

## SELECTION AND GENETIC ANALYSIS OF LABORATORY MUTANTS OF *BOTRYOTINIA FUCKELIANA* RESISTANT TO FENHEXAMID

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### SUMMARY

Ten monoascosporic strains of *Botryotinia fuckeliana* were used in mycelium growth tests to evaluate baseline sensitivity to Fenhexamid, a fungicide recently introduced in the control of grey mould. Their response to the fungicide was  $EC_{50} = 0.1-0.3 \mu\text{g ml}^{-1}$  and  $MIC = 0.3-3 \mu\text{g ml}^{-1}$ . Eight laboratory mutants resistant to the fungicide were obtained from UV-irradiated or unirradiated conidia plated on Fenhexamid-amended medium ( $3 \mu\text{g ml}^{-1}$ ). Mutation ratios were  $1.7 \cdot 10^{-6}$  of survivor conidia for UV-induced mutations and  $0.6 \cdot 10^{-8}$  for spontaneous mutations. Two levels of resistance to Fenhexamid were distinguished in mycelium growth tests: low ( $1 < EC_{50} > 10 \mu\text{g ml}^{-1}$ ) and high resistance ( $EC_{50} > 100 \mu\text{g ml}^{-1}$ ). In conidial germination tests, Fenhexamid proved to be a powerful inhibitor of germ tube elongation ( $EC_{50} \sim 1 \mu\text{g ml}^{-1}$ ) even for mutants displaying high resistance in mycelium tests. Resistant mutants were crossed with Fenhexamid-sensitive reference strains to derive meiotic progeny and to assess the mode of inheritance of resistant phenotypes. Segregation of resistant/sensitive phenotypes in ascospore offspring indicated that resistant phenotypes were due to mutations in single major genes inherited in Mendelian fashion and unlinked with the *Mbc1* and *Daf1* genes, responsible for resistance to benzimidazole and dicarboximide fungicides, respectively.

*Key words:* *Botrytis cinerea*, grey mould, fungicide resistance, genetics.

### INTRODUCTION

*Botryotinia fuckeliana* (de Bary) Whetz. (teleomorph of *Botrytis cinerea* Pers.) incites grey mould disease on at least 250 different host plants (Jarvis, 1980). The pathogen is well known for causing heavy yield losses on many economically important crops, including grapevine and horticultural and ornamental crops, especially in temperate areas worldwide (Jarvis, 1980).

The control of grey mould is not simple because fungicide sprays are often required at times close to harvest and hence there is a risk of leaving high levels of residues on fruits and vegetables. Furthermore, *B. fuckeliana* is well known for its broad variability and adaptation; it is recognised as a "high-risk" pathogen for its ability to acquire resistance to fungicides, causing low effectiveness of sprays in the field (Brent, 1995; Brent and Hollomon, 1998). Although several groups of fungicides with different modes of action are available against the pathogen, the widespread occurrence of acquired resistance has indeed been experienced with most of them.

Due to acquired resistance, benzimidazoles lost their utility in the control of grey mould only few years after their introduction (Smith, 1988). Dicarboximide-resistant mutants are quite common in greenhouses and vineyards, but the chemicals are still in use, although in a low number of sprays per season (Brent, 1995; Brent and Hollomon, 1998). Resistance to the multi-site fungicide Dichlofluanid has been reported in the laboratory as well as in the field (Leroux and Fritz, 1984; Gjaerum and Munthe, 1985; Malatrakis, 1989; Rewal *et al.*, 1991; Washington *et al.*, 1992; Pollastro *et al.*, 1996a). Isolates resistant to anilinopyrimidines have been detected in different countries soon after their introduction (Rüegg *et al.*, 1997; Santomauro *et al.*, 1998, 2000; Leroux *et al.*, 1999; Moyano *et al.*, 2004), and losses of effectiveness have been reported (Forster and Staub, 1996; Hilber and Hilber-Bodmer, 1998; Petsikos-Panayotarou *et al.*, 2003).

Genetic studies on fungicide resistance have shown that resistance to benzimidazoles is caused by the polyallelic major genes *Mbc1*, coding for  $\beta$ -tubuline, with at least four classes of alleles responsible for sensitivity or different levels of resistance variously accompanied by hypersensitivity to *N*-phenylcarbamates (Faretra *et al.*, 1989; Faretra and Pollastro, 1991, 1993a; Pollastro and Faretra, 1992; Yarden and Katan, 1993; Davidse and Ishii, 1995).

