A PCR-based Technique for Identification of *Fusicoccum* sp. from Pistachio and Various Other Hosts in California

Zhonghua Ma and Themis J. Michailides, Department of Plant Pathology, University of California Davis, Kearney Agricultural Center, 9240 South Riverbend Ave., Parlier, CA 93648

ABSTRACT

Ma, Z., and Michailides, T. J. 2002. A PCR-based technique for identification of *Fusicoccum* sp. from pistachio and various other hosts in California. *Plant Dis.* 86:515-520.

Botryosphaeria panicle and shoot blight of pistachio, caused by *Fusicoccum* sp. is a destructive disease in California. In this study, a pair of group-specific polymerase chain reaction (PCR) primers BDI and BDII, was developed for identification of *Fusicoccum* sp. from pistachio and other hosts in California based on the sequences of the rDNA internal transcribed spacer (ITS) region. The primers amplified a 356-bp DNA fragment for all 73 tested isolates of *Fusicoccum* sp. collected from pistachio and other hosts throughout California in different years, but not for the other 33 fungal species isolated from pistachio and the eight isolates of *Fusicoccum* sp. obtained from pistachio trees in Greece. The PCR assay using this pair of primers was sensitive enough to detect 5 pg of genomic DNA of *Fusicoccum* sp. A simple DNA extraction procedure was developed that led to the rapid identification of *Fusicoccum* sp. from pistachio and other host plants in California.

Additional keywords: *Botryosphaeria dothidea*, nut disease, *Pistacia vera*

Botryosphaeria Ces. & De Not. is a species-rich genus with a worldwide distribution and causes diseases of many woody plants. Species identification in *Botryosphaeria* is complicated and difficult because the teleomorphs of these fungi are rarely encountered in nature, and teleomorphic characteristics vary little among species. Furthermore, morphological characteristics of the anamorphs are also similar among some *Botryosphaeria* species and can be strongly influenced by the substrate on which they are produced (3). Additionally, difficulties in inducing sporulation of some *Botryosphaeria* species in culture have resulted in confusion relating to identification of *Botryosphaeria* species (21). Moreover, these fungi are not easily differentiated by host range because host range may be extensive, and fruiting structures of two to three *Botryosphaeria* spp. have been found together on a single host (9).

Botryosphaeria panicle and shoot blight of pistachio (*Pistacia vera* L.) has become a major threat to California pistachio industry since the late 1980s (13,14). The pathogen causing panicle and shoot blight of pistachio was identified as the anamorph of *B. dothidea*, and referred to as a species of *Dothiorella* (14). However, Smith et al.

(20) compared isolates of *Fusicoccum* sp. from pistachio with isolates of *B. dothidea* and *B. ribis* and found the pistachio isolates separated as a taxonomic clade that was between *B. dothidea* and *B. ribis* isolates. At the time when Michailides (14) identified the species causing *Botryosphaeria* panicle and shoot blight of pistachio, *B. dothidea* and *B. ribis* were considered synonymous (2). Currently, considerable controversy still exists surrounding the taxonomic status of *B. dothidea* and *B. ribis*. Some researchers regard the two species as synonyms, while others have treated them as separate taxa (21). Identification of *Fusicoccum* sp. from California pistachio is usually based on mycelial and pycnidiospore characteristics (14) since this pathogen has not been observed to produce sexual structures on artificial media and on pistachio (12). However, pseudothecia of *B. dothidea* were discovered in blighted shoots of pyracantha, olive, and blackberry grown next to infested pistachio orchards, and in avocado orchards in California (15). Production of pycnidiospores of *Fusicoccum* sp. isolates from pistachio requires 2 weeks. More importantly, identification of *Fusicoccum* sp. based on morphological characteristics requires an experienced observer. Thus, it is necessary to develop a simple, rapid, and reliable method for identification of *Fusicoccum* sp. from California pistachio.

