Antagonism of *Paenibacillus lentimorbus* to *Botryosphaeria dothidea* and Biological Control of Panicle and Shoot Blight of Pistachio

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ABSTRACT

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A potential microbial fungicide, *Paenibacillus lentimorbus* isolate CBCA-2, against *Botryosphaeria dothidea*, the pistachio panicle and shoot blight fungus, was obtained from healthy pistachio leaves by both in vitro and in vivo screening techniques. CBCA-2 caused 100% inhibition of pycnidiospore germination after 24 h incubation at 25°C. Malformation of pycnidiospores and hyphae, and lysis and swollen pycnidiospores of *B. dothidea* occurred in the presence of cell suspensions of CBCA-2. Among the five media tested, nutrient yeast dextrose broth significantly increased the production of antifungal compounds. Application of culture filtrates of CBCA-2 suppressed disease on detached pistachio leaves, but washed bacterial cells did not inhibit lesion development. Development of lesions on excised dormant stems was inhibited only when the culture filtrate was applied before fungal inoculation. Survival of the CBCA-2 after treatment with azoxystrobin (Abound), benomyl (Benlate), tebuconazole (Elite), propiconazole (Break), or trifloxystrobin (Flint) at the highest recommended concentration was not affected, but survival was affected by iprodione (Rovral). Spraying a suspension of CBCA-2 on pruning wounds before inoculation with pycnidiospores of *B. dothidea* significantly reduced infection compared with the unsprayed, inoculated controls.

Additional keywords: antagonistic microorganism, *Pistacia vera*

Panicle and shoot blight caused by *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & de Not. is a yield-devastating disease of pistachio that frequently threatens growers (17). Disease affects leaves, panicle rachises, fruits, shoots, and buds. Environmental conditions such as temperature, wetness duration, and relative humidity are important factors influencing disease development (19). Conventional control measures of *Botryosphaeria* panicle and shoot blight are based upon repeated applications of fungicides. Fungicides are applied on a 1-month schedule. Under standard production conditions, as many as five to six sprays are needed for acceptable disease control (20). However, the use of a fungicide may be dependent upon registration, and there are additional concerns about residues of fungicide in the fruit and the environment, as well as possible development of fungicide-tolerant or -resistant

B. dothidea populations (14). Chemical control programs are also threatened by increasingly stringent regulation of pesticide use and disposal.

Biological control may offer an environmentally friendly supplement or alternative to chemical control. Antagonistic microorganisms (AM) have shown potential to reduce disease on many crops (2,3), and thus their use to reduce panicle and shoot blight of pistachio also needs to be investigated. Cultures of potential antagonistic microorganisms were isolated on acidified potato dextrose agar (APDA) from buds, shoots, fruits, and leaves of pistachio, and some of these AM (different from CBCA-2, which is used in the current work) were shown to greatly reduce the incidence of *B. dothidea* in naturally infected buds (18). The objectives of this study were to (i) identify an AM(s), and (ii) determine if an AM itself or its metabolites can reduce panicle and shoot blight of pistachio in both laboratory and field tests. A preliminary report has been published (6).

MATERIALS AND METHODS

Collection of potential antagonistic organisms. Samples of buds, fruits, rachises, shoots, and leaves were collected periodically during the growing season from pistachio orchards located in Fresno,

Yolo, San Joaquin, and Glenn counties in California. A total of 760 microorganisms were collected from PDA plates in which washings from pistachio buds, fruits, and leaves were spread. Potential AMs were also collected during the procedure of plating pistachio buds when performing BUDMON (a technique of monitoring pistachio buds to predict panicle and shoot blight disease) (21).

Laboratory screening tests. Twelve isolates of *B. dothidea* were recovered from naturally infected leaves and shoots of pistachio collected from an orchard in Glenn County in 2000, and the pathogenicity of each isolate was tested on detached pistachio leaves in a plastic container under high (>95%) relative humidity. To inoculate leaves, a mycelial plug with a diameter of 7 mm was placed on each of the five leaves of pistachio (cv. Kerman) for each isolate. After incubation at room temperature (23 ± 2°C) for 10 days, the diameter of resulting lesions was measured and averaged for each isolate. The isolate of *B. dothidea* that caused the largest lesions (presumably the most virulent one, CBD 8, deposited at the culture collection of the Department of Plant Pathology, Kearney Agricultural Center) was chosen for use in laboratory screening tests for potential AMs.