Resistance to dicarboximides is caused by the polyallelic major gene *Daf1* coding for a histidine kinase, with at least five classes of alleles responsible for sensitivity, different levels of resistance variously accompanied by resistance to phenylpyrrole fungicides and reduced tol-

erance to high osmotic pressure (Faretra and Pollastro, 1991, 1993a, b, c; Leroux and Descotes, 1996; Oshima *et al.*, 2002; Vignutelli *et al.*, 2002; Baroffio *et al.*, 2003).

Resistance to Dichlofluanid is determined by two major genes, named *Dic1* and *Dic2*, responsible for high or low resistance, probably involved in a detoxifying mechanism and in glutathione regulation (Pollastro *et al.*, 1996a; Leroux *et al.*, 2002).

Anilinopyrimidines interfere with the biosynthesis of methionine (Masner *et al.*, 1994; Milling and Richardson, 1995), and their primary target site should be cystathionine  $\beta$ -lyase (Fritz *et al.*, 2003); besides they inhibit the secretion of hydrolytic enzymes associated with pathogenesis (Miura *et al.*, 1994). Genetic analysis of resistant strains from field populations of *B. fuckeliana* showed that resistance to anilinopyrimidines is caused by single mutations in at least three major genes, two of which cause multidrug resistance (MDR) also to dicarboximides, phenylpyrroles and several inhibitors of sterol biosynthesis (Chapeland *et al.*, 1999; Leroux *et al.*, 1999, 2002). Santomauro *et al.* (2000) and De Miccolis *et al.* (2002) selected field isolates carrying mutations conferring low resistance that proved lethal in homokaryon.

Fenhexamid belongs to the class of sterol biosynthesis inhibitor (SBI) fungicides, but shows a novel mode of action, inhibiting the enzymatic complex 3-keto reductase, involved in sterol C-4 demethylation (Debieu *et al.*, 2001). It is useful for the control of grey mould due to its high effectiveness and absence of positive cross-resistance with other groups of fungicides (Suty *et al.*, 1999). Knowledge on the risk of resistance to the fungicide is so far scant, although limiting the number of sprays per season is recommended (FRAC, 2006). Field isolates with reduced sensitivity to Fenhexamid have been reported in France and Switzerland, but so far loss of the fungicide's effectiveness has never been observed (Leroux *et al.*, 1998, 1999; Suty *et al.*, 1999; Baroffio *et al.*, 2003).

The present paper deals with selection, description and genetic characterization of Fenhexamid-resistant laboratory-mutants of *B. fuckeliana* in order to clarify the genetic basis of resistance and to explore its possible relationship with resistance to dicarboximides and benzimidazoles, with the aim of contributing to resistance risk assessment and definition of preventive measures.

## MATERIALS AND METHODS

The genetic nomenclature used here follows the genetic symbols and terminology reported by Faretra and Grindle (1992).

**Fungicides.** A commercial formulation of Fenhexamid (Teldor WP50, Bayer Crop Science AG, Leverkusen, Germany) was suspended in sterile water. Benomyl (Du

Pont de Nemours and Co., Wilmington, DE, USA) and Vinclozolin (BASF AG, Limburgerhof, Germany), technical grade, selected as representative of benzimidazole and dicarboximide fungicides, respectively, were dissolved in dimethylsulphoxide. The fungicides were added to autoclaved media cooled to 45-50°C. The concentration of the solvent in media never exceeded 1  $\mu\text{l ml}^{-1}$ .

**Media.** The following media were used (ingredients per litre of distilled water; media were amended with 20 g l<sup>-1</sup> agar Oxoid n. 3 if no otherwise indicated): dextrose agar (DA; 10 g dextrose), water agar (WA; 25 g agar Oxoid n. 3), malt extract agar (MEA; 20 g Oxoid malt extract), potato dextrose agar (PDA; infusion from 200 g peeled and sliced potatoes kept at 60°C for 1 h, 20 g dextrose, adjusted at pH 6.5).

**Fungal isolates.** Monoasporic strains genetically characterized for mating type (*MAT1*) and for resistance to benzimidazoles (*Mbc1*) and dicarboximides (*Daf1*) were used. In particular, the reference strains SAS56 (*MAT1-1 Mbc1S Daf1S*) and SAS405 (*MAT1-2 Mbc1HR Daf1LR*), and eight near-isogenic strains of SAS56 with different responses to benzimidazoles and dicarboximides (Pollastro *et al.*, 1996a, b) were used. The strains and their characteristics are reported in Table 1. All isolates were stored at -80°C and transferred to MEA just before use.