Advances in molecular biology, particularly the polymerase chain reaction (PCR), have provided new opportunities for detection of plant pathogens. PCR with species-specific primers can provide a rapid and precise way to detect fungal plant patho-

gens both in culture and in plant tissues. PCR assays may be based on DNA sequences of the internal transcribed spacer (ITS) region (4,8,10,11,25), randomly amplified regions (7,11,17), or species-specific repetitive sequences (1,16). To date, a PCR-based technique has not been developed for identification of *Fusicoccum* sp. In previous studies (12, Z. Ma and T. J. Michailides, *unpublished data*), we observed a low level of genetic variation among the isolates of *Fusicoccum* sp. collected from pistachio and other host plants in California. This low genetic variation may allow us to develop molecular methodologies for detection of this pathogen. The objective of this study was to develop a reliable and simple PCR-based assay for the identification of *Fusicoccum* sp. from pistachio and other hosts in California.

MATERIALS AND METHODS

DNA sequences of ITS region and primer design. Five representative isolates (CE347, FP497, RE282, TP565, and WA373) of *Fusicoccum* sp. were differentiated by microsatellite-primed (MP)-PCR (Z. Ma, and T. J. Michailides, *unpublished data*) and selected for analysis of the sequences of the ITS region.

The ITS regions of these five isolates of *Fusicoccum* sp. were amplified using fungal universal primers ITS1 and ITS4 (24). PCR was performed in a 100- μ l volume containing 10 ng fungal genomic template, 1.0 μ M of each primer, 0.2 mM of each dNTP, 2.0 mM MgCl₂, 1 $\times$ *Taq* Buffer and 1.5 U of *Taq* Polymerase (Promega, Madison, WI). The PCR was performed using the following parameters: an initial preheat for 3 min at 95°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1.5 min, and terminated with a final extension at 72°C for 10 min. The PCR products were verified on a 1.5% agarose gel in Tris-acetate (TAE) buffer. The amplifications were purified using the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA) and sequenced by DBS Sequencing Inc. (Division of Biological Sciences, University of California at Davis). The ITS sequences of other related *Botryosphaeria* species (*B. corticis*, *B. obtusa*, *B. parva*, *B. ribis*, *B. stevensii*, and *F. luteum*) were obtained from GenBank databases.

The complete sequences of the ITS regions (ITS1, 5.8S gene, and ITS2) of *Fusicoccum* sp. isolates CE347, FP497, RE282, TP565, WA373 (GenBank Acces-

Corresponding author: T. J. Michailides
E-mail: themis@ucdavis.edu

Accepted for publication 27 December 2001.

sion Nos. AF452701 – AF452705), and other related fungal species were aligned using the computer program Clustal W 1.81 (European Bioinformatics Institute, Cambridge, UK). A pair of primers (BDI and BDII) specifically targeting *Fusicoccum* sp. rDNA (Fig. 1) was constructed using a computer program, Web Primers (<http://alces.med.umn.edu/webprimers.htm>). The primers were synthesized by Invitrogen (Life Technologies, Grand Island, NY).

Specificity and sensitivity of primers BDI and BDII. Other hosts of *Fusicoccum* sp. can serve as potential inoculum sources for pistachio. Isolates of *Fusicoccum* sp. from pistachio and other host plants in California are related genetically (12). In this study, 73 isolates of *Fusicoccum* sp. collected from pistachio and other hosts throughout California in different years, as well as eight isolates of *Fusicoccum* sp. collected from pistachio trees in Greece, and 33 different fungal species associated with pistachio were used for specificity tests (Table 1). The morphological identification of *Fusicoccum* sp. isolates was based on the characteristics of mycelia growing on potato dextrose agar (PDA, Microtech Scientific, Orange, CA) and pycnidiospores produced on autoclaved pistachio leaves (12). The isolates of other species were identified based on standard morphological characteristics. Fungal genomic DNA of each isolate was extracted using FastDNA Kit (BIO 101, Vista, CA). In sensitivity tests, serial amounts of genomic DNA (50 fg to 5 ng) of the *Fusicoccum* sp. isolates FP497, TP565, and CE347 were used as DNA template for PCR amplifications.