In vitro tests were performed to determine the effects of selected AMs on mycelial growth of *B. dothidea*. Petri plates (9 cm diameter) containing PDA (39 g of PDA [Difco Laboratories, Detroit, MI] per liter of medium) were streaked with cells of each potential AM. Inoculum of potential AMs was obtained from a 3-day-old actively growing colony at 25°C. Two agar disks of 5 mm diameter with mycelia of the most virulent *B. dothidea* were obtained from a culture growing on PDA and placed at the opposite sides (30 mm from the line of the AM). The plates were incubated in the dark at 25°C for 4 days. Each AM was tested in triplicate plates, and each experiment was repeated once. AMs that secrete an effective antifungal substance may prevent the growth of *B. dothidea* mycelia and result in an inhibition zone. Inhibition zones were measured at the narrowest point and recorded. Those isolates that showed significant inhibition to mycelial growth of *B. dothidea* were chosen for further examination on detached leaves.

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Screening antagonistic microorganisms on detached leaves. Healthy pistachio (cv. Kerman) leaves were collected from an orchard at Kearney Agriculture Center with no history of Botryosphaeria blight or fungicide sprays. For each isolate tested, three leaves were placed on plastic mesh (1 cm square opening) platforms resting in plastic containers (23 × 31 × 10 cm [length × width × height]). The leaves were surface-sterilized in 10% bleach for 10 min, rinsed twice with sterilized water, dried on clean paper towels in a sterile hood, and then inoculated with a 7.5- μ l drop of the cell suspension (10^8 CFU/ml solution) of each of the 39 potential AMs at a distance of 10 mm from the midrib. The cell suspension was prepared by shaking a culture of each isolate in 50 ml of potato dextrose broth (PDB) in a 250-ml Erlenmeyer flask in a shaker at 100 rpm for 2 days under laboratory conditions. A 7.5- μ l drop of spore suspension (5×10^5 spores per ml, prepared in PDB) of *B. dothidea* was added at the same site immediately after the application of the AM. Control leaves were treated with a 7.5- μ l drop of spore suspension alone. To favor infection by *B. dothidea*, 200 ml of water was poured into the plastic containers to create high relative humidity (>95%), and the containers were covered and incubated at room temperature ($23 \pm 2^\circ\text{C}$). All the treatments and control leaves were assigned according to a completely randomized design, with three replicates per treatment, and the experiment was repeated once. Disease was monitored after 10 days incubation by measuring the diameter of the resultant lesions. From this screening, a bacterial strain CBCA-2 proved to be the most inhibitory to development of leaf lesions and was selected for further evaluation. CBCA-2 was identified as *Paenibacillus lentimorbus* (IMI number 388261, identification report H50/02/YU2) by CABI Bioscience Identification Services (International Mycological Institute, UK).

Effect of AMs on mycelium and pycnidiospores of *B. dothidea*. *Hyphal morphology.* A mycelial plug (7 mm diameter) from a 3- to 4-day-old PDA culture of *B. dothidea* was transferred to the center of a petri plate containing 15 ml of PDB and incubated at 25°C in the dark. When mycelial growth on the surface of the liquid reached a diameter of about 10 mm, a loopful with a cell suspension from a 2-day-old PDB culture of CBCA-2 was transferred to the plates, and the plates were incubated at 25°C for 3 more days. Hyphal strands at the edge of the *B. dothidea* colony were removed and examined under a microscope for abnormalities and hyphal lysis. Cultures of *B. dothidea* in PDB in the absence of AM served as controls. Treatment and control plates were arranged in a completely randomized design with three replicated plates for each treatment, and the experiment was repeated once.