**Selection of resistant mutants.** Conidia of SAS56 were obtained from 7-10-day-old colonies grown on PDA with 12 h d<sup>-1</sup> exposure to lighting from a combination of daylight lamps (Osram, L36W/20) and NUV lamps (Osram, L36W/73). Conidia were collected by scraping the surface of colonies with a sterile loop, then suspended in sterile water containing 0.05% Tween 20, and filtered through Miracloth (Calbiochem, La Jolla, CA, USA) to remove mycelial fragments. For mutagenic treatments, 10 ml aliquots of conidial suspensions were distributed in 100-mm Petri dishes and exposed to UV irradiation (150 mJ, causing 99% lethality) in a GS Gene Linker UV chamber (Bio-Rad Laboratories, Hercules, Canada). Irradiated or non-irradiated conidia were added to MEA amended with 3  $\mu\text{g ml}^{-1}$  Fenhexamid (active ingredient, a.i.), a concentration inhibiting the growth of wild-type strains. The final concentration of conidia was from 4·10<sup>7</sup> to 1·10<sup>8</sup> l<sup>-1</sup>. Petri dishes were kept at 21±1°C and colonies developing within 15-20 days were counted and individually transferred to fresh MEA amended with 3  $\mu\text{g ml}^{-1}$  Fenhexamid. Tests were repeated at least three times and the total number of conidia used was 3.6·10<sup>8</sup> for both UV-induced and spontaneous mutations.

**Phenotypic characterization of mutants.** Putative mutants were tested for mycelial growth on MEA and MEA amended with Fenhexamid (7 concentrations from 0.1 to

**Table 1.** *Botryotinia fuckeliana* Fenhexamid-sensitive reference strains.

| Strain   | Origin | Genotype <sup>(a)</sup> |                         |             | EC <sub>50</sub> <sup>(b)</sup> | MIC <sup>(b)</sup> |
|----------|--------|-------------------------|-------------------------|-------------|---------------------------------|--------------------|
|          |        | <i>Mbc1</i>             | <i>Daf1</i>             | <i>MAT1</i> |                                 |                    |
| SAS56    | --     | <i>S</i>                | <i>S</i>                | 1           | ~0.1                            | 3                  |
| SAS405   | --     | <i>HR</i>               | <i>LR</i>               | 2           | 0.3-1                           | 3                  |
| SAR10993 | SAS56  | <i>HR</i>               | <i>LR</i>               | 2           | 0.1-0.3                         | 1                  |
| SAR10995 | SAS56  | <i>HR</i>               | <i>LR</i>               | 1           | 0.1-0.3                         | 1                  |
| SAR11004 | SAS56  | <i>S</i>                | <i>S</i>                | 2           | 0.1-0.3                         | 3                  |
| SAR11003 | SAS56  | <i>HR</i>               | <i>S</i>                | 2           | 0.1-0.3                         | 0.3                |
| SAR11047 | SAS56  | <i>LR</i>               | <i>S</i>                | 1           | 0.1-0.3                         | 1                  |
| SAR11088 | SAS56  | <i>HR<sup>ID</sup></i>  | <i>S</i>                | 2           | 0.1-0.3                         | 3                  |
| SAR11010 | SAS56  | <i>S</i>                | <i>LR</i>               | 2           | 0.1-0.3                         | 0.3                |
| SAR11126 | SAS56  | <i>S</i>                | <i>HR</i>               | 1           | ~0.1                            | 3                  |
| SAR11194 | SAS56  | <i>S</i>                | <i>HR<sup>IPO</sup></i> | 1           | 0.1-0.3                         | 3                  |

<sup>(a)</sup> *Mbc1* alleles: *S* = wild-type sensitivity; *LR* = low resistance and normal sensitivity to *N*-phenylcarbamates; *HR* = high resistance and hypersensitivity to *N*-phenylcarbamates; *HR<sup>ID</sup>* = high resistance and normal sensitivity to *N*-phenylcarbamates. *Daf1* alleles: *S* = sensitivity, *LR* = low resistance, *HR* = high resistance and hypersensitivity to high osmolarity; *HR<sup>IPO</sup>* = high resistance and almost normal sensitivity to high osmotic pressure.

<sup>(b)</sup> EC<sub>50</sub>: Effective Concentration 50%; MIC: Minimal Inhibitory Concentration.

