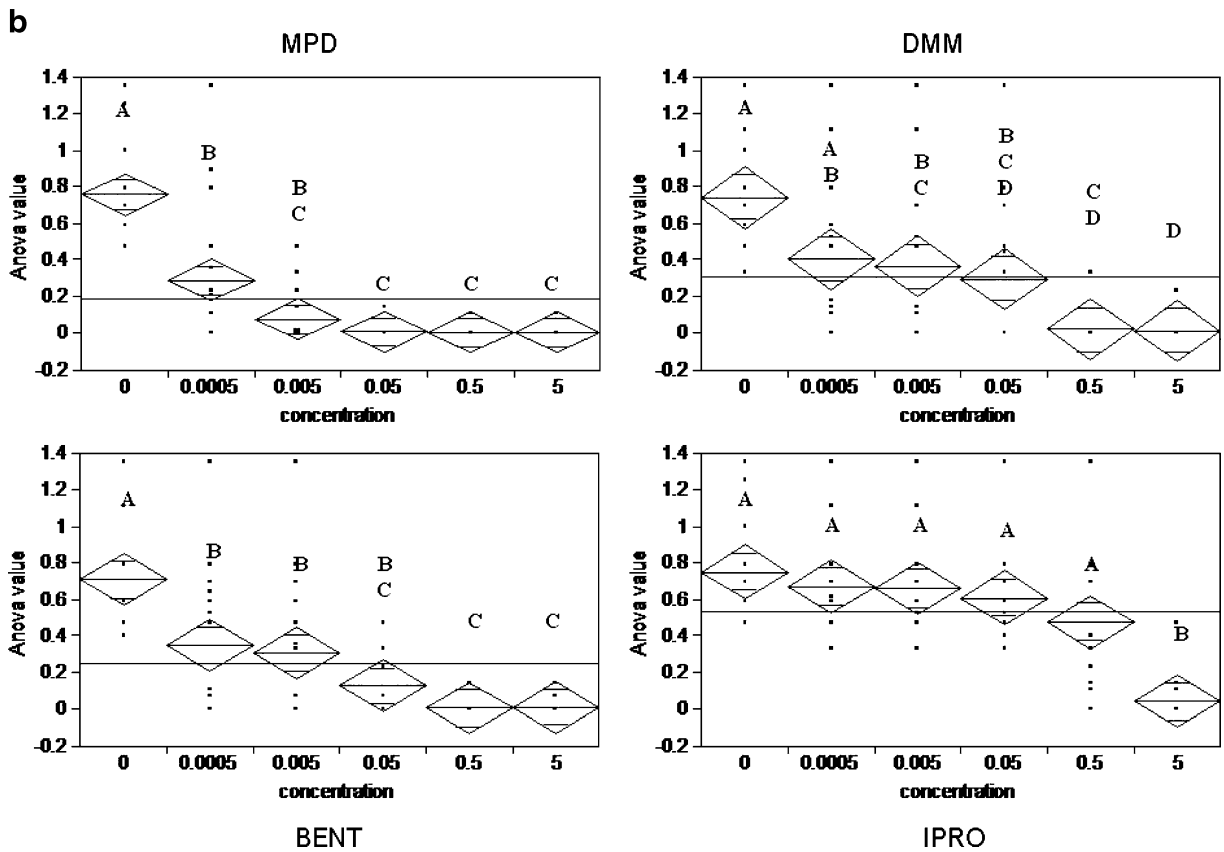
5
BL-18
BL-21
BL-22
BL-24
BL-25
IL-60


b
MPD
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BENT
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0.0005
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5

indicated in Tables 2 and 3. UV light (254 nm) was supplied to spores suspended in ice water at the doses and time periods indicated in Tables 2 and 3.

Sensitivity to CAAs

Sensitivity of isolates to CAAs was tested at the germination stage and the infection stage as described before (Cohen et al. 2008). For germination, spores were mixed with technical CAA of 0.001 to 100 μg ml^{-1} (1:1, vol/vol) and applied to depressions in glass slides (20 μl/depression) and germination was examined at×40 magnification after 20 h of incubation at 15°C in the dark. For infection, spores (2–3× 10^4 ml^{-1}) were mixed (1:1, vol/vol) with technical grade CAA of 0.001 to 100 μgml^{-1} (×10 step dilutions) and inoculated onto the lower surface of detached cotyledons, 20 μl droplet/cotyledon, 4–12 cotyledons/dose/fungicide. The proportion of sporulating cotyledons and/or the sporulation intensity (0–3 visual scale) on each cotyledon was recorded at 7 dpi for wild type isolates and up to 21 dpi for mutant isolates. Sensitivity to CAAs of mutant isolates was also tested in intact cotyledons growing in pots. Plants were sprayed to run-off with formulated CAAs of 0.78 to 50 or 100 μg ml^{-1} (×2 step dilutions), one pot with 30–40 plants per dose, and thereafter spray-inoculated with spores of the mutant isolate. Inoculated plants were placed at 100% relative humidity at 18°C in the dark for 20 h to ensure infection, and then in a growth chamber at 15–18°C (12 h light/day, 100 μE $m^{-2}s^{-1}$).

Crosses

Crosses between isolates of opposite mating types were made in the manner described for *P. infestans* (Rubin and Cohen 2006). WT isolates were crossed with mutants expressing unstable resistance to CAAs to possibly obtain homozygous resistant progeny isolates. Crosses were also made between resistant mutants. To make a cross, spores (asexual) of the two parental isolates were mixed at 1:1 ratio and inoculated onto detached cotyledons or first true leaves in Petri dishes. At 7–10 dpi, when plenty of oospores were produced, leaves were homogenized in ice water, the homogenate was poured into 9 cm Petri dishes, 10 ml/plate, incubated in a ventilated hood to dry out, re-suspended in water and dried again to ensure the killing of the vegetative structures of the pathogen. Oospores in water suspension (1-5× 10^3 ml^{-1}) were inoculated onto intact cotyledons growing in pots, 20 μl droplet/cotyledon, and the pots sealed in 1 l transparent plastic containers incubated for 1–4 weeks in a 15°C growth chamber, until progeny spores were formed on the infected cotyledons (usually 1–5 sporulating cotyledons out of about 100 inoculated). Oospores of progeny isolates were collected and used for sensitivity tests as described above. In one cross, single spore isolates were produced, propagated in the absence of fungicides, and each was tested for sensitivity to CAAs.