The PCR reaction was performed in a 50- μ l volume containing 5 μ l (approximately 15 ng) fungal genomic DNA extracted by FastDNA Kit, 0.2 μ M of each primer (BDI and BDII), 0.2 mM of each dNTP, 2.0 mM MgCl₂, 1 $\times$ Promega Taq Polymerase Buffer and 1.5 U of Promega Taq Polymerase. The PCR was performed using the following parameters: an initial preheat for 3 min at 95°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 69°C for 1 min, extension at 72°C for 1.5 min, and terminated with a

final extension at 72°C for 10 min. PCR products (10 μ l per sample) were verified on 1.5% agarose gels in TAE buffer.

MP-PCR fingerprinting for the isolates of *Fusicoccum* sp. collected from pistachio trees in California and Greece. Eight isolates from Greece, as well as 11 California isolates selected randomly, were used for analysis of DNA fingerprinting produced by the microsatellite primer M13 (GAG GGT GGC GGT TCT), T₃B (AGG TCG CGG GTT CGA ATC C), or (CTC)₄RC. The PCR reactions were performed in 50- μ l volume containing: 50 ng fungal genomic template, 1.0 μ M of microsatellite primer, 0.2 mM of each dNTP, 2.0 mM MgCl₂, 1 $\times$ Promega Taq Polymerase Buffer (10 mM Tris-Cl, pH 9.0, 50 mM KCl, 0.1% Triton X-100) and 1.5 U of Promega Taq Polymerase. The PCR was performed using the following parameters: an initial preheat for 3 min at 95°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1.5 min, and terminated with a final extension at 72°C for 10 min. The PCR products (10 μ l per sample) were separated on 1.5% agarose gels in TAE buffer and photographed after staining with ethidium bromide.

Rapid extraction of DNA for identification of *Fusicoccum* sp. To rapidly identify isolates of *Fusicoccum* sp., approximately 20 mg (fresh weight) of mycelia were collected by gently scraping the surface of a 3-day-old culture on PDA with a sterilized loop and placed in a 1.5-ml microcentrifuge tube containing 100 μ l of 1 M Tris-HCl (pH 8.0). Samples were boiled at 98°C for 15 min and immediately placed on ice for 5 min. A 5- μ l aliquot of a 1:10 or 1:100 dilution of resulting supernatant was used for PCR amplification.

Double-blind test. Ten isolates of *Fusicoccum* species that were similar in colony morphology on PDA were used for a double-blind test. Extraction of DNA was conducted using the method of boiling mycelia described above. A 5- μ l aliquot of a 1:10 supernatant dilution of each isolate was used for PCR amplification with the primers BDI and BDII. To identify these isolates based on the characteristics of pycnidiospores, mycelial plugs of each

isolate were transferred on autoclaved pistachio leaves that were placed on the surface of agar in plates supplemented with 5 ml of 2% agar in the bottom (12). After incubation under light at room temperature (25 $\pm$ 2°C) for 2 weeks, mature pycnidia were produced on pistachio leaves, and the characteristics of pycnidiospores for each isolate were examined under a light microscope.

Detection of *Fusicoccum* sp. from natural infected pistachio leaves. During the 2001 growing season, 20 pistachio leaves showing putative incipient infections of *Fusicoccum* sp. were collected from an orchard at the Kearney Agricultural Center, University of California in Parlier. A small piece of tissue (0.5 $\times$ 0.5 cm) was taken from the margin of each infection point and surface-disinfested with 0.525% sodium hypochlorite for 3 min (10% commercial bleach), rinsed with sterile water, and placed on acidified (2.5 ml of 25% vol/vol lactic acid per liter) potato dextrose agar (APDA, Microtech Scientific). After incubation at 30°C for 4 days, approximately 10 mg (fresh weight) of mycelia were collected and placed in a 1.5-ml microcentrifuge tube containing 50 μ l of 1 M Tris-HCl (pH 8.0). Extraction of DNA was conducted using the boiling method described above. A 5- μ l aliquot of a 1:10 dilution of each sample was used for PCR amplification using the primers BDI and BDII. Morphological identification of *Fusicoccum* sp. from these samples was performed according to the protocol described above.