100 µg ml<sup>-1</sup> a.i.) to evaluate EC<sub>50</sub> (effective concentration 50%) and MIC (minimal inhibitory concentration), as described by Faretra and Pollastro (1991). Two Fenhexamid-sensitive strains and eight putative resistant mutants were also tested for conidial germination. Conidia, obtained as described above, were suspended in sterile water containing 0.05% Tween 20. Aliquots (10 µl) of conidial suspension (10<sup>6</sup> conidia ml<sup>-1</sup>) were spotted on DA and Fenhexamid-amended DA (9 concentrations from 0.01 to 100 µg ml<sup>-1</sup> a.i.). Conidia were kept at 21±1°C for 24 h, with the exception of the two mutants BAR121 and BAR122 that due to their slow germination rate were incubated for 48 h; germinating conidia were fixed with lactophenol-cotton blue. Random samples of 100 conidia on each of three spots per treatment were observed at x125 magnification, and germinated conidia were counted. Germ tube length was measured in a sample of 25 conidia for each treatment by using micrometer oculars.

#### Derivation of meiotic progeny and genetic analysis.

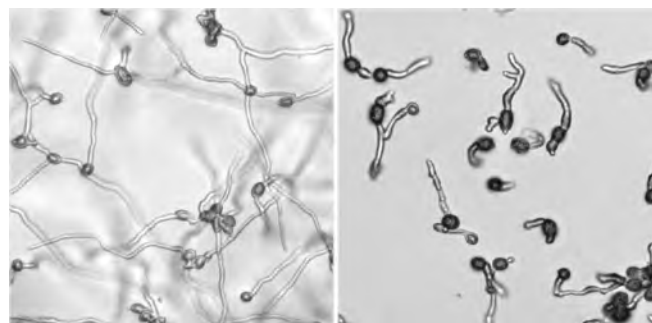
Mutants, all from SAS56 and likely *MAT1-1*, were mated with the three reference strains, SAS405 (*MAT1-2*), SAR10993 (*MAT1-2*), and SAR10995 (*MAT1-1*), all resistant to benzimidazoles and dicarboximides (*Mbc1HR Daf1LR*). Mutants were mated either as sclerotial or spermatizing partner in the cross to obtain apothecia as described by Faretra *et al.* (1988). Ascospores from individual apothecia were spread at low density on WA, singly collected under a dissection microscope and subcultured on MEA. The colonies obtained were tested as described by Faretra and Pollastro (1991) for growth on MEA and on MEA singly amended with Fenhexamid (10 µg ml<sup>-1</sup> a.i.), Vinclozolin (5 µg ml<sup>-1</sup>) or Benomyl (10 µg ml<sup>-1</sup>). Resistant phenotypes were distinguished from

sensitive ones by colony appearance after 2-3 days of incubation at 21±1°C. Data obtained from single apothecia were statistically analysed for segregation of phenotypic characters and distribution of alleles by means of the  $\chi^2$  test, as corrected by Yates (1934).

## RESULTS

Preliminary tests showed that mycelial growth of 10 monoascosporic strains was inhibited by concentrations between 0.3 and 3 µg ml<sup>-1</sup> Fenhexamid, with EC<sub>50</sub> ranging from 0.1 to 0.3 µg ml<sup>-1</sup> (Table 1).

Fenhexamid did not affect conidial germination but did reduce the length of germ tubes, which appeared swollen, distorted and strongly branched (Fig. 1). For wild-type sensitive strains a strong reduction in germ tube length (73-84%) was observed starting from 0.1 µg ml<sup>-1</sup>.



**Fig. 1.** Germination of conidia of the wild type strain SAS56 after 24 h of incubation on DA (left) and on DA+0.1 µg ml<sup>-1</sup> Fenhexamid (right). The fungicide causes abnormal shape of germ tubes that appear swollen, bent, and branched.

**Table 2.** Putative Fenhexamide-resistant mutants compared with two wild-type sensitive reference strains.

| Strain | Origin <sup>(a)</sup> | Phenotype Fen <sup>(b)</sup> | EC <sub>50</sub> <sup>(c)</sup> | MIC <sup>(c)</sup> |
|--------|-----------------------|------------------------------|---------------------------------|--------------------|
| SAS56  | --                    | S                            | ~0.1                            | 3                  |
| SAS405 | --                    | S                            | 0.3-1                           | 3                  |
| BAR121 | UV                    | LR                           | ~10                             | >100               |
| BAR122 | UV                    | LR                           | 1-3                             | >100               |
| BAR123 | UV                    | LR                           | 1-3                             | >100               |
| BAR124 | UV                    | HR                           | >100                            | >100               |
| BAR125 | UV                    | HR                           | >100                            | >100               |
| BAR126 | UV                    | LR                           | 1-3                             | >100               |
| BAR127 | SM                    | LR                           | ~1                              | >100               |
| BAR128 | SM                    | LR                           | 0.3-1                           | >100               |