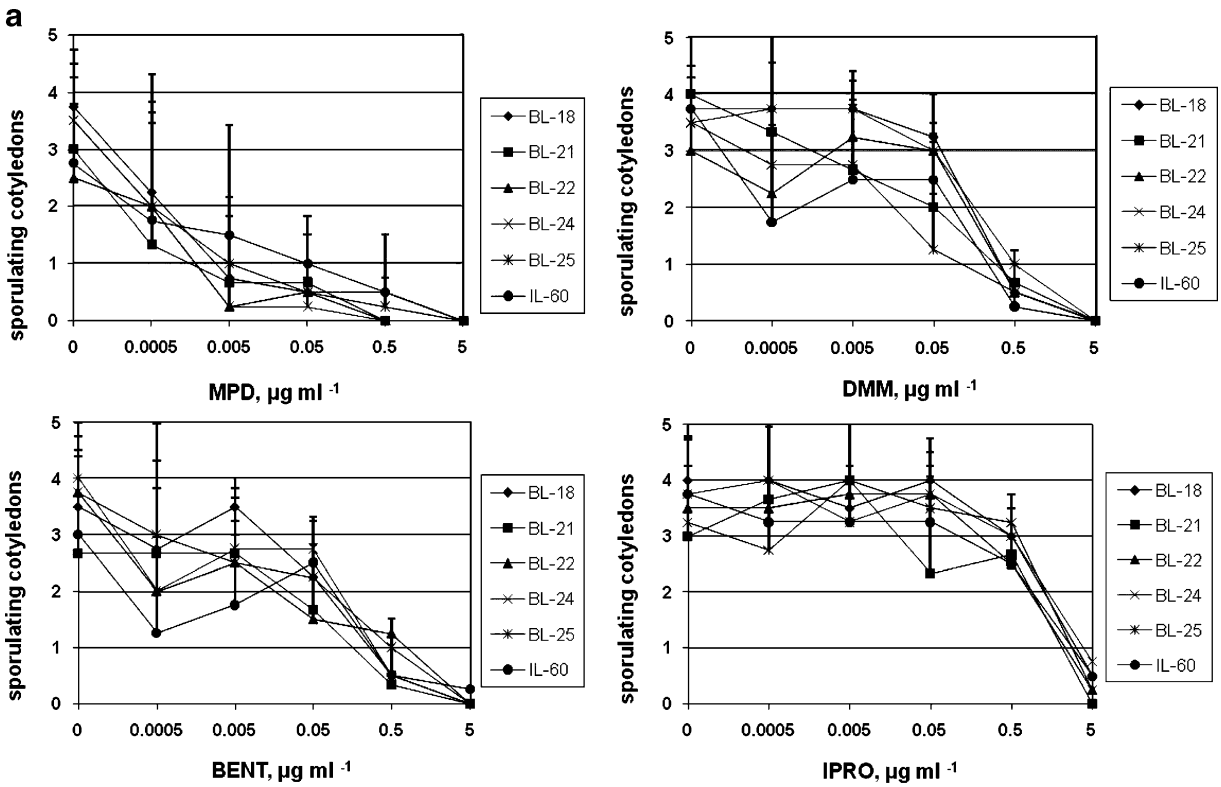
Fig. 2 **a**. The effect of CAA fungicides on infection of lettuce cotyledons with six wild type isolates of *Bremia lactucae*. Spores (about 200 in 20 μl) were mixed with technical grade CAA and inoculated onto detached lettuce cotyledons in Petri dishes. Plates were incubated at 15°C for 20 h in the dark and then at 15°C under 12 h light/day. The number of sporulating cotyledons was determined at 7 dpi. Vertical bars represent standard deviation of the mean (n=4). **b**. One-way Anova analysis of the infection data. Anova unit=square root of arc-sin infected cotyledons. Horizontal diagonal=mean; vertical diagonal=standard error. In each panel, diagonals sharing the same letter are not significantly different (Tukey-Kramer test, α=0.05)