Pycnidiospore germination. Pycnidiospores of *B. dothidea* oozing from pycnidia produced on pistachio leaves that had been inoculated with *B. dothidea* isolate CBD 8 were transferred with a fine needle into a 1.5-ml Eppendorf tube containing 500 μ l of sterile distilled water. The tube was vigorously shaken, incubated for 1 h, and shaken again for 5 min. Pycnidiospore concentration was adjusted to 1×10^6 spores per milliliter with a hemacytometer under a compound microscope. Germination of pycnidiospores as affected by the AM was determined on double-depression microscope slides. A PDB suspension of CBCA-2 was made from a 48-h shake culture at 100 rpm in a 250-ml flask, and 200 μ l of the antagonist was added separately to each depression well of a double-depression slide. A 20- μ l drop of a pycnidiospore suspension of *B. dothidea* was added to each well; control slides received 200 μ l of PDB without the AM and 20 μ l of the *B. dothidea* (CBD 8) spore suspension. Slides were incubated at 25°C in a sterile petri dish containing filter paper moistened with distilled water. Germination was determined by counting 100 spores per well per treatment after 8 h incubation. This experiment was repeated three times.

Fungicide resistance of CBCA-2. Because fungicide application may affect the activity of CBCA-2, fungicides commonly applied to trees in the orchard were tested for inhibitory effect. A CBCA-2 culture was grown in PDB on a rotary shaker at 100 rpm and $23 \pm 2^\circ\text{C}$ for 48 h. Subsequently, a 0.15-ml aliquot of the cell suspension was spread with an L-shaped glass rod on the PDA surface. A sterilized filter paper disk (7.5 mm diameter) was placed on the agar surface, and a 25- μ l volume of the fungicide at the recommended field rate was added to each of five replicate disks in separate plates. The fungicides tested and the corresponding recommended active ingredient (a.i.) and product (in parentheses) rates per hectare were as follows: benomyl, 1,120 g a.i. (or 2,240 g Benlate); tebuconazole, 280 g a.i. (or 560 g Elite); propiconazole, 280 g a.i. (or 290 ml Break); trifloxystrobin, 140 g a.i. (or 280 g Flint); iprodione, 560 g a.i. (or 1,170 ml Rovral); and azoxystrobin, 225 g a.i. (or 900 ml Abound) (20). After incubation at 27°C for 48 h, inhibition of growth of CBCA-2 was evaluated by measuring the width of the clear zone (if present) between the edge of the paper disk and the bacterial growth.

Disease inhibition by CBCA-2 on detached pistachio leaves. Isolate CBCA-2 was grown in 50 ml PDB in a 250-ml Erlenmeyer flask for 2 days, with shaking as described above. The culture filtrate was obtained by centrifuging at $2,100 \times g$ for 10 min. The pellet of cells was washed twice by resuspension in distilled water and centrifugation as described above. A suspension of washed bacterial cells (10^8

CFU/ml) was made. Healthy pistachio leaves were collected from a pistachio (cv. Kerman) orchard at Kearney Agricultural Center in Parlier, surface-disinfested in 10% bleach (5.25% sodium hypochlorite solution), and inoculated with a spore suspension of *B. dothidea* and CBCA-2 cell suspension in the same way as described above. Ten days after incubation in a high humidity plastic container as described earlier, disease inhibition by treatment with each component of CBCA-2 (washed bacterial cells, culture filtrate, and incubation mixture) was recorded. Disease development was evaluated based on the diameter of lesions on leaves. The test had 10 replicates and was repeated twice. Lesion diameter was used to compare the effect of culture filtrate and cell suspension of CBCA-2 on lesion development.