RESULTS

Primers design for *Fusicoccum* sp. Sequences from the ITS regions obtained from five isolates of *Fusicoccum* sp. collected from pistachio and other hosts in California were very similar (99.6% identical). A pair of primers specifically targeting the *Fusicoccum* sp. ITS region was designed as BDI (5'-CTC GAC TCT CCC ACC CTA TGT-3') and BDII (5'-GAG GTG TTT TCT ACT ACG CTC GA-3') (Fig. 1). This pair of primers amplified a 356-bp DNA fragment from the isolates CE347, FP497, RE282, TP565, and WA373 of *Fusicoccum* sp.

Specificity and sensitivity of the primers BDI and BDII. Using a high annealing temperature (69°C), a 356-bp DNA fragment was amplified consistently from all 73 tested *Fusicoccum* sp. isolates collected from pistachio and other hosts throughout California in different years, but not from 33 other fungal species isolated from pistachio trees in California and the eight isolates of *Fusicoccum* sp. collected from pistachios in Greece (Table 1). The eight isolates of *Fusicoccum* sp. collected from pistachio trees in Greece were significantly different from the California isolates in DNA fingerprinting produced by MP-PCR (Fig. 2). Examples of PCR products ampli-

Fig. 1. Schematic presentation of locations of the pair of group-specific polymerase chain reaction (PCR) primers (BDI and BDII) developed from the sequences of the rDNA internal transcribed spacer (ITS) region for identification of *Fusicoccum* sp. from pistachio and other hosts in California. The primers ITS1 and ITS4 are the universal primers.

fied from genomic DNA of various fungal species by the primers BDI and BDII are presented in Figure 3.

In a sensitivity test with serial DNA dilutions of three *Fusicoccum* sp. isolates FP497, TP565, and CE347, the primers BDI and BDII were effective at detecting five pg of fungal genomic DNA. Examples of PCR products amplified from genomic

DNA of the isolate FP497 using the primers BDI and BDII are presented in Figure 4.

PCR with DNA from the rapid extraction procedure. DNA from boiling mycelial supernatant was suitable for PCR amplification. Using either 1:10 or 1:100 aliquot dilution of supernatant obtained by boiling 20 mg mycelia in 100 µl 1 M Tri-

HCl, the primers BDI and BDII yielded the expected amplification product (356 bp) for each of four tested *Fusicoccum* sp. isolates (Fig. 5).

Double-blind test. After 4 days of incubation on PDA, 10 tested isolates were very similar in colony morphology. However, the PCR assay showed 6 of 10 isolates to be *Fusicoccum* sp. These six iso-

Table 1. List of the isolates of *Fusicoccum* sp. and other fungal species isolated from pistachio and other hosts used in this study