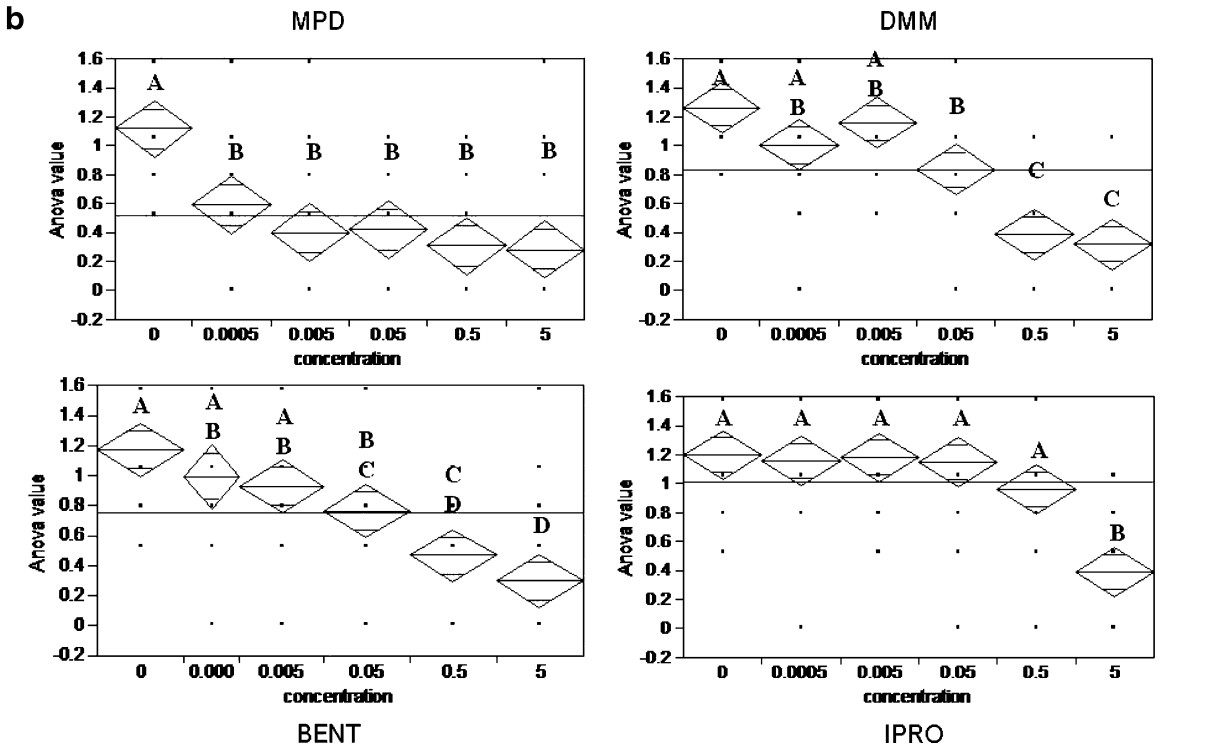
DNA extraction

Samples for DNA extraction were obtained by collecting spores (of WT, RM, F1's and BC's isolates) from infected lettuce cotyledons or leaves. Samples were immediately frozen in liquid nitrogen and stored at −20°C until used. DNA extraction was done using MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin, USA) following manufacturer instructions.

PCR manipulations and analysis of the *CesA3* gene fragment

PCR amplifications were performed in a TGradient Thermocycler (Biometra, Göttingen, Germany); primers were obtained from Syntezza (Jerusalem, Israel). PCR reactions were done in a total volume of 30 μl containing 50 ng of extracted gDNA, 1.25 U of GoTaq DNA polymerase (Promega), 0.2 mM dNTP and 0.2 μM primers (BlCesA3fw TGTGTGGCGTGCACAGCAGACG, BlCesA3rev CGTACATGGCTACACTTTGGCTACACTTCC) in the polymerase manufacturer's buffer. The PCR program was as follows: first 4 min initial denatur-


a
sporulating cotyledons
MPD, µg ml -1
DMM, µg ml -1
BENT, µg ml -1
IPRO, µg ml -1
BL-18
BL-21
BL-22
BL-24
BL-25
IL-60
0
0.0005
0.005
0.05
0.5
5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ation at 94°C, then 35 cycles of 30 s at 94°C, 30 s at 59°C, 45 s at 72°C followed by a final extension at 72°C for 5 min. Prior to sequencing, PCR products were analysed on 1.5% agarose gels and purified using the NucloeSpin Extract II kit (Macherey-Nagel).

Sequencing reactions were done with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacturer's instructions using sequencing primer (BlCesA3seq TATTCAGGCTCGTGCGA). Sequence analysis was performed on a 3130 Genetic Analyzer (Applied Biosystems) and further processed using Lasergene software (DNASTAR, Inc.).

Results

Sensitivity of wild-type isolates to CAAs

The wild-type isolates were highly sensitive to CAAs. When spores were incubated in the presence of technical CAAs in vitro, germination was strongly suppressed (Fig. 1a). Anova (Fig. 1b) revealed MIC values of 0.005, 0.5, 0.5 and 5 $\mu g ml^{-1}$ for MPD, DMM, BENT and IPRO, respectively. Similar results were obtained when spores were mixed with CAAs and inoculated onto cotyledon leaves (Fig. 2a and b). Two-way Anova analysis showed that inhibition of infection was significantly affected by the CAA, its dose and their combination but not by the isolate (Table 1). Due to its low efficacy, IPRO was not included in further experiments.

The effect of UV light or EMS on development of resistance in wild type isolates

Table 2 summarizes the results of 43 mutagenesis experiments conducted to induce resistance in wild type isolates of *B. lactucae* against CAAs. EMS applied to WT spores of *B. lactucae* at 0.15, 0.17, 0.2, 0.5 or 0.66% caused a reduction in infection frequency of about 50, 60, 70, 80 and 90%, respectively. Spores exposed to UV 254 irradiation at 100 $mJcm^{-2}$ for 5 or 10 min lost about 50 and 90% of their infection frequency, respectively.

Exposure to EMS or UV light enabled spores of *B. lactucae* to infect lettuce in the presence of 50 $\mu g ml^{-1}$ CAA at generation 0. Control experiments repeatedly showed that when mutagenic treatments were not employed, spores failed to infect in the presence of$\geq$0.5–5 $\mu g ml^{-1}$ technical CAAs or$\geq$ 0.78–3.12 $\mu g ml^{-1}$ formulated CAAs. The infection frequency at generation 0 of spores mixed with EMS and CAA was much higher for DMM or BENT as compared to MPD (Fig. 3a) and was inversely related to the concentration of MPD (Fig. 3b), DMM or BENT (see Table 2 for details).

The data presented in Table 2 show that mutagenesis did not result with the formation of stable resistance against CAAs, *vis*. isolates that can repeatedly infect plants in the presence of$\geq$50 $\mu g ml^{-1}$ CAA. Nonetheless, in many experiments, resistant mutants (spores germinating at up to 12.5 $\mu g ml^{-1}$ CAA and infecting leaves treated with up to 50 $\mu g ml^{-1}$ CAA) did show up at generation 0 (using spores immediately after mutagenesis treatment). However, resistance diminished after one or a few inoculation transfers on CAA-treated lettuce leaves. Two examples are given in Fig.4. In the first (Fig. 4a), spores that were exposed to UV light and thereafter mixed with CAA were infective to detached cotyledons in the presence of up to 50 $\mu g ml^{-1}$ BENT (Table 2 Exp.1–9). Resistance to BENT (and viability) diminished after 5 transfer inoculations in the presence of CAA (Fig. 4a). In the second (Fig. 4b), spores that were mixed with 0.5% EMS

Table 1 Two-way Anova analysis of the single and combined effects of the compound (CAA), dose, isolate and their combinations on infection of lettuce cotyledons with wild type isolates of *Bremia lactucae*. The data in Fig. 2 were used for analysis

Source	Parameters	DF	Sum of squares	F ratio	Prob>F
Compound	3	3	9.234470	26.5434	<.0001*
Concentration	5	5	22.772119	39.2735	<.0007*
Compound X Concentration	15	15	4.628105	2.6606	0.0007*
Isolate	5	5	0.612663	1.0566	0.3840
Isolate x Compound	15	15	0.873199	0.5020	0.9395
Isolate x concentration	25	25	1.892276	0.6527	0.9009
Isolate x Compound X concentration	75	75	3.727254	0.4285	1.0000