Inhibition of lesion development on excised dormant stems by CBCA-2. Dormant pistachio 1-year-old stem sections (diameter approximately 20 mm, length 20 cm) were surface-sterilized with 10% bleach (5.25% sodium hypochlorite solution) for 10 min, followed by two rinses with sterile water. The ends of the stems were sealed with Parafilm. A piece of bark (6 mm diameter) was removed with a cork borer, and a cavity of 6 mm in diameter was created. An aliquot of 20 μ l of CBCA-2 culture or PDB was placed into the cavity. Three groups were made: A, control 1 (only wounded); B, control 2 (wounded and inoculated with 20 μ l PDB); and bio-control treatment C (wounded and inoculated with 20 μ l of a cell suspension of CBCA-2). These groups were further divided into two classes: (i) fungal challenge simultaneously after application of CBCA-2, and (ii) fungal challenge 3 days after application of CBCA-2. Seven replications were made for each treatment. A 6-mm-diameter agar plug carrying actively growing mycelia of CBD 8 isolate of *B. dothidea* was placed into the cavity. Inoculated dormant stems were incubated on plastic screens raised 2 cm above the bottom of plastic containers at $24 \pm 1^\circ\text{C}$ in the dark. To favor infection by *B. dothidea*, 200 ml of water was poured into each container to create high (>95%) relative humidity. A randomized complete block design was used, and the experiment was conducted twice. The lesion length was measured after 9 days incubation. Lesion length was used to compare the antagonistic effect of *P. lentimorbus*.

Effect of culture media on production of antifungal compounds by CBCA-2. To determine the effect of incubation medium on the antifungal compounds' production, CBCA-2 was incubated with 50 ml each of PDB, PPDB (potato dextrose broth amended with 0.5% polypeptone), LB (Luria-Bertani, Difco), KB (King's medium B: 20 g of proteose peptone no. 3, 1.5 g of K_2HPO_4 , 1.5 g of MgSO_4 , and 10 ml of glycerin in 1 liter of distilled water),

or NYDB (nutrient yeast dextrose broth: 8 g of nutrient broth, 5 g of yeast extract, and 10 g of dextrose in 1 liter of distilled water) in a 250-ml Erlenmeyer flask and shaken at 100 rpm for 3 days at $23 \pm 2^\circ\text{C}$ in the dark. A 5-ml aliquot of culture filtrate from each cell suspension was obtained by centrifuging at $2,100 \times g$ and filter ($0.45 \mu\text{m}$) sterilization. The aseptic filtrate ($20 \mu\text{l}$) was placed onto a small mycelial block (approximately 1 mm^3) of *B. dothidea* in a PDA plate. Twofold, fourfold, and eightfold dilutions of the filtrate in sterile distilled water were also placed onto another mycelial block. After incubation for 2 days at 27°C in the dark, antifungal activity of each dilution of the filtrate was recorded by measuring the colony diameter of *B. dothidea* developed from the mycelial block. The test included five replicates, and the experiment was repeated once. Colony diameter was used to compare the effect of different media on the production of antifungal compounds of CBCA-2.

Field test for control of *B. dothidea*. CBCA-2 was grown in PDB at $23 \pm 2^\circ\text{C}$ on a rotary shaker for 2 days. Bacterial cells were harvested by centrifuging at $2,100 \times g$ for 10 min. Treatments were azoxystrobin (225 g a.i./ha), azoxystrobin (225 g a.i./ha) plus cells (10^8 CFU/ml) of *P. lentimorbus*, culture fluids alone, cells (10^8 CFU/ml) in water, washed bacterial cells (10^8 CFU/ml) in PDB, unwashed cell suspension (10^8 CFU/ml) of *P. lentimorbus* in PDB, and a distilled water control. A 30-year-old pistachio (cv. Kerman) orchard at Kearney Agricultural Center was used for this experiment. Wounds were made on shoots by pruning. All treatments were applied with a household sprayer directly onto wound surfaces immediately after pruning until runoff. Each pruning wound was inoculated with $100 \mu\text{l}$ of a suspension of *B. dothidea* pycnidiospores (10,000 spores per ml) 5 h after treatment, and wounds were covered with Parafilm to prevent desiccation. Sixteen pruning cuts (wounds) per tree on three trees were inoculated for each treatment, and the treatments were replicated arbitrarily in a randomized design. Wounds varied between 8 and 16 mm in diameter. Eight months after inoculation, stem pieces were cut 30 mm below the inoculated wound. These pieces were split in quarters lengthwise, surface-sterilized in 10% bleach solution for 10 min, rinsed twice in sterilized water, and plated on petri plates (three pieces in each) containing APDA (PDA and 2.6 ml of a 25% [vol/vol] solution of lactic acid). After 3 days incubation at 25°C , plates were examined for typical mycelial growth of *B. dothidea*; the number of colonies of *B. dothidea* was recorded by its colony morphology; and the incidence of infected pruning wounds (%) was determined and used for data analysis. This experiment was conducted in 2001 and repeated in 2002, except that stem pieces were col-

lected 5 months after the application of CBCA-2 and inoculation of the fungus.

