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# Characterizing the production of a wild-type and benomyl-resistant *Fusarium lateritium* for biocontrol of *Eutypa lata* on grapevine

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**Benomyl-resistant (BR) and wild-type (WT) strains of *Fusarium lateritium* were examined for their tolerance to benomyl on potato dextrose agar (PDA) containing benomyl and control of the *Eutypa lata* in grapevine bioassays. The WT strain grew on PDA containing 1 $\mu$g/ml benomyl at 13, 26 and 29°C. The BR strain grew on PDA containing 10 $\mu$g/ml benomyl at 4°C, on PDA containing 100 $\mu$g/ml benomyl at 29°C, and on PDA containing 1000 $\mu$g/ml benomyl at 13°C and 26°C. The BR strain was also able to colonize grapevine segments and control *E. lata* in the presence of 1000 $\mu$g/ml benomyl. Both strains were amenable to production *via* liquid fermentation and both achieved 100% control of *E. lata* in grapevine bioassays. Neither the duration of fermentation nor incubation temperature during grapevine bioassays influenced the efficacy of either strain against *E. lata*. The results suggest that application of BR *F. lateritium* alone or in combination with benomyl may provide good control of *E. lata*.** *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 151–155.

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## Introduction

Although there has been considerable progress in the methods and techniques involved in the development of biological control agents (BCAs), the field has continuously suffered an arduous transition to wide-scale field application [6]. One significant reason for the delay in the adoption of broad-scale biocontrol is that potential end-users doubt the efficacy of such measures as opposed to more traditional chemical control measures. Furthermore, issues associated with the effective production and delivery of BCAs have also limited their adoption.

Researchers have identified the fungus *Fusarium lateritium* as a BCA against *Eutypa lata* [4], a very opportunistic and highly destructive pandemic fungal phytopathogen that causes the disease *Eutypa* dieback in grapevines. In California, it is estimated that as much as 20% of the total vineyard acreage (750,000 acres) is infected, to some extent, by *E. lata* (Andrew Walker, personal communication). This disease occurs when the pruning wounds of grapevines become infected with spores from *E. lata*. If not prevented, infection can be lethal and replanting becomes the only course of action. Although *F. lateritium* has been identified as a BCA, its adoption for the control of *E. lata* has been limited due to inconsistent performance in the field.

Over the last 30 years, benomyl, a broad-spectrum fungicide, has been the sole means of controlling *E. lata*. Benomyl application is somewhat effective for short-term control of the disease. However, more long-term strategies for *E. lata* control are needed since wounds are susceptible for as long as 4 weeks after pruning, and no post-infection eradication method currently exists. Additionally, application of benomyl in the field is often a laborious process and is complicated by the need to make multiple applications over a short period of time to achieve effective control. Furthermore, tolerance of fungi to low levels of benomyl (0–10 $\mu$g/ml) has been reported and the gene conferring this tolerance has been identified in several fungi [1,2,5,8]. Clearly, given these reasons, a more effective means of control of *E. lata* is needed. Application of BCAs that colonize the pruning wounds might offer more long-term control of *E. lata*, either by themselves or in combination with benomyl. Indeed, the biocontrol efficacy of certain fungi and bacteria against *E. lata* increases with wound colonization time [4].

The purpose of this study was to isolate a benomyl-resistant (BR) strain (i.e., one able to grow in the presence of benomyl) of *F. lateritium* and subsequently characterize the growth and efficacy of both the wild-type (WT) and BR strains for the control of *E. lata*. In so doing, we sought to identify differences in the growth characteristics of the strains and the effect of fermentation culture age on efficacy. Because benomyl is the preferred means of control of *E. lata*, dosage–response studies on both the WT and the BR strains of *F. lateritium* were also conducted.

## Materials and methods

### Isolation of the BR F. lateritium strain

A classical random mutagenesis and selection approach was taken to isolate the BR *F. lateritium* strain. First, potato dextrose agar (PDA) plates (Difco) were prepared using 0, 12 and 60 $\mu$g/ml of benomyl. Benomyl was added as a powder to the PDA

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after autoclaving PDA and prior to pouring. WT *F. lateritium* was grown for 4 days in 100 ml of potato dextrose broth (PDB) in a 500-ml flask shaken at 220 rpm at room temperature. One-milliliter samples of culture were removed and centrifuged in sterile 1.5-ml microcentrifuge tubes. The supernatant was discarded and the cell pellets aseptically transferred to four of each type of benomyl plate. The first plate of each benomyl concentration was exposed to two 15-W UV light sources for 2 min, the second plate for 1 min, and the third plate for 30 s. The remaining plates were left as controls. The UV light source was positioned 60 cm above each plate. After UV exposure, the plates were wrapped with aluminum foil and incubated for 3–4 days at 28°C. Growth on the plates was noted and transferred onto PDA plates containing 0, 12 or 60 $\mu$g/ml benomyl. These plates were also incubated at 28°C for 3–4 days. Benomyl resistance was maintained during repeated plate-to-plate transfers.