Table 2 Description of the experiments conducted to induce resistance against carboxylic acid amides (CAAs) in seven wild type isolates of *Bremia lactucae*

Exp.	Isolate	Spores× 1000	Mutagen	Mutagen dose	CAA, $\mu g ml^{-1}$	Cotyledons inoculated	Cotyledons sporulating	dpi	Infection frequency	Resistant generations
1	BL-18	nd	UV	100 mJ/cm2, 5 min	MPD 1, 10	36	1	25	nd	4
2	BL-22	nd	UV	100 mJ/cm2, 5 min	MPD 1, 10	36	5	25	nd	4
3	BL-24	nd	UV	100 mJ/cm2, 5 min	MPD 1, 10	36	3	25	nd	4
4	BL-18	nd	UV	100 mJ/cm2, 5 min	DMM 5, 10	36	1	25	nd	4
5	BL-22	nd	UV	100 mJ/cm2, 5 min	DMM 5, 10	36	5	25	nd	4
6	BL-24	nd	UV	100 mJ/cm2, 5 min	DMM 5, 10	36	3	25	nd	4
7	BL-18	nd	UV	100 mJ/cm2, 5 min	BENT 5, 10	36	18	25	nd	4
8	BL-22	nd	UV	100 mJ/cm2, 5 min	BENT 5, 10	36	23	25	nd	4
9	BL-24	nd	UV	100 mJ/cm2, 5 min	BENT 5, 10	36	10	25	nd	4
10	BL-18	nd	UV	100 mJ/cm2, 5 min	DMM 5	300	34	11	nd	2
11	BL-21	nd	UV	100 mJ/cm2, 5 min	DMM 5	150	4	11	nd	2
12	BL-22	nd	UV	100 mJ/cm2, 5 min	DMM 5	150	3	15	nd	2
13	BL-24	nd	UV	100 mJ/cm2, 5 min	DMM 5	150	8	15	nd	2
14	BL-25	nd	UV	100 mJ/cm2, 5 min	DMM 5	150	9	11	nd	2
15	IL-60	nd	UV	100 mJ/cm2, 5 min	DMM 5	150	2	15	nd	2
16	BL-25	3250	EMS	0.15%	MPD 5	942	28	10	8.6E-6	2
17	BL-25	3250	EMS	0.15%	DMM 5	938	307	10	94.5E-6	1
18	BL-25	3250	EMS	0.15%	BENT 5	946	55	10	16.9E-6	1
19	BL-18	1690	EMS	0.15%	MPD 5	402	18	12	10.7E-6	1
20	BL-21	1250	EMS	0.15%	MPD 5	450	42	12	33.6E-6	2
21	BL-22	440	EMS	0.15%	MPD 5	456	5	12	11.4E-6	1
22	BL-24	352	EMS	0.15%	MPD 5	434	12	12	34.1E-6	2
23	BL-25	530	EMS	0.15%	MPD 5	402	7	12	13.2E-6	1
24	IL-60	424	EMS	0.15%	MPD 5	368	4	12	9.4E-6	1
25	CH	182	EMS	0.15%	MPD 5	408	2	12	11.0E-6	1
26	BL-18	3600	EMS	0.15%	DMM 5	786	236	11	65.6E-6	1
27	BL-21	4180	EMS	0.15%	DMM 5	804	380	11	90.9E-6	1
28	BL-22	3740	EMS	0.15%	DMM 5	876	366	11	97.9E-6	1
29	BL-24	3500	EMS	0.15%	DMM 5	902	309	11	88.3E-6	1
30	BL-25	2565	EMS	0.15%	DMM 5	802	256	11	99.8E-6	1
31	IL-60	2090	EMS	0.15%	DMM 5	814	264	11	126.3E-6	1
32	CH	1600	EMS	0.15%	DMM 5	860	190	11	118.8E-6	1
33	BL-18	664	EMS	0.15%	BENT 5	537	80	11	120.5E-6	1
34	BL-21	826	EMS	0.15%	BENT 5	552	84	11	101.7E-6	1

Table 2 (continued)

Exp.	Isolate	Spores× 1000	Mutagen	Mutagen dose	CAA, μgml^{-1}	Cotyledons inoculated	Cotyledons sporulating	dpi	Infection frequency	Resistant generations
35	BL-22	1080	EMS	0.15%	BENT 5	852	97	11	89.8E-6	1
36	BL-24	1020	EMS	0.15%	BENT 5	819	83	11	81.4E-6	1
37	BL-25	675	EMS	0.15%	BENT 5	636	46	11	68.1E-6	1
38	IL-60	270	EMS	0.15%	BENT 5	531	30	11	111.1E-6	1
39	CH	560	EMS	0.15%	BENT 5	600	90	11	160.7E-6	1
40	CH	2000	EMS	0.50%	MPD 5	600	5	14	2.5E-6	7
41	CH	2000	EMS	0.50%	DMM 5	600	40	14	20.0E-6	9
42	CH	2000	EMS	0.50%	BENT 5	600	30	14	15.0E-6	10
43	Il60	8000	EMS	0.20%	MPD 12.5	800	4	15	500.0E-9	nd
43	Il60	8000	EMS	0.20%	MPD 25	800	2	15	250.0E-9	nd
43	Il60	8000	EMS	0.20%	MPD 50	800	1	15	125.0E-9	nd
43	Il60	8000	EMS	0.20%	MPD 100	800	0	15	000.0E+0	nd

Resistant generations=the number of successful transfer inoculations of detached cotyledons in the presence of 0.5–5 μgml^{-1} technical CAA or 3.12–25 μgml^{-1} formulated CAA in intact seedlings

and 5 μgml^{-1} CAA were infective to plants treated with up 25–50 μgml^{-1} CAAs but this resistance was gone after 7–10 transfer inoculations on CAA-treated plants (Exp.40–42, Table 2.).