Data analysis. Analysis of variance was performed for each experiment using the general linear models (GLM) procedure, and treatment means for the effect of culture media on the production of antifungal compounds by CBCA-2 were separated by the Duncan's multiple range test at $P = 0.05$ and for all other mean comparisons by the least significant difference (LSD) test at $P = 0.05$ with SAS software (SAS Institute, Cary, NC). Since results of the repeated experiments for disease inhibition on detached leaves, inhibition of lesion development on excised dormant stems, and effect of culture media on the production of antifungal compounds were similar, the averages of the two replicated experiments were presented. The data of effects of AMs on mycelium and pycnidiospores of *B. dothidea*, and the fungicide resistance data were not subjected to statistical analyses.

RESULTS

Screening of microbial antagonists against *B. dothidea* in vitro. A total of 154

bacterial isolates were recovered and screened for their antagonistic effects on the growth of the panicle and shoot blight fungus of pistachio. Thirty-nine isolates (25.3%) expressed inhibitory action against the growth of *B. dothidea* in dual culture, and nine isolates (5.8%) inhibited lesion development by coinoculation of *B. dothidea* and the bacterium on detached leaves. Isolate CBCA-2 of *P. lentimorbus* obtained from healthy pistachio leaves was the most efficient isolate and was used for further tests. This isolate completely inhibited the growth of *B. dothidea* when it was coinoculated with the pathogen on a detached pistachio leaf bioassay.

Effect of CBCA-2 on mycelial growth and pycnidiospore germination of *B. dothidea*. *Mycelium morphology.* After introduction of *P. lentimorbus* into the PDB, very little additional growth of *B. dothidea* was observed. Hyphal tips of *B. dothidea* showed malformations, formation of chlamydospores, and hyphae were thickened and vacuolated (Fig. 1B and F) compared with hyphae not treated with an AM (Fig. 1E). Many swellings occurred in

Fig. 1. Interaction of *Botryosphaeria dothidea* (isolate CBD 8) and *Paenibacillus lentimorbus* isolate CBCA-2 in dual culture. **A** and **C**, ungerminated, swollen and lysed pycnidiospores in the presence of CBCA-2 after 8 h; **B** and **F**, thickened hyphae tips and vacuoles in hyphae; **D**, occurrence of bubbles in hyphae; **E**, normal hyphae in the absence of CBCA-2 in potato dextrose broth (PDB) for 3 h; **G**, germinated pycnidiospores in the absence of CBCA-2 in PDB for 3 h; and **H**, germinated pycnidiospores in the absence of CBCA-2 in PDB for 8 h, stained with cotton blue. (Magnification for **A** to **G** is $\times 400$ and for **H** is $\times 250$.)

the hyphae or at the hyphal tips (Fig. 1D), whereas normal hyphal walls were smooth without swellings or vacuoles. No lysis of hyphal strands was noticed.

Pycnidiospore germination. Pycnidiospores of *B. dothidea* did not germinate in the presence of CBCA-2, whereas an average of 51.4% germination rate was recorded in the controls (without CBCA-2) after 3 h, and 100% germination was recorded after 8 h incubation (Fig. 1G and H). In the presence of CBCA-2, pycnidiospores of *B. dothidea* were swollen but did not germinate, even after 24 h, and two to five septa per spore developed (Fig. 1A and C). Vacuolation, lysis, and irregular texture in the cytoplasm of the spores were also observed (Fig. 1C).

Fungicide resistance. Among the six fungicides tested, only iprodione (Rovral) showed inhibition to the growth of CBCA-2, and this inhibition was slight and temporary. Mean width of the inhibition zone was 3.5 mm at 24 h after incubation at 27°C. However, the inhibition zone was reduced to 1.5 mm (57.1% reduction) after 48 h incubation. If registered, iprodione (Rovral) sprays on pistachio could have some temporary negative effect on the activity of the *P. lentimorbus* strain CBCA-2 to act as an antagonist against *B. dothidea*.