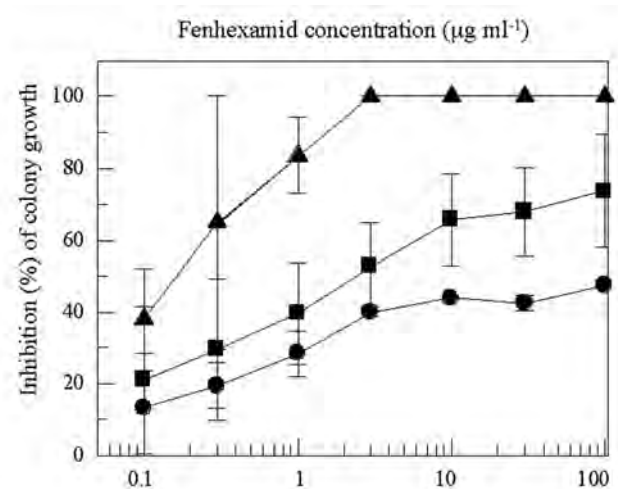
<sup>(a)</sup> Putative resistant mutants derived from UV-irradiated conidia (UV) or spontaneous mutations (SM).

<sup>(b)</sup> S: sensitive reference strains; LR: low-resistant mutants; HR: high-resistance mutants.

<sup>(c)</sup> EC<sub>50</sub>: Effective Concentration 50%; MIC: Minimal Inhibitory Concentration.

Mutants were obtained from both unirradiated and UV-irradiated conidia. Mutation rates were  $1.7 \cdot 10^{-6}$  survivor conidia ( $3.6 \cdot 10^{-8}$  total conidia) for UV-induced mutations and  $0.6 \cdot 10^{-8}$  for spontaneous mutations. In total, eight mutants were obtained: six from UV-irradiated conidia and two from unirradiated conidia. On the control medium (MEA), the putative mutants BAR121, BAR122 and BAR126 showed slower growth than the others, reduced of about 40% as compared to SAS56.

The response of putative mutants to Fenhexamid was evaluated. According to EC<sub>50</sub> values for mycelial growth, at least two classes of mutants were detected: low resistance (LR),  $1 < EC_{50} > 10 \mu\text{g ml}^{-1}$  (BAR121, BAR122, BAR123, BAR126, BAR127, BAR128) and high resistance (HR),  $EC_{50} > 100 \mu\text{g ml}^{-1}$  (BAR124 and

**Fig. 2.** Different responses of sensitive reference strains to Fenhexamid (▲), low resistance (■) and high resistance (●) mutants.

BAR125). For all mutants MIC was higher than  $100 \mu\text{g ml}^{-1}$  (Table 2; Fig. 2).

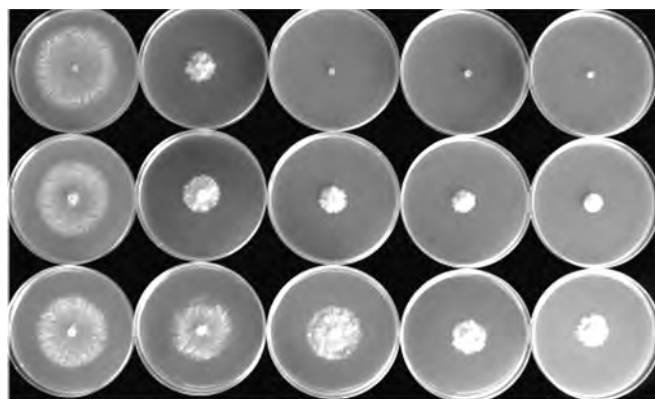
Fenhexamid did not affect conidial germination but inhibited germ-tube elongation even for resistant mutants. The EC<sub>50</sub> values for germ-tube elongation were estimated as  $0.03-0.1 \mu\text{g ml}^{-1}$  for wild-type sensitive reference strains. The data for putative mutants differed slightly from those of the colony-growth tests. The LR mutants, BAR123, BAR127 and BAR128, showed EC<sub>50</sub> values of  $0.03-0.1 \mu\text{g ml}^{-1}$ . The HR mutants, BAR124 and BAR125, and the LR mutant BAR126 showed EC<sub>50</sub> values of  $1-3 \mu\text{g ml}^{-1}$ . The mutants BAR121 and BAR122 showed slow conidial germination and observations could only be made after 48 h of incubation; under these conditions their EC<sub>50</sub> was estimated to be  $10-100 \mu\text{g ml}^{-1}$ .