Response of sexual progeny isolates to CAAs

The Israeli isolates IL60 and BS (Bet-Shaan) belonged to the same mating type as they produced no oospores when mixed-inoculated onto lettuce leaves. Similarly, all European isolates shared the same mating type as they all did not produce oospores when mixed-inoculated onto lettuce leaves in all possible combinations. However, the Israeli isolates did mate with any European isolate to produce oospores, indicating that IL and EU isolates belong to opposite mating types. We took advantage of this fact to cross mutants belonging to opposite mating types in order to possibly produce homozygous resistance to CAAs. Altogether, 42 crosses were made: 10 F1's between wild types IL60 or BS and resistant mutants (RM) derived from EU isolates (the mating type of isolates have not changed after mutagenic treatments); 3 BC's between F1's and IL60; 12 BC's between F1's and RM's; and 17 F2's between BC's. Bioassays showed that most progeny isolates were sensitive to CAAs (data not shown) but a few were resistant. For example, the BC x F1 and the BC x BC progenies presented in Fig. 5 were resistant to 6.25–

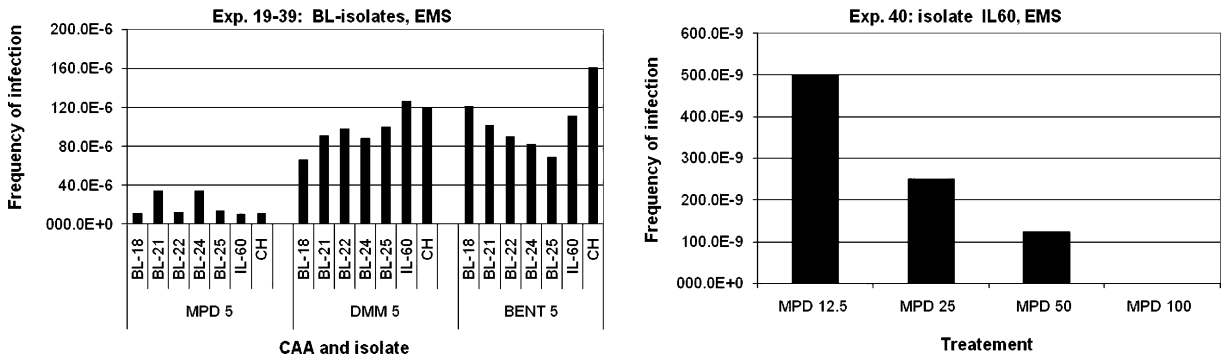


Fig. 3 Infection frequency of EMS-treated spores of *Bremia lactucae* in the presence of CAAs. **a**. Exposure of 7 WT isolates to 5 μgml^{-1} MPD, DMM or BENT. **b**. Exposure of isolate IL60 to increasing concentrations of MPD. More details in Table 2

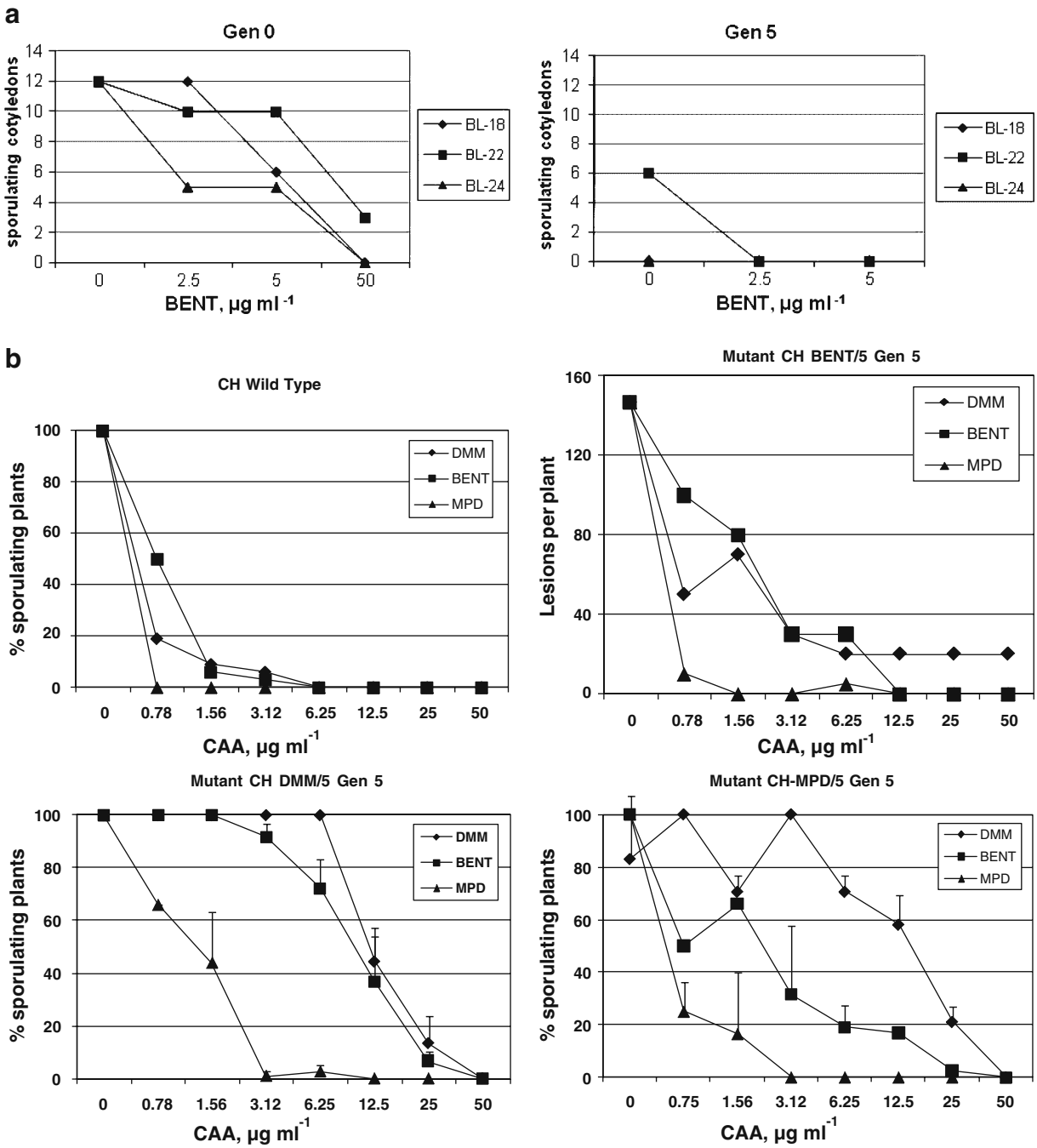


Fig. 4 **a**. Persistence of resistance against benthiavalicarb (BENT) induced by UV light (Exp.7–9, Table 2) during 5 generations in three wild type isolates of *Bremia lactucae.* Only Gen 0 and Gen 5 are shown. The spores produced at each infection cycle were all collected, mixed with benthiavalicarb at the indicated concentrations and inoculated onto detached cotyledons to produce the next generation. Note that two isolates lost infectivity at Gen 5. **b**. Efficacy of formulated CAAs against CH wild type and resistant isolates derived from it by EMS at Generation 5 (Exp.40–42, Table 2). The resistance of these mutants was diminished after 7–10 transfer inoculations on CAA-treated plants. See Table 2 for more details