Effect of CBCA-2 on lesion development on detached pistachio leaves. The experiment of co-culture of bacteria with *B. dothidea* on detached pistachio leaves (Fig. 2) proved the effectiveness of the antagonistic capacity of CBCA-2. Development of lesions on pistachio leaves was completely inhibited by treatment with the cell suspension and the culture filtrates (Fig. 3). However, washed bacterial cells did not inhibit lesion development compared with the water control (Fig. 3).

Fig. 2. Effect of isolate CBCA-2 of *Paenibacillus lentimorbus* on lesion development by *Botryosphaeria dothidea* CBD 8 on detached pistachio (cv. Kerman) leaves after treatment with CBCA-2 (left), or no treatment (right). Bacteria were applied at a concentration of 10^8 CFU/ml at the same time as the pycnidiospore suspension (5×10^5 spores per ml). (A drop of 15 μ l of water was applied (left) to indicate the inoculation site before photography.)

Inhibition of lesion development on dormant stems. The experiment of co-culturing the bacteria with *B. dothidea* on dormant pistachio stems proved the effectiveness of the antagonistic capacity of isolate CBCA-2 (Fig. 4). CBCA-2 reduced significantly ($P < 0.05$) the lesion length in pistachio stems treated with the bacteria 3 days prior to fungal challenge. Application of bacteria and mycelium of *B. dothidea*

simultaneously did not result in significant symptom reduction compared with control 1 (treated only by mycelial plugs of *B. dothidea*). The CBCA-2 isolate caused an 80% reduction of lesion length compared with control 2 when applied 3 days before inoculating with *B. dothidea* (Fig. 4).

Effect of culture media on the production of antifungal compounds. Figure 5 shows the effect of media on the produc-

Fig. 3. Diameter of leaf lesions by *Botryosphaeria dothidea* on pistachio leaves treated with fractions of shaken cultures of *Paenibacillus lentimorbus* isolate CBCA-2. Treatment 1, whole cell suspension; treatment 2, culture filtrate; treatment 3, washed bacterial cells; treatment 4, potato dextrose broth (control); treatment 5, water control. Leaves were inoculated with a 5×10^5 spores per ml suspension of *B. dothidea* at the same time as bacterial cells. Each value represents the mean of 10 replicates. The experiment was repeated twice. The values of bars with the common letters are not significantly different at $P = 0.05$ according to the least significant difference (LSD) test.

Fig. 4. Antagonistic effect of application of *Paenibacillus lentimorbus* isolate CBCA-2 on lesion length in dormant stems of pistachio caused by *Botryosphaeria dothidea*. Control 1 was only wounded, control 2 was wounded and inoculated with 20 μ l of potato dextrose broth. Bars topped with different letters are significantly different at $P = 0.05$ according to the least significant difference (LSD) test. Black bar represents bacteria and fungus applied simultaneously; white bar represents *B. dothidea* applied 3 days after bacteria application.

tion of antifungal compounds by CBCA-2. The type of culture medium strongly affected the activity of the culture filtrate against *B. dothidea*. Culture filtrates obtained from NYDB and PPD completely inhibited mycelial growth at twofold dilution, and filtrate obtained from NYDB diluted eightfold still strongly suppressed the radial growth of *B. dothidea*. Treatment of *B. dothidea* with each of the media without the CBCA-2 bacteria did not affect growth of the panicle and shoot blight pathogen. The undiluted filtrates and two-fold dilution obtained from LB and PDB cultures significantly ($P < 0.05$) inhibited radial growth of *B. dothidea* compared with the culture filtrate obtained from KB, although activity was diminished when these were diluted fourfold or eightfold (Fig. 5).

Biological control of *B. dothidea* in the orchard. Under field conditions, the infection of pistachio pruning cuts by *B. dothidea* was reduced by all the treatments compared with the water control in both 2001 and 2002 trials (Fig. 6). Both culture filtrate and culture fluid plus bacterial cells significantly ($P < 0.05$) reduced infection by *B. dothidea* compared with the other treatments. The other four treatments also showed significant reduction of infection compared with the water control. The highest suppression was obtained with treatment 7 (bacterial cell suspension), which reduced the infection rate from 14.8 to 2.2% (an 85% reduction rate) in 2001, and from 13.5 to 3.7% (a 72.6% reduction rate) in 2002 (Fig. 6).