### Culture maintenance

Both WT and BR *F. lateritium* strains were maintained in shake flask cultures containing PDB at 30°C and 150 rpm, and on PDA at 26°C. The BR *F. lateritium* strain was also maintained on PDA containing 100 $\mu$g/ml benomyl at 26°C.

### Benomyl tolerance assays on PDA

In this study, tolerance to benomyl is considered to mean the ability to grow in the presence of 10 $\mu$g/ml benomyl or more. Tolerance assays were carried out in 15-cm-diameter Petri dishes containing different levels of benomyl in PDA. Media were prepared aseptically by adding benomyl suspended in 10 ml of acetone to 990 ml of autoclaved PDA cooled to 60°C. The benomyl concentrations tested were 0, 1, 10, 100 and 1000 $\mu$g/ml. Media were held overnight at 4°C prior to inoculation.

Inoculation was completed using 21 $\mu$l of 3-day shake flask cultures of BR and WT *F. lateritium* in three 7-$\mu$l aliquots. Each benomyl concentration was tested twice. Once inoculated, plates were placed in incubators for 3 days at 4, 13, 26 or 29°C. Colony diameter was measured after the 3-day incubation period using a computer imaging system (Alpha Innotech, San Leandro, CA). Mean colony diameters and standard errors about the mean were calculated for each treatment.

### Fermentation studies

Fermentation studies were carried out using both the WT and BR strains of *F. lateritium*. Inoculum for each fermentation was prepared aseptically by transferring a 1–2 $cm^2$ colonized piece of maintenance PDA culture into 100 ml of sterile PDB in a 500-ml shake flask. The shake flasks were incubated for approximately 4 days at 220 rpm at 27°C.

Shake flask cultures were used to inoculate 4.5 l of sterile PDB in a fully instrumented Bioflow 3000 fermenter (New Brunswick Scientific, New Brunswick, NJ). Dissolved oxygen and pH were measured on-line using sterilized dissolved oxygen probes and pH electrodes. Agitation rate was maintained at 300 rpm and aeration was maintained at 4.5 l/min. Samples were removed daily for analysis of cell morphology, biomass concentration, carbohydrates and nitrogen (both ammonia and primary amino nitrogen). Biomass concentration was monitored using 10-ml samples of fermentation both. Samples were filtered through a preweighed filter, washed with 10 ml of water and then dried in a microwave oven to constant weight (approximately 4 min). Fermentations were also sampled at 16, 40, 144 and 240 h after inoculation for biological efficacy analyses. Efficacy was evaluated using the bioassay described below.

### Grapevine bioassays

Bioassays were conducted in a manner similar to that described by Munkvold and Marois [4]. Bioassays used 4- to 5-day-old cultures of *E. lata* produced in PDB shake flask culture at 30°C and 150 rpm. These cultures yielded approximately $1\times10^5$ cells/ml. 1.5-cm long segments of 1-year-old Zinfandel grapevine canes were used in all bioassays. Canes were obtained from the UC Davis campus vineyard and stored at −20°C until needed. Bark was removed from the segments before they were autoclaved for 30 min. Autoclaved segments were then embedded upright in water agar in 15-cm-diameter Petri dishes (five segments per dish). First, segments were inoculated with 25 $\mu$l of broth from the *F. lateritium* fermentations. Two days after the inoculation of *F. lateritium*, segments were inoculated with 25 $\mu$l of broth from the *E. lata* shake flask cultures and then incubated for 10 days at either 26°C or 10°C.

Bioassays were also completed to evaluate the tolerance of the BR *F. lateritium* strain and *E. lata* to benomyl when they were applied to grapevine segments. Benomyl concentrations tested included 0, 10, 100 and 1000 $\mu$g/ml (50 $\mu$l/segment). Bioassays were inoculated with 20-$\mu$l aliquots of broth from 4- to 5-day-old shake flask cultures of BR *F. lateritium* and *E. lata*. The *F. lateritium* shake flask cultures yielded approximately $4.5\times10^5$ cells/ml. Segments were inoculated first with benomyl and then with *E. lata* and BR *F. lateritium*. All inoculations were completed within 1 h in a laminar flow hood. These bioassays were incubated at 26°C for 10 days.