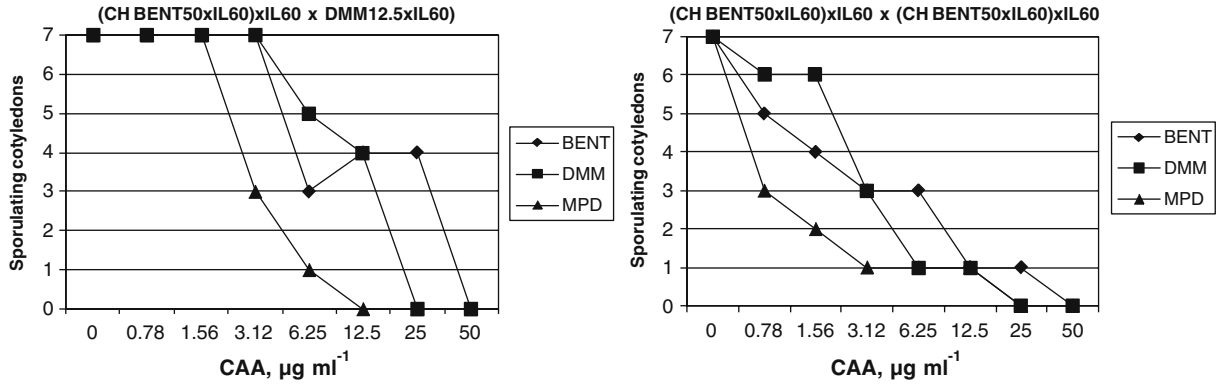


Fig. 5 Sensitivity of two sexual progeny isolates of *Bremia lactucae* to CAAs. Spores were mixed with formulated CAAs and drop-inoculated onto detached cotyledons. The number of sporulating cotyledons was counted at 18dpi. **a**. Cross 13. **b**. Cross 32. More details in Table 3

25 µgml^{-1} formulated CAA, but subsequent transfer inoculations of these progenies on CAA-treated plants revealed sensitive isolates only.

Single spore isolates were derived from an F1 progeny of a cross between the mutant CH BENT50 and the wild type BS, and each was tested for resistance to technical CAAs in detached cotyledons. Results are presented in Fig. 6. The five isolates that grew in the presence of 5 or 50 µgml^{-1} CAA lost resistance in the subsequent transfer cycle. Unfortunately, all single spore isolates belonged to the EU mating type (or were sterile) so that F2 progenies could not be generated.

Mutagenesis of RM and sexual progeny isolates

EMS or UV light was applied to resistant mutants or sexual progeny isolates with an attempt to bring resistance to the homozygous state. Data presented in Table 3 show that in 17 out 23 experiments, mutagenesis induced infection in cotyledons treated with 50 µg ml^{-1} MPD or BENT and 100 µgml^{-1} DMM. However,

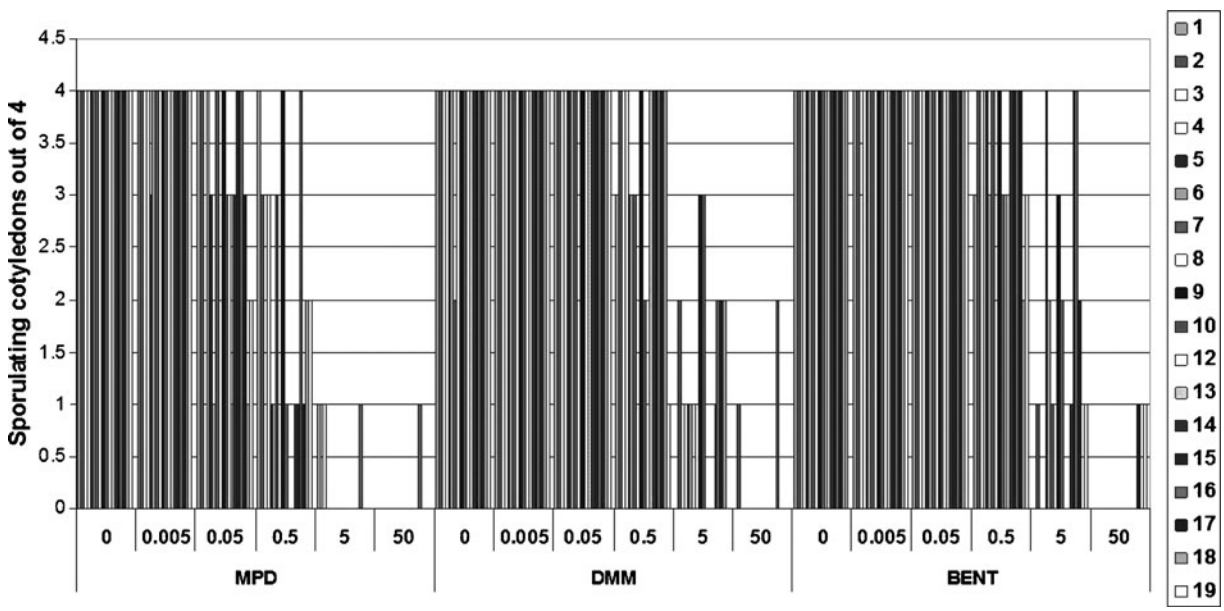


Fig. 6 Sensitivity to CAAs of 19 single-spore isolates derived from the F1 progeny of a cross between the resistant mutant CH BENT50 and the wild type isolate BS. Note that a few isolates could infect leaves in the presence of 50 µgml^{-1} CAA

Table 3 Description of the experiments conducted to induce resistance against carboxylic acid amides (CAAs) in mutants and hybrid isolates of *Bremia lactucae*

