BOTRYTIS TOLERANCE TO 6-PENTYL-ALPHA-PYRONE AND MASSOIALACTONE

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ABSTRACT

The potential for *Botrytis* populations exposed to UV radiation to develop tolerance to the *Trichoderma* metabolite 6-pentyl-alpha-pyrone (6-PAP) and the 6-PAP analog massoiolactone was determined using 14 different *Botrytis* isolates. There was significant isolate variation in germination of *Botrytis* conidia after UV exposure. Plating conidia onto sublethal doses of 6-PAP yielded no 6-PAP tolerant mutants. After UV irradiation, two *Botrytis* mutants grew on normally lethal doses of 6-PAP, while 59 mutants grew on normally lethal doses of massoiolactone. 6-PAP mutants were cross resistant to normally lethal doses of massoiolactone, whereas massoiolactone mutants were not able to grow on normally lethal doses of 6-PAP. After one generation of growth on kiwifruit slices, 6-PAP mutants lost tolerance to normally lethal doses of 6-PAP. However after three generations of growth on kiwifruit slices, 6-PAP plus massoiolactone mutants retained tolerance to normally lethal doses to massoiolactone.

Keywords: UV radiation, mutagenicity, cross resistance, 6-PAP.

INTRODUCTION

The ability of the *Trichoderma harzianum* Rifai metabolite 6-pentyl-alpha-pyrone (6-PAP) to control *Botrytis cinerea* Pers.: Fr. growth was reported by Merlier *et al.* (1984) who suggested it be used as a potential fungicide in fruit crops. Massoiolactone, an analog compound of 6-PAP, is derived from the bark of the tree *Cryptocaria massoa*. Massoiolactone showed greater antifungal activity than 6-PAP (Parker *et al.* 1999) and can be biosynthesised (Hiroyuki *et al.* 1997). Both compounds, 6-PAP and massoiolactone, are available commercially and are used as food flavouring compounds (Parker *et al.* 1999).

In New Zealand *Botrytis* stem end rot of kiwifruit (*Actinidia deliciosa*) in cool storage is a major cause of fruit loss for the kiwifruit industry (Pennycook 1985; Elmer *et al.* 1997). In the early 1990s the New Zealand Kiwifruit Marketing Board initiated research into postharvest application of natural products for the control of *Botrytis* stem end rot. A major limitation to the successful application of these natural products, 6-PAP and massoiolactone, as commercial postharvest treatments, would be the development of resistant strains of *B. cinerea*. In this study, we report on the potential for populations of *B. cinerea* exposed to UV radiation to develop tolerance to 6-PAP and massoiolactone.

MATERIALS AND METHODS

Isolates and inoculum

A total of 14 *B. cinerea* isolates (Table 1) were used in this study. Fungal maintenance and production of conidial suspensions have been described by Boyd-Wilson *et al.* (1998).

TABLE 1: Description of *Botrytis* isolates used in this study.

Code	<i>B. cinerea</i> isolate ¹	Fungicide resistance ²	Source
1	BC 82	DrBr	Nelson
2	BC 109	DsBs	Auckland (single ascospore isolate) ³
3	BC 135	-	Tauranga
4	BC 141	-	Tauranga
5	BC 144	-	Tauranga
6	BC 145	DsBs	Auckland (single conidium isolate) ³
7	BC 146	DsBr	Auckland (single conidium isolate) ³
8	BC 147	DrBs	Auckland (single conidium isolate) ³
9	BC 148	DrBr	Auckland (single conidium isolate) ³
10	BC 18	DrBs	Nelson
11	BC 3	DsBs	Nelson
12	BC 24	DrBr	Nelson
13	BC 149	DsBr	Auckland (single ascospore isolate) ³
14	BC 139	-	Tauranga

¹Isolate code for HortResearch Culture Collection, Lincoln, New Zealand.

²As described by Beever *et al.* (1989). Abbreviation: D=Dicarboximide; B=Benzimidazole; r=resistant; s=sensitive.

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Agar emulsion

The effect of 6-PAP (Sigma) and massoiolactone (International Frutarom Corp.) concentration on *Botrytis* colony formation, colony size, germination and germ tube growth was investigated *in vitro*. Potato dextrose agar (PDA, Difco) amended with triton (PDAt, 4 ml triton/litre PDA) to restrict colony growth (Suntornsuk and Hang 1994) was autoclaved and cooled to approximately 60°C in a hot water bath. 6-PAP and massoiolactone were diluted to 10% with acetone and stored in the freezer. An aliquot of the chemical solution was pipetted into a sterile universal, 10 ml PDAt added and aseptically emulsified for 1 min using an Ultra Turrex homogeniser. The milky, frothy emulsion was then mixed with 190 ml PDAt and poured into sterile petri dishes.