After incubation, segments were removed from the water agar, split in half and surface-disinfected for 30 min in a 2.5% sodium hypochlorite solution. Split segments were plated on PDA and monitored for *E. lata* presence after 5 days. All bioassays were performed in triplicate. Positive and negative controls were performed for each bioassay. Mean percent infection levels and standard errors about the mean were calculated for each treatment.

## Results

### Isolation of the BR strain of F. lateritium

In order to isolate a BR strain of *F. lateritium*, several attempts were made to find a natural mutant in the WT population. WT *F. lateritium* was streaked onto PDA plates containing 1200 $\mu$g/ml of benomyl, and the plates were incubated at 28°C for 3–4 days. No growth was observed in this study and two subsequent studies. Benomyl concentrations were lowered to 300, 120, 60 and 12 $\mu$g/ml, but growth was still not observed on these plates. Higher inocula produced by centrifuging 1 ml of 4-day flask cultures were also not successful in producing a natural mutant.

Therefore, UV mutagenesis was performed on 4-day cultures of *F. lateritium* after spreading approximately $1\times10^7$ conidia from 1 ml of centrifuged cells on PDA plates containing 0, 12 or 60 $\mu$g/ml benomyl. Growth appeared on plates containing 0 or 12 $\mu$g/ml benomyl, but not on the 60 $\mu$g/ml plates. Strains were transferred to other plates containing benomyl to confirm the resistance and for storage.

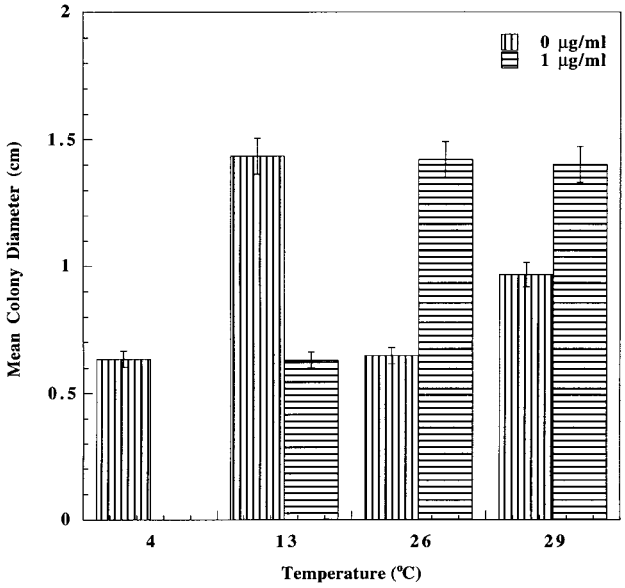


**Figure 1** Mean colony diameter measurements for WT *F. lateritium* taken after a 3-day incubation period. Growth was not observed at benomyl concentrations greater than 1 $\mu$g/ml. At 4°C, growth was not observed at 1 $\mu$g/ml benomyl.

## *Benomyl tolerance of WT and BR F. lateritium on PDA containing benomyl*

Colony diameter data for WT and BR *F. lateritium* strains after incubation for 3 days on PDA plates containing benomyl are illustrated in Figures 1 and 2. WT *F. lateritium* did not grow at benomyl levels above 1 $\mu$g/ml (data not shown). At 4°C, no growth of WT *F. lateritium* was observed in the presence of 1 $\mu$g/ml benomyl. When grown on PDA containing no benomyl, the WT *F. lateritium* mean colony diameters increased as temperature increased from 4°C to 13°C, but decreased as temperature increased above 13°C. When WT *F. lateritium* was grown on PDA containing 1 $\mu$g/ml benomyl, mean colony diameters were greatest at 26°C and 29°C.

The BR *F. lateritium* strain grew in the presence of high concentrations of benomyl over a range of temperatures (Figure 2). However, the BR strain did not grow at 100 $\mu$g/ml or greater when the incubation temperature was 4°C. Growth was also inhibited on PDA containing 1000 $\mu$g/ml when the incubation temperature was 29°C. Mean colony diameters for the BR strain were greatest at 26°C for almost all benomyl concentrations tested. For experiments conducted at 13, 26 or 29°C, colony diameters tended to decrease as the concentration of benomyl increased. There was a slight decrease in colony diameter at 4°C as the benomyl concentration increased from 0 to 10 $\mu$g/ml.