DISCUSSION

An antagonist sought for use on a plant surface, in soil, or within a plant tissue is most likely to develop in that niche if it was originally obtained from a similar niche (7). Based on a large-scale screening of antagonists from leaves, fruits, and buds of pistachio, an isolate of *P. lentimorbus* recovered from the washings of healthy pistachio leaves was found to be a promising antagonistic microorganism. Cell suspensions of the AM showed strong inhibition on the mycelium growth and pycnidiospore germination by causing malformation and formation of chlamydospores in the hyphae, pycnidiospore swelling, vacuolation, lysis, and irregular texture in the cytoplasm of the spores. Moreover, a strong inhibition of pycnidiospore germination (the failure of *B. dothidea* pycnidiospores to germinate after 24 h exposure to *P. lentimorbus*) was observed by the application of the cell suspension of CBCA-2. Mycelial malformation was probably due to the antibiotic substances interfering with normal growth processes. A toxic metabolite may penetrate a cell and inhibit its activity by chemical toxicity (7). It is well documented that strains of *Bacillus* spp. produce antibiotic substances that can influence the morphology of fungal mycelium and spores (1,8,16).

A high incidence of *B. dothidea* in pruning wounds was reported (22). The pathogen can enter through the pruning cuts and establish itself. In orchards with a high inoculum level of *B. dothidea*, wounds created by pruning of pistachio trees during

winter need to be protected, because the fungus uses these wounds as an infection court (22). Botryosphaeria canker of apple caused by *B. dothidea* was associated with poor pruning techniques in the southeastern United States (4), and a fungicide was

Fig. 5. Colony diameter of *Botryosphaeria dothidea* treated with dilutions of culture filtrates of *Paenibacillus lentimorbus* isolate CBCA-2 obtained from five growth media. Undiluted culture filtrate was considered as onefold. The media used include: King's medium B (diamond), potato dextrose broth amended with polypeptone (multiplication symbol), Luria-Bertani (triangle), potato dextrose broth (square), and nutrient yeast dextrose broth (asterisk). Each value plotted represents the average of three replicates. Points with same letters for each dilution are not significantly ($P < 0.05$) different according to Duncan's multiple range test.

Fig. 6. Effect of various treatments on infection of pistachio pruning cuts by *Botryosphaeria dothidea*. Treatment 1, azoxystrobin (225 g a.i./ha); treatment 2, control (water); treatment 3, bacterial cells (10^8 CFU/ml) of CBCA-2 of *Paenibacillus lentimorbus* plus azoxystrobin (225 g a.i./ha); treatment 4, bacterial cells (10^8 CFU/ml) of CBCA-2 in potato dextrose broth; treatment 5, bacterial cells (10^8 CFU/ml) of CBCA-2 in water; treatment 6, culture filtrate alone; and treatment 7, culture fluid plus cells (10^8 CFU/ml) of CBCA-2. Five hours after treatment, wounds were inoculated with a suspension of 10,000 spores of *B. dothidea* per ml. Percentage of infection of the wounds was determined 8 months later in 2001, and 5 months later in 2002. Bars topped with the same letter (lowercase for 2001, uppercase for 2002) are not significantly different ($P < 0.05$) according to the least significant difference (LSD) test.

applied after pruning to prevent infection before callus formation. In pistachio inoculations, incidence of *B. dothidea* was reduced significantly by spraying a cell suspension of CBCA-2 on pruning wounds of pistachio, suggesting that CBCA-2 could be used to protect pruning cuts from infection by *B. dothidea* in the orchard. The value of several other AMs was demonstrated in field trials on protecting the pruning wounds of grapevine against *Eutypa dieback* in California (24) and South Africa (8).