Dose-response assays

The doses of 6-PAP and massoiolactone required to prevent *Botrytis* germination and/or mycelial growth were determined using dose-response assays. Germination was assessed by counts of colonies formed and mycelial growth by measuring colony diameter on emulsified agar plates. Emulsified plates were inoculated with 1 ml of conidia suspension (approximately 100 conidia/ml) of *Botrytis* isolates 1-10, 12, 13 for germination and 1-14 for mycelial growth assessments. 6-PAP concentrations were 0, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07 and 1%. Massoiolactone concentrations were 0, 0.005, 0.01, 0.02, 0.03 and 0.04%. Control treatments consisted of PDAt (equivalent to 0% compound) and PDAt amended with acetone (PDAt + acetone) to reach a final acetone concentration of 10%. The numbers of colonies formed were counted after 1 week of incubation in the dark at 20°C and converted to a percentage relative to the PDAt + acetone control to allow for variation in the initial conidia number. To determine the effect of 6-PAP on mycelial growth (colony size) the colony diameter was measured for 10 colonies (randomly chosen) per plate. All treatments were completely randomised and set up in triplicate.

The effect of 6-PAP on germ tube growth was determined by inoculating emulsified agar plates with *Botrytis* isolates 3 and 6 with three 30 µl droplets of conidia suspension (10⁶ conidia/ml) onto a plate. 6-PAP concentrations were 0, 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07 and 1%. Control treatments consisted of PDAt (equivalent to 0% compound) and PDAt + acetone. Assessing only conidia on the

periphery of the droplets, germination and germ tube growth was measured after 20 h of incubation at 20°C in the dark. Germ tube length was assessed for 10 germinated conidia/droplet chosen at random.

Effect of UV radiation on germination

The LT₉₅ and LT_{99.9} (time taken for UV radiation to reduce spore germination by 95% and 99.9% respectively compared to non-exposed controls) was determined for each *Botrytis* isolate as previously described by Boyd-Wilson *et al.* (1998). Briefly, agitated spore suspensions (10⁶ conidia/ml) were exposed to artificial UV light (254 nm; Philips TUV, 30W/G30 T8) in the laminar flow cabinet. An aliquot of 100 µl was removed at 1 min intervals for 12 min and three 30 µl droplets pipetted onto PDA. Germination was assessed after 20 h incubation in the dark at 20°C.

Tolerance formation (mutagenicity) on sublethal and lethal doses of 6-PAP and massoiolactone

Botrytis suspensions (10 ml, 10⁶ conidia/ml) were UV irradiated for 6 min as described above. All irradiated conidia then were plated (100 ml/plate) onto sublethal (0.04% 6-PAP) or lethal (0.05% 6-PAP, 0.02% massoiolactone) doses of 6-PAP and massoiolactone. Plates were incubated in the dark at 20°C for 1 week and assessed for the presence of compound tolerant mutant colonies. *Botrytis* isolates (2, 4, 5, 9, 11, 13, 14) were studied individually for mutagenicity on sublethal compound doses, and in a mixed suspension (1-14, in equal amounts) for lethal doses. All irradiated treatments were compared with non irradiated control treatments.

Stability of mutants

Stability of mutants, i.e. isolates which express increased tolerance to normally lethal doses of 6-PAP or massoiolactone, was determined. Ripe kiwifruit were cut into slices (approximately 5 mm) and placed onto filter paper in glass petri dishes which were then autoclaved. On cooling, conidia from the mutant colonies were transferred onto the sterile fruit slices using a sterile loop. Fruit slices were incubated at room temperature under the light bank (12 h photoperiod, Philips TLD 58W/33) and allowed to grow and sporulate. This was classed as the first generation. Conidia from the first generation were then looped onto fresh autoclaved kiwifruit slices and classed as the second generation. This was repeated for five generations.

For each generation, after mutants had totally colonised the kiwifruit slices and produced conidia, spores were harvested, conidia concentration adjusted to 10⁶ conidia/ml and plated (100 ml) in duplicate onto both 0.05% 6-PAP and 0.02% massoiolactone emulsified agar. Plates were then incubated in the dark at 20°C for 1 week before being assessed for the presence of mutant *Botrytis* colonies tolerant to normally lethal doses of 6-PAP and massoiolactone. By plating each mutant suspension onto normally lethal doses of both 6-PAP and massoiolactone emulsified agar, cross resistance between compounds of mutant isolates was determined.

Statistical analysis

Analysis of variance (ANOVA) was used to detect differences between isolates in dose-response assays. Inconsistent trends between isolates made it inappropriate to fit regression lines to the data. LT₉₅ and LT_{99.9} values were calculated from non-linear regression equations.

RESULTS

6-PAP concentration significantly affected germination, germ tube growth, colony formation and colony size ($P < 0.001$) of all *Botrytis* isolates tested. Compared to 0% compound concentration, acetone control treatments had no effect on colony formation and size ($P > 0.05$), nor on germtube growth ($P > 0.05$). On the 6-PAP amended agar, germination as measured by colony formation (Fig. 1) and mycelial growth (Fig. 2) was different between isolates ($P < 0.001$). However, germination and mycelial growth were completely inhibited at concentrations of 0.05% 6-PAP or higher. Mycelial growth was more sensitive to the concentration of 6-PAP than germination.