**Table 3.** Phenotypes and number of ascospore progenies from crosses between Fenhexamid-resistant mutants and Fenhexamid-sensitive reference strains SAS405 or SAR10993.

| Resistant isolates <sup>(a)</sup> |               | N. of analysed apothecia | Ascospores progeny N. |            | $\chi^2$ value for a 1:1 segregation <sup>(b)</sup> |      |
|-----------------------------------|---------------|--------------------------|-----------------------|------------|---|------|
| Ref. N.                           | Phenotype Fen |                          | Total                 | Phenotypes |   |      |
|                                   |               |                          |                       | FenS       | FenR  |      |
| BAR123                            | LR            | 2                        | 253                   | 142        | 111   | 3.56 |
|                                   |               | 1                        | 54                    | 54         | 0   | n.a. |
| BAR124                            | HR            | 3                        | 251                   | 127        | 124   | 0.02 |
| BAR125                            | HR            | 2                        | 229                   | 123        | 106   | 1.12 |
|                                   |               | 3                        | 130                   | 130        | 0   | n.a. |
| BAR126                            | LR            | 5                        | 485                   | 248        | 237   | 0.20 |
|                                   |               | 1                        | 68                    | 68         | 0   | n.a. |
| BAR127                            | LR            | 5                        | 391                   | 191        | 200   | 0.16 |
|                                   |               | 8                        | 400                   | 400        | 0   | n.a. |
| BAR128                            | LR            | 3                        | 255                   | 135        | 120   | 0.76 |
|                                   |               | 1                        | 71                    | 71         | 0   | n.a. |

<sup>(a)</sup> Mutants showed low (LR) or high resistance (HR) to Fenhexamid.

<sup>(b)</sup>  $\chi^2$  at  $P=0.05$  level of probability is 3.84; n.a.: not appropriate.



**Fig. 3.** Examples of colony growth of sensitive, low resistance and high resistance strains (from top to bottom) on MEA (control) and MEA containing 0.1, 1, 10 or 100  $\mu\text{g ml}^{-1}$  Fenhexamid (from the left to the right).

As expected, all mutants were sensitive to benzimidazole and dicarboximide fungicides (*Mbc1S Daf1S*) like the parental strain SAS56. All the mutants were crossed with the reference strains SAS405 (*MAT1-2*), SAR10993 (*MAT1-2*) and SAR10995 (*MAT1-1*), and as expected they were fertile only with the *MAT1-2* reference strains like the parental strain SAS56.

The ascospore progeny of fertile crosses were analysed. The majority of apothecia collected from each

cross yielded ascospore progeny in which sensitive (*FenS*) and resistant (*FenR*) phenotypes were in statistically significant 1:1 ratios (Table 3; Fig. 3). This suggests that single major genes were responsible for resistant phenotypes of laboratory mutants. In most of crosses, however, few apothecia yielded only sensitive ascospores (Table 3).

The mutants BAR121 and BAR122 in all the crosses yielded only few apothecia and ascospores, and 134 ascospores examined from two apothecia of the cross SAR10993  $\times$  BAR121 were not viable. Ascospores from one of the two analysed apothecia from the cross SAR10993  $\times$  BAR123 showed co-segregation between resistance and a morphological trait, snow-white mycelium, not shown by either parent. Co-segregation between resistance and a morphological feature was also observed in the progenies from crosses SAS405  $\times$  BAR127 and SAR10993  $\times$  BAR128: all colonies of resistant progenies were smaller than those of either parents or sensitive progenies and showed abundant aerial mycelium.

The genotypes involved in crosses (*FenR Mbc1S Daf1S*  $\times$  *FenS Mbc1HR Daf1LR*) were suitable for examining re-assortment in ascospore progeny for resistance to Fenhexamid, benzimidazole and dicarboximide fungicides. *Fen* markers segregated independently from *Mbc1* and *Daf1* markers in ascospore progeny, the ratio of recombinant to parental phenotypes being not significantly different from 1:1 (Tables 4, 5).

**Table 4.** Recombination between traits of resistance to Fenhexamid and Vinclozolin in ascospore progeny of *B. fuckeliana*.