Species	Isolate	Host	Location	PCR assay ^z
<i>Fusicoccum</i> sp.	MP1, MP2, MP3, MP4, MP14	Pistachio	Montgomery, Butte County	+
<i>Fusicoccum</i> sp.	CP2, CP3, CP5, CP6	Pistachio	Chico, Butte County	+
<i>Fusicoccum</i> sp.	HP7, HP8, HP10, HP13	Pistachio	Hansen, Glenn County	+
<i>Fusicoccum</i> sp.	SP 81, SP132, SP133, SP200	Pistachio	San Joaquin County	+
<i>Fusicoccum</i> sp.	KP 94, KP95, KP96, KP97	Pistachio	Kings County	+
<i>Fusicoccum</i> sp.	MAP103, MAP106, MAP108, MAP111	Pistachio	Madera County	+
<i>Fusicoccum</i> sp.	BP169, BP170, BP171, BP172	Pistachio	Butte County	+
<i>Fusicoccum</i> sp.	FP497, FP498, FP499, FP579, FP624, FP719	Pistachio	Fresno County	+
<i>Fusicoccum</i> sp.	TP565, TP566, TP567, TP764, TP766	Pistachio	Tulare County	+
<i>Fusicoccum</i> sp.	AGP116, AGP118, AGP123, AGP124	Pistachio	Aegina, Greece	–
<i>Fusicoccum</i> sp.	TGP128, TGP129, TGP130, TGP131	Pistachio	Thessaloniki, Greece	–
<i>Fusicoccum</i> sp.	AV8, AV10	Avocado	Riverside City	+
<i>Fusicoccum</i> sp.	CE347, CE348, CE403, CE412	Cedar	Fresno County	+
<i>Fusicoccum</i> sp.	EU353, EU356, EU402	Eucalyptus	Fresno County	+
<i>Fusicoccum</i> sp.	JA358	Jasmine	McCall, Fresno County	+
<i>Fusicoccum</i> sp.	JU38, JU39, JU40, JU394, JU395	Juniper	Clovis, Fresno County	+
<i>Fusicoccum</i> sp.	PE361	Pear	Stockton, San Joaquin County	+
<i>Fusicoccum</i> sp.	RA363	Raywood ash	Clovis, Fresno County	+
<i>Fusicoccum</i> sp.	RE276, RE279, RE365	Redwood	Reedley, Fresno County	+
<i>Fusicoccum</i> sp.	RE282, RE288	Redwood	Madera County	+
<i>Fusicoccum</i> sp.	RE289, RE290, RE291, RE292	Redwood	Hanford, Kings County	+
<i>Fusicoccum</i> sp.	WA370, WA372, WA373	Walnut	Fresno County	+
<i>Fusicoccum</i> sp.	WA371	Walnut	Los Banos, Madera County	+
<i>Fusicoccum</i> sp.	WA389	Walnut	San Joaquin County	+
<i>Fusicoccum</i> sp.	WA408, WA409	Walnut	Merced County	+
<i>Alternaria alternata</i>	CH1	Pistachio	Fresno County	–
<i>Aspergillus ochraceus</i>	2568	Pistachio	Colusa County	–
<i>A. parasiticus</i>	2535	Pistachio	San Joaquin County	–
<i>A. terreus</i>	2570	Pistachio	Colusa County	–
<i>Bipolaris spicifera</i>	CH2	Pistachio	Glenn County	–
<i>Botryosphaeria obtusa</i>	307	Pistachio	Fresno County	–
<i>B. obtusa</i>	308	Apple	Fresno County	–
<i>B. obtusa</i>	309	Apple	Fresno County	–
<i>B. obtusa</i>	312	Apple	San Joaquin County	–
<i>B. rhodina</i>	315	Pistachio	Stanislaus County	–
<i>Botrytis cinerea</i>	549	Pistachio	Fresno County	–
<i>Cladosporium cladosporioides</i>	1887	Pistachio	Fresno County	–
<i>Curvularia inaequalis</i>	CH3	Pistachio	Glenn County	–
<i>C. lunata</i>	CH4	Pistachio	San Joaquin County	–
<i>Chaetomium</i> sp.	CH5	Pistachio	Yolo County	–
<i>Drechslera biseptata</i>	CH6	Pistachio	San Joaquin County	–
<i>Emericella</i> sp.	2536	Pistachio	Glenn County	–
<i>Epicoccum purpurascens</i>	CH7	Pistachio	Glenn County	–
<i>Exserohilum rostrata</i>	CH22	Pistachio	Glenn County	–
<i>Fusarium acuminatum</i>	CH8	Pistachio	San Joaquin County	–
<i>F. culmorum</i>	CH9	Pistachio	Yolo County	–
<i>F. moniliforme</i>	CH10	Pistachio	Glenn County	–
<i>F. dimerum</i>	CH11	Pistachio	Glenn County	–
<i>Humicola grisea</i>	CH12	Pistachio	San Joaquin County	–
<i>Monilia</i> sp.	608	Pistachio	...	–
<i>Mucor</i> sp.	2541	Pistachio	Tulare County	–
<i>Nigrospora</i> sp.	CH13	Pistachio	Glenn County	–
<i>Paecilomyces lilacinus</i>	CH14	Pistachio	Yolo County	–
<i>Penicillium expansum</i>	CH15	Pistachio	San Joaquin County	–
<i>P. chrysogenum</i>	CH16	Pistachio	Glenn County	–
<i>Phomopsis</i> sp.	CH17	Pistachio	Yolo County	–
<i>Rhizomucor</i> sp.	1886	Pistachio	Kings County	–
<i>Stemphylium botryosum</i>	CH18	Pistachio	Glenn County	–
<i>Torula</i> sp.	CH19	Pistachio	San Joaquin County	–
<i>Trichoderma</i> sp.	CH20	Pistachio	Glenn County	–
<i>Ulocladium atrum</i>	CH21	Pistachio	San Joaquin County	–