Germtube growth was completely inhibited at a 0.04 and 0.05% 6-PAP concentration for *Botrytis* isolate 6 and 3, respectively. Increasing 6-PAP concentration

FIGURE 1: Dose-response for 6-PAP and colony formation for several *Botrytis* isolates after 1 week of incubation in the dark at 20°C. Colony counts were converted to a percentage relative to the acetone control to allow for variation in the initial conidia number.

FIGURE 2: Dose-response for 6-PAP and mycelial growth for several *Botrytis* isolates after 1 week of incubation in the dark at 20°C.

from 0 to 0.04% and 0.05% resulted in an linear decrease of germtube growth for isolate 6 (slope = -2650, $R^2 = 0.91$) and isolate 3 (slope = -2068, $R^2 = 0.91$).

Differences ($P < 0.001$) between isolates were also found for the effect of massoialactone on colony formation. No colonies formed on massoialactone emulsified agar at concentration of 0.02% or greater (Fig. 3).

Germination of all *Botrytis* isolates was affected ($P < 0.05$) by exposure to UV radiation. Depending on the isolate, irradiation of 300-420 s at a distance of 220 mm from the UV source killed 95% - 99.9% of all conidia (Fig. 4).

No isolates with increased 6-PAP tolerance resulted from UV irradiation of conidial suspensions from seven *Botrytis* isolates for 360 s and their subsequent incubation on sublethal doses of 6-PAP (0.04%). Approximately 1.4×10^8 conidia were irradiated.

FIGURE 3: Dose-response for massoialactone and colony formation for several *Botrytis* isolates after 1 week of incubation in the dark at 20°C. Colony counts were converted to a percentage relative to the acetone control to allow for variation in the initial conidia number.

FIGURE 4: Effect of UV radiation time (s) on germination of *Botrytis* isolates 1-14 after 20 h incubation in the dark at 20°C.

UV irradiation for 360 s of a mixed conidia suspension of all 14 *Botrytis* isolates and their subsequent incubation on lethal doses of 6-PAP (0.05%) and massoialactone (0.02%) resulted in two 6-PAP tolerant isolates, and 59 massoialactone tolerant isolates. These are referred to as 6-PAP and massoialactone mutants respectively. Approximately 2×10^7 conidia were irradiated.

The two 6-PAP mutants were found to be cross resistant to normally lethal doses of massoialactone. Twenty five massoialactone mutants were randomly selected from the 59 mutants and tested for 6-PAP cross resistance. None of the isolates tested were able to form colonies on 6-PAP emulsified PDA at agar (0.05%).

After 1 generation on kiwifruit host tissue (fruit slices) conidia from both 6-PAP mutants were sensitive to lethal doses of 6-PAP (0.05%). However, conidia of the 6-PAP mutants and massoialactone mutants remained tolerant to lethal doses of

massoialactone (0.02%) after 3 generations on host tissue. No tolerance to 0.02% massoialactone was observed after the 5th generation on kiwifruit host tissue.

DISCUSSION

Our findings confirm fungicidal activity of 6-PAP and massoialactone. Only small doses are required to completely inhibit *Botrytis* colony formation.

UV irradiation of *Botrytis* conidia resulted in the formation of *Botrytis* isolates tolerant to normally lethal doses of 6-PAP and massoialactone. The mutation rate for 6-PAP and massoialactone tolerance was very high at approximately 6×10^{-6} conidia for massoialactone and 2×10^{-7} conidia for 6-PAP. This mutation rate occurred at a higher level than previously reported for high-level benzimidazole tolerance of 2×10^{-8} conidia (Polach and Molin 1975). Therefore it may be concluded that field applications of 6-PAP or massoialactone may result in widespread tolerance of *Botrytis* isolates to these compounds as occurred with benzimidazoles (Geeson 1976, 1978).

UV irradiation readily induced tolerance formation to the compounds tested, thus natural tolerance to either compound is likely to occur. If 6-PAP is to be applied as a postharvest treatment the natural occurrence of 6-PAP tolerance in kiwifruit orchards needs to be established to provide baseline sensitivities of resistant *Botrytis* isolates (Braithwaite *et al.* 1995). These base line sensitivities may aid in predictions of the success rate of 6-PAP application to the picking wound for control of *Botrytis* stem end rot in cool storage.

Although, the two 6-PAP mutants were not found to be tolerant to normally lethal doses of 6-PAP after one generation on kiwifruit host tissue, massoialactone mutants were still tolerant to normally lethal doses of massoialactone after 3 generations indicating that tolerance may be inherited. A larger sample size of 6-PAP mutants is needed to verify whether 6-PAP tolerance may be inherited. Further research is also needed to evaluate the stability of 6-PAP and massoialactone mutants when exposed to sublethal and/or normally lethal compound doses on host tissues.

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