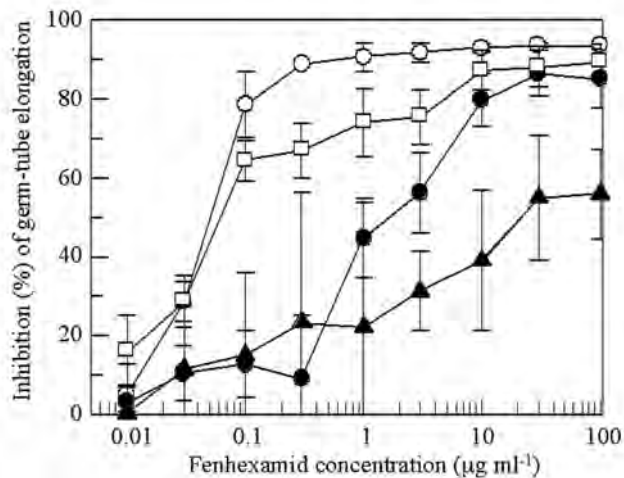
| Cross                         |                               | N. of apothecia analysed | Ascospore progeny |                      |      |     | Parental: recombinant | $\chi^2$ for independent segregation of markers <sup>(a)</sup> |      |
|-------------------------------|-------------------------------|--------------------------|-------------------|----------------------|------|-----|-----------------------|--|------|
| Reference strain (FenS DafLR) | Resistant isolate (FenR DafS) |                          | Total             | Phenotypes (Fen-Daf) |      |     |                       |  |      |
|                               |                               |                          |                   | S-S                  | S-LR | R-S | R-LR                  |  |      |
| SAR10993                      | BAR123                        | 2                        | 253               | 69                   | 73   | 42  | 69                    | 115:138  | 1.91 |
| SAR10993                      | BAR124                        | 3                        | 251               | 58                   | 69   | 64  | 60                    | 133:118  | 0.78 |
| SAR10993                      | BAR125                        | 2                        | 229               | 62                   | 61   | 49  | 57                    | 110:119  | 0.28 |
| SAR10993                      | BAR126                        | 3                        | 296               | 74                   | 77   | 76  | 69                    | 153:143  | 0.27 |
| SAS405                        | BAR127                        | 2                        | 168               | 36                   | 45   | 49  | 38                    | 94:74  | 2.15 |
| SAR10993                      | BAR128                        | 2                        | 198               | 47                   | 51   | 52  | 48                    | 103:95   | 0.25 |
| Total                         |                               | 14                       | 1,395             | 346                  | 376  | 332 | 341                   | 708:687  | 0.28 |

<sup>(a)</sup>  $\chi^2$  at P=0.05 level of probability is 3.84.

**Table 5.** Recombination between traits of resistance to Fenhexamid and Benomyl in ascospore progeny of *B. fuckeliana*.

| Cross                         |                               | N. of apothecia analysed | Ascospore progeny |                      |      |     | Parental: recombinant | $\chi^2$ for independent segregation of markers <sup>(a)</sup> |      |
|-------------------------------|-------------------------------|--------------------------|-------------------|----------------------|------|-----|-----------------------|--|------|
| Reference strain (FenS MbcHR) | Resistant isolate (FenR MbcS) |                          | Total             | Phenotypes (Fen-Mbc) |      |     |                       |  |      |
|                               |                               |                          |                   | S-S                  | S-HR | R-S | R-HR                  |  |      |
| SAR10993                      | BAR123                        | 2                        | 253               | 68                   | 74   | 48  | 63                    | 122:131  | 0.25 |
| SAR10993                      | BAR124                        | 3                        | 251               | 60                   | 67   | 61  | 63                    | 128:123  | 0.06 |
| SAR10993                      | BAR125                        | 2                        | 229               | 53                   | 70   | 52  | 54                    | 122:107  | 0.85 |
| SAR10993                      | BAR126                        | 3                        | 296               | 78                   | 73   | 78  | 67                    | 151:145  | 0.08 |
| SAS405                        | BAR127                        | 2                        | 168               | 36                   | 45   | 40  | 47                    | 85:83  | 0.01 |
| SAR10993                      | BAR128                        | 2                        | 198               | 38                   | 60   | 48  | 52                    | 108:90   | 1.46 |
| Total                         |                               | 14                       | 1,395             | 333                  | 389  | 327 | 346                   | 716:679  | 0.92 |

<sup>(a)</sup>  $\chi^2$  at P=0.05 level of probability is 3.84.



**Fig. 4.** Percentage inhibition of germ-tube elongation caused by Fenhexamid in sensitive strains (○) (SAS 56, SAS 405), and in the derived mutants: BAR124, BAR125, BAR126 (□,  $EC_{50} = 1-3 \mu\text{g ml}^{-1}$ ); BAR127, BAR128, BAR123 (●,  $EC_{50} = 0.03-0.1 \mu\text{g ml}^{-1}$ ); BAR121, BAR122 (▲,  $EC_{50} = 10-100 \mu\text{g ml}^{-1}$  after 48 h).