Exp.	Isolate(s)	Spores× 1000	Mutagen	Mutagen dose	CAA, μgml^{-1}	Cotyledons inoculated	Cotyledons sporulating	dpi	Infection frequency	Resistant generations
1	CH BENT 50	3000	EMS	0.50%	DMM 40	1000	54	8	18.0E-6	14
2	CH BENT 5	5000	EMS	0.66%	BENT 50	3000	37	11	7.4E-6	5
3	mix hybrids	6000	EMS	0.20%	MPD 50	600	9	11	1.5E-6	3
4	mix hybrids	1200	EMS	0.20%	DMM 50	90	4	11	3.3E-6	3
5	mix hybrids	6000	EMS	0.20%	BENT 50	800	14	11	2.3E-6	3
6	mix hybrids	5000	EMS	0.20%	DMM 40	600	20	8	4.0E-6	2
7	mix hybrids	5000	EMS	0.20%	BENT 40	600	0	8	000.0E+0	0
8	mix hybrids	5000	EMS	0.20%	MPD 40	600	0	8	000.0E+0	0
9	hybrid 5	2000	EMS	0.17%	DMM 100	600	2	8	1.0E-6	2
10	hybrid 13	2000	EMS	0.17%	DMM 100	600	10	8	5.0E-6	2
11	hybrid 14	2000	EMS	0.17%	DMM 100	600	2	8	1.0E-6	2
12	hybrid 15	2000	EMS	0.17%	DMM 100	600	0	8	000.0E+0	0
13	hybrid 17	2000	EMS	0.17%	DMM 100	600	25	8	12.5E-6	2
14	hybrid 18	2000	EMS	0.17%	DMM 100	600	15	8	7.5E-6	2
15	hybrid 19	2000	EMS	0.17%	DMM 100	600	0	8	000.0E+0	
16	hybrid 32	2000	EMS	0.17%	DMM 100	600	25	8	12.5E-6	2
17	hybrid 5×7	2000	EMS	0.17%	DMM 100	600	5	8	2.5E-6	2
18	hybrid 8×8	2000	EMS	0.17%	DMM 100	600	0	8	000.0E+0	0
19	hybrid 18×7	2000	EMS	0.17%	DMM 100	600	3	8	1.5E-6	2
20	hybrid 8×8	1000	UV	100 mJ/cm2, 10 min	DMM 50	600	0	10	0	0
21	mix hybrids	1500	UV	100 mJ/cm2, 10 min	DMM 50	240	4	16	2.7E-6	1
22	mix hybrids	1500	UV	100 mJ/cm2, 10 min	BENT 50	240	7	16	4.7E-6	1
23	CH B50 Gen 11	2000	UV	100 mJ/cm2, 10 min	DMM 6.25	360	1	10	500.0E-9	3

Resistant generations=the number of successful transfer inoculations of detached cotyledons in the presence of 0.5–5 μgml^{-1} technical CAA or 3.12–25 μgml^{-1} formulated CAA in intact seedlings

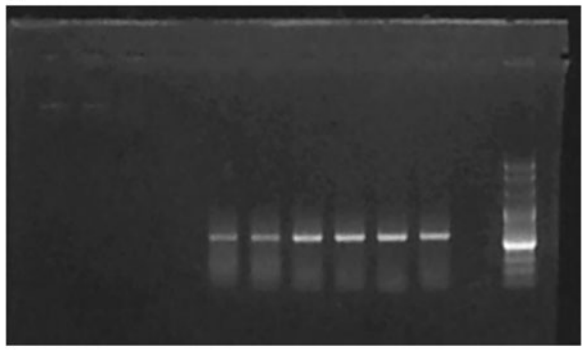
here again, resistance diminished after 1–14 subsequent transfer inoculation cycles.

Sequencing of *CesA3* gene fragment in WT, RM and mixed hybrid isolates

DNA was extracted from spores of 26 isolates of *B. lactucae*: six WT, four RM, ten F1's and six BC's isolates. DNA of the 10 F1's was mixed before used. *CesA3* sequence analysis revealed no difference in the 582 bp fragment (Figure 7a.). No mutation was found at position 1105 that was shown to affect sensitivity to CAAs in *Plasmopara viticola* (Blum et al. 2010a) All isolates displayed Gly1105 encoded by codon GGC (as exemplified in Fig. 7b).

Discussion

This paper reports on three major findings. The first, EMS or UV treatments enabled spores of *B. lactucae* to infect lettuce leaves in the presence of up to 100 μg ml^{-1} DMM or 50 μgml^{-1} BENT or MPD. For MPD, this is×1000 the dose required to completely inhibit infection by WT isolates. The second, resistance to CAAs in *B. lactucae* was not linked to mutation in the



b

CH-WT-*CesA3* sequence

CGAAGGTTAGTCGTGGGCGAAACTGGTGTGGGCAGAAGAATCATG
GACGGAAATCCCTAATGTGCTGTTCTTTTTCCGCTTATGTTTAGCCA
AGTCGTGGCGCTCATTCGTTTCTTTGAGTACGAGAATGCAACGAAT
CCGTGGAATTACGTGTCAGCAATGTTTTTTGGCTTTTTCGTCATGAG
CCAATTCTATCCCATGGTCAAGATGAGTATCACGGAATATTGTGGAT
GGGACCACACGGCCGCCACGTTCACGGCCAATGTCTTC**GGC**TCC
TTGTTGGTCGTATACGTTGTCGTGTTCGTACAGCTATGGCAAGTGT
ATTACGAGGGCAACTTGTTAGTTGCTCAAGGTGCTGATGGCGGTGG
TGGCGCTGAGACTGCTACTGCGGTGTAACCGAGAAGCGTGTTCGG
ATCAATTGCTATGACGATTAGGCGGAGAGGTAAGGGTGCAACAAAA
ACGGAAGTGTAGCCAAAGTGTAGCATGGTACGACGTGTGGCGTCC
TCCGCAGACGACGGGGGAACCAAAATGTCGTCGTGTACGAAGTGT
GCCCCCCCACGTAACGTGTTGCGTCTACTGCACACCACGTTGGAG
CGGAGGTGG