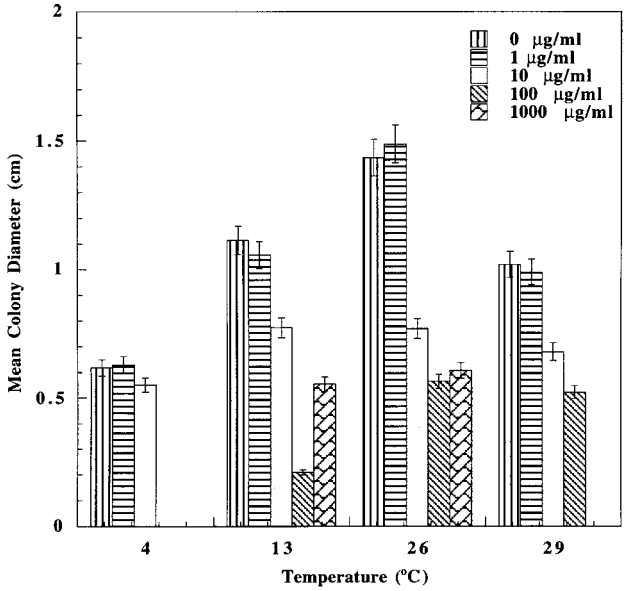


**Figure 2** Mean colony diameter measurements for BR. *F. lateritium* taken after a 3-day incubation period. Growth was not observed at 4°C when the benomyl concentration exceeded 10 $\mu$g/ml and at 29°C when the concentration exceeded 100 $\mu$g/ml.

## *Fermentation studies*

After determining that the isolated strain was indeed BR, it was important to examine the fermentation characteristics and efficacy of the newly isolated strain. Parallel 4.5-L (working volume) fermentations using PDB for medium were inoculated in instrumented fermenters — one with the WT strain and one with the BR strain. As illustrated in Figure 3a, fermentation characteristics such as pH and cell concentration for the two strains were

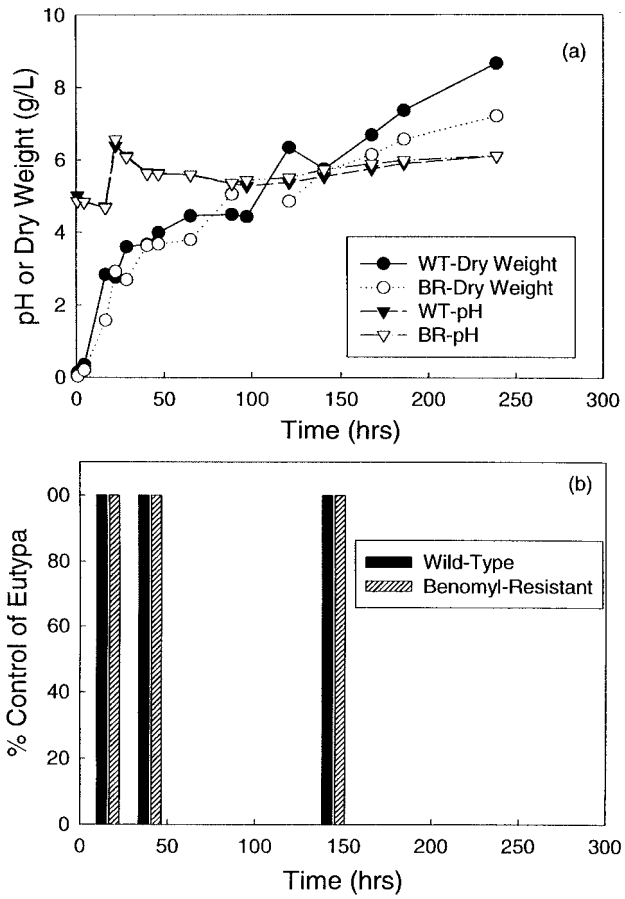


**Figure 3** (a) Fermentation characteristics of the WT and BR *F. lateritium* strains produced in PDB. (b) *E. lata* control levels for bioassays conducted using WT and BR *F. lateritium* fermentation samples at 10°C. All bioassays were performed in triplicate.