^z + denotes that the primer pair BDI and BDII yielded the expected amplification product (356 bp) from the isolate, and – denotes that the primers did not amplify the product from the isolate.

lates were also confirmed as *Fusicoccum* sp. based on the characteristics (hyaline, nonseptate) of pycnidiospores produced on autoclaved pistachio leaves after 2 weeks of incubation at room temperature. The other four isolates were identified as *B. obtusa* based on pycnidiospore characteristics (dark, nonseptate spores).

Detection of *Fusicoccum* sp. from naturally infected pistachio leaves. Identification of *Fusicoccum* sp. from putative incipient infected pistachios based on PCR assay was in agreement with the morphological identification. After 4 days of incubation on APDA plates, mycelial cultures

were initiated from 16 of 20 leaf samples. PCR assay of 16 mycelial samples showed that 14 of 16 samples were identified as *Fusicoccum* sp. These 14 of 16 mycelial samples were further confirmed as *Fusicoccum* sp. based on the characteristics of pycnidiospores produced on autoclaved pistachio leaves after 2 weeks of incubation.

DISCUSSION

In this study, a pair of group-specific PCR primers, BDI and BDII, was developed based on ITS sequences for identification of *Fusicoccum* sp. from pistachio

and other host plants in California. The primers were capable of identifying all 73 tested *Fusicoccum* sp. isolates collected from pistachio and other hosts throughout California in different years (Table 1), but they did not amplify the expected DNA fragment from the eight isolates of *Fusicoccum* sp. obtained from pistachios in Greece. This result is further supported by the DNA fingerprinting tests, in which the eight isolates from Greece were significantly different from the isolates obtained from pistachio and other host plants in California (Fig. 2). Similar group-specific primers were also reported on other pathogens (7,10). Several distinct ITS groups have been reported for isolates of *Fusicoccum* sp. collected from different locations (5,9,21, Z. Ma and T. J. Michailides, unpublished data). Thus, it may be very difficult to develop a pair of PCR primers just based on ITS sequences to identify all the isolates of this pathogen worldwide.

The PCR-based technique has advantages over the traditional culture methods used for identification of *Fusicoccum* sp. Unlike culture methods that require experience in identification of *Fusicoccum* species, the PCR-based method is easy to learn and can provide reliable results, even when persons who conduct these tests have no previous experience with this pathogen. Additionally, the results of identification of *Fusicoccum* sp. based on morphological characteristics can be influenced strongly by the substrate on which the fungus was grown (3). However, the PCR-based identification should not be affected by the substrate on which the pathogen grows. Finally, the PCR-based identification of *Fusicoccum* sp. may be performed as soon as there is sufficient fungal material (10 mg fresh weight) without the need to wait 2 weeks for the formation of mature pycnidia and pycnidiospores. Thus, the PCR-based assay presented in this study should be a useful method for identification of *Fusicoccum* sp. from pistachio and other hosts in California, especially for those researchers who do not have enough experience with *Fusicoccum* species. Recently, this PCR-based technique has been used for identification of a number of isolates of *Fusicoccum* sp. collected from commercial pistachio orchards in various counties in California (N. Ntahimpera and T. J. Michailides, unpublished data).