M50-*CesA3* sequence

GCCGGCCACCAACACATCATGGACGGAAATCCCTAATGTGCTGTTC
TTTTTACGCTTATGTTTAGCCAAGTCGTGGCGCTCATTCGATTCTTT
GAGTACGAGAATGCAACGAATCCGTGGAATTACGTGTCAGCAATGT
TTTTTGGCTTTTTCGTCATGAGCCAATTCTATCCCATGGTCAAGATG
AGTATCACGGAATATTGTGGATGGGACCACACGGCCGCCACGTTC
ACGGCCAATGTCTTC**GGC**TCCTTGTTGGTCGTATACGTTGTCGTG
TTCGTACAGCTATGGCAAGTGTATTACGAGGGCAACTTGTTAGTTG
CTCAAGGTGCTGATGGCGGTGGTGGCGCTGAGACTGCTACTGCGG
TGTAACCGAGAAGCGTGTTCGGATCAATTGCTATGACGATTAGGCG
GAGAGGTAAGGGTGCAACAAAAACGGAAGTGTAGCCAAAGTGTAG
CCATGTACGAC

Mixed-hybrids-*CesA3* sequence

GGAATGGTCAGTGGGCGAACACTGGTGCTGGGCAGAAGAATCATG
GACGGAAATCCCTAATGTGCTGTTCTTTTTCCGCTTATGTTTAGCCA
AGTCGTGGCGCTCATTCGTTTCTTTGAGTACGAGAATGCAACGAAT
CCGTGGAATTACGTGTCAGCAATGTTTTTTTGGCTTTTTCGTCATGAG
CCAATTCTATCCCATGGTCAAGATGAGTATCACGGAATATTGTGGAT
GGGACCACACGGCCGCCACGTTCACGGCCAATGTCTTC**GGC**TCC
TTGTTGGTCGTATACGTTGTCGTGTTCGTACAGCTATGGCAAGTGT
ATTACGAGGGCAACTTGTTAGTTGCTCAAGGTGCTGATGGCGGTGG
TGGCGCTGAGACTGCTACTGCGGTGTAACCGAGAAGCGTGTTCGG
ATCAATTGCTATGACGATTAGGCGGAGAGGTAAGGGTGCAACAAAA
ACGGAAGTGTAGCCAAAGTGTAGCAT

Fig. 7 **a**. PCR product of a 582 bp DNA fragment of *CesA3* of *Bremia lactucae* derived from spores of the wild type isolate CH (2 lanes), the resistant mutant M50 at generation 0 (2 lanes), and mixed hybrid isolates (2 lanes). Each DNA sample was loaded twice in order. **b**. Sequence analysis of the 582 bp DNA fragment of *CesA3* of *Bremia lactucae*. Triplett at position 1105 (underlined, bold) is identical in CH WT, the resistant mutant M50, and the mixed hybrid isolates

cellulose synthase 3 (*CesA3*) gene. In *P. viticola* and *P. cubensis*, resistance to CAAs was associated with a mutation at position 1105 of this gene (Blum et al. 2010a, b). The third, resistance to CAAs in *B. lactucae* was unstable, diminishing after one or several transfer inoculations on CAA-treated plants. Attempts to stabilize resistance by producing sexual progenies have failed, as did repeated mutagenesis treatments applied to mutants or hybrid isolates. These findings indicated that the likelihood of *B. lactucae* to develop stable resistance against CAA fungicides is rather small.

We assume that the failure of *B. lactucae* to continue growing on CAA-treated plants at subsequent generations after mutagenesis, as well as the failure to bring resistance to homozygosity, resulted from the lack of mutation in the *CesA3* gene. Cellulose synthesis is crucial for germ-tube development in oomycetes and hence for infection (Blum et al. 2010c). CAA fungicides were shown to inhibit cellulose biosynthesis, interfering with cellulose synthase, and avoiding germ-tube development and infection (Blum et al. 2010c). A mutation at SNP 1105 of *CesA3* alters the protein making it insensitive to CAAs, thus allowing spore germination and infection in the presence of CAAs (Blum et al. 2010a, b).

While we may understand the reason for the failure of *B. lactucae* to continue growing on CAA-treated plants at subsequent generations after mutagenesis (lack of mutation), we do not understand the mechanism which governs the appearance of resistant mutants in the first generation after mutagenic treatments (Generation 0). In fact, this resistance could not be inherited by sexual crosses as it was observed for resistance in *P. viticola* (Gisi et al. 2007) and may therefore be related to mechanisms other than mutations at the target site *(CesA3 gene)* of CAA fungicides. One explanation might be that the stress imposed by the mutagenic agents temporarily interferes with the binding of CAA to the CesA3 protein. This is probably a rare phenomenon, which may have happened in only a few spores. Another, remote, possibility is that the stress imposed by EMS or UV have activated the expression of CAA-insensitive *Ces* genes, probably via promoter methylation. Because these cellulose synthase enzymes are not as efficient as Ces3A during spore germination and infection, a re-set mechanism reactivates *Ces3A* during progressive generations, restoring sensitivity to CAAs.

The data presented here are similar to those we published recently for *P.infestans* (Rubin et al. 2008). There we showed that UV or chemical mutagens applied to sporangia induced the appearance of mutants resistant to CAAs, but all showed erratic, unstable resistance in planta, diminishing after 1 to 8 asexual infection cycles, and failed to grow on CAA-amended medium. However, mutants with stable resistance to mefenoxam or a shift in mating type were obtained, suggesting that *P. infestans* could be artificially mutated for resistance against mefenoxam and a shift in mating type, but not against CAAs (Rubin et al. 2008). Indeed, based on those and other findings, mandipropamid was released to the market as a solo product *Revus* for controlling late blight in potatoes.

Our failure to produce stable CAA resistant mutants of *B. lactucae* in the laboratory, together with our failure to enforce selection of resistant isolates by heavy and continuous sprays of CAAs onto downy mildew-infected lettuce plants growing in closed plastic tunnels (Y.Cohen, unpublished data) suggest that CAA-resistant mutants of *B. lactucae* are unlikely to appear soon in nature. Although CAA fungicides (especially dimethomorph) have been applied since many years to control downy mildew in lettuce under commercial conditions, no resistant isolates have been detected (Syngenta internal information, and CAA FRAC working group). In contrast, mefenoxam resistance is known to be present in *B. lactucae* populations in several countries since many years.

More research is required to understand the mechanisms responsible for the temporary appearance of resistance to CAAs in *B.lactucae* after mutagenic treatments.

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