**Table 1** Colonization of grapevine segments and control of *E. lata* by the BR *F. lateritium* in the presence of benomyl

| Benomyl concentration ($\mu$g/ml) | Treatment (number per treatment) | *E. lata* colonization (%) | *F. lateritium* colonization (%) |
|---|---|---|---|
| 0 | *F. lateritium* (12) | 0 | 100 |
| | *E. lata* (12) | 100 | 0 |
| | *F. lateritium* and *E. lata* (3) | 0 | 100 |
| 10 | *F. lateritium* (3) | 0 | 100 |
| | *E. lata* (3) | 100 | 0 |
| | *F. lateritium* and *E. lata* (3) | 0 | 100 |
| 100 | *F. lateritium* (3) | 0 | 100 |
| | *E. lata* (3) | 90 | 0 |
| | *F. lateritium* and *E. lata* (3) | 0 | 100 |
| 1000 | *F. lateritium* (3) | 0 | 100 |
| | *E. lata* (3) | 0 | 0 |
| | *F. lateritium* and *E. lata* (3) | 0 | 100 |

nearly identical. Biological assays were also performed on samples from these two fermentations. Results from the bioassays incubated at 10°C are presented in Figure 3b. Both WT and BR *F. lateritium* fermentation samples from 16, 40, 144 and 240 h were effective in controlling *E. lata*. Similar results were obtained in bioassays incubated at 26°C. In all cases, full control of *E. lata* was observed for both the WT and BR strains of *F. lateritium*, indicating that the newly isolated strain possesses at least comparable efficacy.

It should be noted that after running these two fermentations, samples taken from the fermenters confirmed that the BR strain isolated from the end of the fermentation remained BR.

### *Influence of benomyl concentration on the colonization of grapevine segments and control of E. lata by the BR F. lateritium*

Bioassays and colonization studies were completed on grapevine segments to confirm control of *E. lata* by the BR *F. lateritium* and the ability of both organisms to colonize grapevine segments in the presence of benomyl. Results from these studies are listed in Table 1. The BR *F. lateritium* controlled *E. lata* and colonized grapevine segments for each benomyl concentration tested. *E. lata* did not colonize segments when the benomyl concentration applied to the segments exceeded 100 $\mu$g/ml.

## Discussion

The WT *F. lateritium* strain was moderately tolerant to benomyl. This level of tolerance to benomyl has been observed in other fungi [3,7,8]. Both the WT and BR *F. lateritium* strains displayed some level of cold sensitivity when grown on PDA containing benomyl. The WT strain grew on PDA containing 1 $\mu$g/ml benomyl at 13, 26 and 29°C, but did not grow at 4°C. Likewise, the BR strain grew on PDA containing 100 $\mu$g/ml benomyl at 13, 26 and 29°C, but did not grow at 4°C. Cold sensitivity to benomyl is not uncommon for BR fungi [8]. Cold sensitivity must be considered since the temperature during grapevine pruning, and thus the window for *E. lata* infection, ranges between 5°C and 25°C. Because of the variance in temperature during the window of infection and the observed cold sensitivity of both WT and BR strains, efforts are now under way to determine the behavior of the WT and BR *F. lateritium* in the temperature range of 5–15°C.

At 26°C and 29°C, colony diameters were greater for WT *F. lateritium* grown on PDA containing 1 $\mu$g/ml benomyl than with no benomyl. Furthermore, on PDA containing no benomyl, WT *F. lateritium* colony diameters were smaller at 26°C and 29°C than at 13°C. Benomyl and temperature levels may have affected the biomass density of the colonies, and subsequently, the colony diameters. Since colony densities were not measured in this study, it is difficult to conclude whether or not WT *F. lateritium* colonized PDA plates better in the presence or absence of 1 $\mu$g/ml benomyl at 13, 26 or 29°C.

The suggested field application concentration of benomyl for control of *E. lata* in grapevine is 12,000 $\mu$g/ml (1.6 oz/gal) (Andrew Walker, personal communication). The concentrations in studies presented in this paper ranged from 0 to 1000 $\mu$g/ml. Although the benomyl tolerance levels of the BR strain were lower than the benomyl application levels presently employed in the field, the observed tolerance levels were still quite high. If the benomyl application concentrations in the field were lowered below the suggested level to 1000 $\mu$g/ml, the BR strain could be applied concurrently in the field with benomyl. This could offer both short-term and long-term control of *E. lata* and reduce the number of benomyl applications needed for control.

The fermentation results demonstrate that both the WT and BR strains of *F. lateritium* are amenable to production *via* liquid fermentation. Other issues related to the development of the fungi as BCAs, such as shelf life, storage and handling, will be considered in future studies.

There were no observed differences in the efficacy of either strain of *F. lateritium* against *E. lata*. Both strains controlled *E. lata* at 10°C and 26°C at several stages of growth. Although control of *E. lata* was achieved with WT and BR *F. lateritium* under a variety of conditions, the results from the bioassays in this study are inconclusive as to the mechanism of biocontrol. Further research is needed in this particular area as it would greatly aid the development of production methods for *F. lateritium* as a BCA.

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