The mechanisms of biological control are generally classified as competition, parasitism or predation, and antibiosis (9). Disease inhibition on detached pistachio leaves by CBCA-2 appears to be due to the antifungal compounds produced in the culture filtrate, because treatment with the washed bacterial cells did not suppress the disease development, while culture filtrate and cell suspension completely inhibited the symptom development. Inhibition of lesion development on excised dormant stems by the cell suspension of CBCA-2 was observed when the bacterium was applied 3 days before, but not simultaneously with, the *B. dothidea* inoculum, suggesting that this bacterium does not have a therapeutic effect but exerts a preventative effect on the disease. Antibiosis may play a major role in the inhibition of panicle and shoot blight by CBCA-2.

Our results, especially the consistency of inhibition by CBCA-2 both in vitro and in vivo, suggest that this isolate of *P. lentimorbus* could be a potential microbial fungicide and could be integrated into currently used practices for panicle and shoot blight management. Survival of this potential microbial fungicide was not affected by azoxystrobin, benomyl, tebuconazole, propiconazole, or trifloxystrobin at the highest possible concentration used in the orchards. Iprodione showed temporary inhibition to the growth of the AM CBCA-2, but the suppressive effect diminished with time; there was no inhibition observed after 3 days. The results indicated that CBCA-2 is compatible with all the fungicides that are currently registered or are candidates for future registration on pistachio. Application of chemical pesticides with biocontrol agents was reported to increase disease control in some cases (3,15). The antifungal products produced by *P. lentimorbus* could play a significant role in preventing disease development while some of these fungicides exhibit a therapeutic effect.

Evaluation of bacteria in vitro as potential antagonists from a wide range of agricultural crops has been reported (1,5,11,23,27). This is, however, the first report in which bacteria from pistachio leaves, buds, and fruits have been isolated and their antagonistic potential evaluated against the panicle and shoot blight pathogen. Biocontrol potential of several tree

diseases by *Bacillus* spp. has been reported (12,29,30,33). *P. lentimorbus* was recently transferred from *Bacillus lentimorbus* based on 16S rRNA gene sequence (25). *P. lentimorbus* was reported to be a pathogen of scarab larvae in Central and South America (10), and chestnut weevil in Turkey (32), as well as a biocontrol agent for gray leaf spot of perennial ryegrass caused by *Pyricularia grisea* (31) and Fusarium dry rot of potato tubers (26). However, its antifungal activity against *B. dothidea* was demonstrated for the first time here. In California, *P. lentimorbus* was also isolated from hemiptera insects that are major pests of pistachio (T. J. Michailides, unpublished).

The possibility of using a naturally occurring AM to control plant diseases remains an attractive and environmentally sound method. A thorough understanding of the mechanisms of microbial antagonists may eventually lead to effective utilization of beneficial microorganisms for control. *Bacillus* spp. are ubiquitous antagonistic bacteria, producing a multitude of broad-spectrum antibiotic compounds. Greenhouse and field control of many plant diseases have been achieved with application of *Bacillus* spp. (1,11,13). Furthermore, this genus includes many attractive candidates used as biocontrol agents, because its species are abundant in soil and aerial parts of plants. These bacteria have the ability to produce drought-resistant endospores that provide long survival, persistence, and resistance against physical stresses (16). The use of *P. lentimorbus* as a microbial fungicide against *B. dothidea* would be an environmentally friendly way to suppress panicle and shoot blight of pistachio.

Commercial acceptance of antagonists depends on their ability to perform as well as or better than commercial fungicides (29). It was evident from our investigation that the bacteria treatment was consistently more effective compared with commercial fungicides and therefore might have a potential to be accepted by pistachio growers. However, additional studies on determining the interactions of the bacteria with pistachio and the effects of environmental factors are needed before an effective and sustainable biocontrol program can be developed on pistachio. Whether this AM would prevent disease development in the new growth of pistachio also needs to be investigated in the orchard. Although NYDB significantly increased the production of antifungal compounds over other media, suitable formulation additives may provide the antagonist with a more constant environment and a competitive advantage (28). The effect of various nutrients and formulation additives on the antagonistic performance of *P. lentimorbus* toward *B. dothidea* needs to be investigated. Antifungal compounds in the culture filtrate need to be isolated and identified. Furthermore, the form in which this bacte-

rium can be used commercially needs further investigation.

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