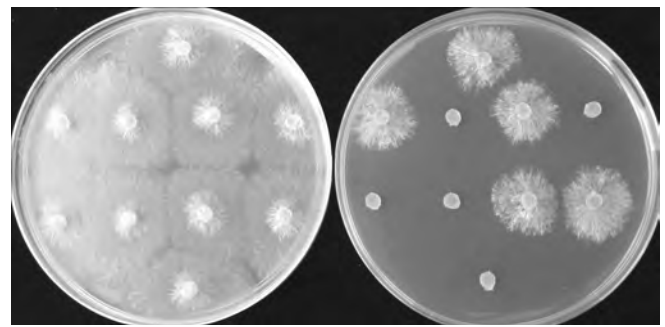
## DISCUSSION

Fenhexamid proved to be a powerful inhibitor of germ-tube elongation associated with alteration of hyphal tips, as already described (Haenssler and Pontzen, 1999; Debieu *et al.*, 2001) and observed for other SBI fungicides (Köller, 1992). For wild-type sensitive *B. fuckeliana* isolates, the fungicide at concentration as low as  $0.1 \mu\text{g ml}^{-1}$  was able to reduce germ-tube elongation notably (up to 84%), while  $1-3 \mu\text{g ml}^{-1}$  were required to reduce mycelial growth up to 50%.

Experiments to select laboratory mutants resistant to Fenhexamid, either spontaneous or UV-induced, produced only few mutants often with aberrant morphology and colony growth; this suggests that resistance is not easily induced in *B. fuckeliana*. Resistant mutants fell into two phenotypic classes in their responses to Fenhexamid (low and high resistance) with prevalence of low resistance.  $EC_{50}$  values for conidial germ tube growth were generally lower than those for colony growth, confirming that Fenhexamid effectively inhibits germ tube elongation rather than mycelial growth.

Genetic analysis of ascospore progenies indicated that the resistant phenotypes of laboratory mutants were due to mutation in single major genes inherited in Mendelian fashion. Resistance failed to be transmitted in all ascospores within a few apothecia, in most of crosses. Failure to transmit parental resistance traits to ascospore progeny is known to occur in *B. fuckeliana* as a result of co-existence of sensitive and resistant nuclei in heterokaryotic parental isolates (Faretra and Pollastro, 1991, 1993a, 1993b; Pollastro *et al.*, 1996b).

The existence of two levels of resistance suggests that distinct mutations in the same locus or in different loci



**Fig. 5.** Monoascospore progeny from a cross between a sensitive reference strain and a resistant mutant grown on MEA (left) and on MEA containing  $10 \mu\text{g ml}^{-1}$  Fenhexamid (right).

are involved. No specific crosses between resistant mutants were possible because they all had the same mating type *MAT1-1*. Further crosses among resistant progenies or molecular analyses will be required to establish if mutants carry mutations in the same or different loci.

The Fenhexamid-resistance gene(s) is/are not linked with the *Mbc1* and *Daf1* genes conferring resistance to benzimidazoles and dicarboximides, respectively. In a few cases, the resistant progenies from crosses between LR mutants and sensitive strains showed slower colony growth and conidial germination than wild-type sensitive progenies, hence showing an association between resistance to Fenhexamid and reduced fitness.

The high effectiveness of Fenhexamid as inhibitor of germ-tube elongation and negative pleiotropic effects (slow growth, reduced conidial germination) of some mutations responsible for resistance, previously reported by Ziogas *et al.* (2003) for laboratory resistant mutants, and by Suty *et al.* (1999) for less sensitive field isolates, would suggest that the fungicide is not at high risk of practical resistance. This is supported by results from field trials. For instance, 3-4 sprays per year with Fenhexamid on grapevine did not reduce effectiveness during a period of 7-8 years, although *B. fuckeliana* isolates less sensitive than normal did appear in the field (Suty *et al.*, 1999). Besides, investigations carried out in France showed that *B. fuckeliana* isolates from grapevine collected even before the use of Fenhexamid showed high resistance to the fungicide in colony-growth tests; such resistance has never caused practical loss of effectiveness (Leroux *et al.*, 1999).

Nevertheless, possible selection in the field of resistant mutants different from those till now observed, and the experience on fungicide-resistance in *B. fuckeliana* acquired, suggest to make best use of Fenhexamid in spray schedules that implement appropriate anti-resistance strategies. Hence, the fungicide should be used in integrated grey mould control in a number of protective sprays per season not exceeding those recommended by FRAC (2006), and in alternation or mixtures with fungicides with different modes of action.

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