Most PCR procedures used for detection of plant pathogens are based on lengthy DNA extraction protocols (6). In this study, we successfully extracted DNA for PCR amplification by boiling mycelia of *Fusicoccum* sp. The boiling extraction protocol worked with both pure and contaminated cultures. Similar extraction protocols have been reported on *Eutypa lata* (11), *Gremmeniella abietina* (8), and *Phoma tracheiphila* (18). In some cases, the boiling method was also used for extraction of DNA directly from infected

Fig. 2. Comparisons of DNA fingerprinting produced by microsatellite primer A, M13, B, T₃B, and C, (CTC)₄RC among isolates of *Fusicoccum* sp. collected from pistachio in California and Greece. M is a molecular weight marker (1-kb DNA ladder, Promega, Madison, WI).

Fig. 3. Specificity of the primer pair BDI and BDII, which amplified a 356-bp fragment from the genomic DNA of the isolates of *Fusicoccum* sp. collected from pistachio and other hosts throughout California. The primers did not amplify the expected polymerase chain reaction product from other fungal species isolated from pistachio and from the isolates of *Fusicoccum* sp. collected from pistachio trees in Greece (such as AGP116 and TGP130). M is a molecular weight marker (1-kb DNA ladder, Promega, Madison, WI).

plant tissues (8,11), although it did not work with latent infections on pistachio leaves in this study.

In this study, an attempt to use PCR assay for directly detecting latent infections of *Fusicoccum* sp. on pistachio tissue was not successful. This was probably because of inhibitors that normally affect PCR assays by inhibiting the *Taq* polymerase. The PCR inhibitors may have prevented the primer pair BDI and BDII from amplifying the fungal genomic DNA (10 pg) mixed with the DNA extracts of infected pistachio leaves, although the primers were sensitive enough to detect five pg of fungal genomic DNA suspended in water (Fig. 4). Reports have been made on inhibitors from other plant pathogens interfering with PCR assays (7,10,11,17,25). To overcome PCR inhibitors, we used: (i) the hexadecyltrimethylammonium bromide (CTAB) method, (ii) the BIO101 FastDNA Kit method, (iii) the method of boiling infected plant tissues (11), and (iv) the NaOH extraction method (23) to extract DNA from pistachio tissues with incipient infections by *Fusicoccum* sp. However, all these tests were unsuccessful.

Several studies showed that increase of fungal biomass by preincubation could overcome the effects of inhibitors (17,19,22). In this study, the PCR assay was also successful and reliable in identi-

fying the isolates of *Fusicoccum* sp. from naturally infected pistachio tissues after 4 days of preincubation on APDA. However, further experiments will be needed to improve the PCR assay so that the technique can directly detect *Fusicoccum* sp. on pistachio and other host plants in California.

ACKNOWLEDGMENTS

This research was supported in part by grants from California Pistachio Commission and the University of California BioSTAR Project. We thank Wei-qun Chen for providing some isolates of other fungal species recovered from pistachio, and George F. Drier for reviewing the manuscript prior to submission.

LITERATURE CITED

- Boehm, E. W. A., Ma, Z., and Michailides, T. J. 2001. Species-specific detection of *Monilinia fructicola* from California stone fruits and flowers. *Phytopathology* 91:428-439.
- Brown, E. A., II, and Britton, K. D. 1986. Botryosphaeria disease of apple and peach in the southeastern United States. *Plant Dis.* 70:480-484.
- Butin, H. 1993. Morphological adaptation and spore pleomorphism in the form-complex *Dichomera-Camarosporium* and *Fusicoccum-Dothiorella*. *Sydowia* 45:161-166.
- Cam, B. L., Devaux, M., and Parisi, L. 2001. Specific polymerase chain reaction identification of *Venturia nashicola* using internally transcribed spacer region in the ribosomal DNA. *Phytopathology* 91:900-904.
- Denman, S., Crous, P. W., Taylor, J. E., Kang, J., Pascoe, I., and Wingfield, M. J. 1999. An overview of the taxonomic history of *Bot-*

ryosphaeria and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. *Sydowia* 52:129-140.

- Faggian, R., Bulman, S. R., Lawrie, A. C., and Porter, I. J. 1999. Specific polymerase chain reaction primers for the detection of *Plasmodiophora brassicae* in soil and water. *Phytopathology* 89:392-397.
- Guzmán, P., Gepts, P., Temple, S., Mkan-dawire, A. B. C., and Gilbertson, R. L. 1999. Detection and differentiation of *Phaeoisariopsis griseola* isolates with the polymerase chain reaction and group-specific primers. *Plant Dis.* 83:37-42.
- Hamelin, R. C., Bourassa, M., Rail, J., Dusa-benyagasan, M., Jacobi, V., and Laflamme, G. 2000. PCR detection of *Gremmeniella abietina*, the causal agent of Scleroderis canker of pine. *Mycol. Res.* 104:527-532.
- Jacobs, K. A., and Rehner, S. A. 1998. Comparisons of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. *Mycologia* 90:601-610.
- Kageyama, K., Ohya, A., and Hyakumachi, M. 1997. Detection of *Pythium ultimum* using polymerase chain reaction with group-specific primers. *Plant Dis.* 81:1155-1160.
- Lecome, P., Péros, J. P., Blancard, D., Bastien, N., and Délye, C. 2000. PCR assays that identify the grapevine dieback fungus *Eutypa lata*. *Appl. Environ. Microbiol.* 66:4475-4480.
- Ma, Z., Boehm, E. W. A., Luo, Y., and Michailides, T. J. 2001. Population structure of *Botryosphaeria dothidea* from pistachio and other hosts in California. *Phytopathology* 91:665-672.
- Ma, Z., Morgan, D. P., and Michailides, T. J. 2001. Effects of water stress on *Botryosphaeria* blight of pistachio caused by *Botryosphaeria dothidea*. *Plant Dis.* 85:745-749.
- Michailides, T. J. 1991. Pathogenicity, distribution, sources of inoculum, and infection courts of *Botryosphaeria dothidea* on pistachio. *Phytopathology* 81:566-573.
- Michailides, T. J., Morgan, D. P., and Felts, D. 2001. Collection and characterization of *Botryosphaeria dothidea* from various hosts and pathogenicity studies on pistachio. *Kearney Agricultural Center Plant Protection Quarterly* 11 (1):3-8.
- Plyler, T. R., Simone, G. W., Fernandez, D., and Kistler, H. C. 1999. Rapid detection of the *Fusarium oxysporum* lineage containing the Canary Island date palm with pathogen. *Phytopathology* 89:407-413.
- Pryor, B., and Gilbertson, R. L. 2001. A PCR-based assay for detection of *Alternaria radicina* on carrot seed. *Plant Dis.* 85:18-23.
- Rollo, F., Salvi, R., and Torchia, P. 1990. Highly sensitive and fast detection of *Phoma tracheiphila* by polymerase chain reaction. *Appl. Microbiol. Biotechnol.* 32:572-576.
- Schaad, N. W., Cheong, S. S., Tamaki, S., Hatziloukas, E., Panopoulos, N. J. 1995. A combined biological and enzymatic amplification (BIO-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. *Phytopathology* 85:243-248.
- Smith, D. R., Michailides, T. J., and Stanosz, G. R. 2001. Differentiation of a *Fusicoccum* species causing panicle and shoot blight on California pistachio trees from *Botryosphaeria dothidea*. *Plant Dis.* 85:1235-1240.
- Smith, H., Crous, P. W., Wingfield, M. J., Coutinho, T. A. and Wingfield B. D. 2001. *Botryosphaeria eucalyptorum* sp. nov., a new species in the *B. dothidea*-complex on *Eucalyptus* in South Africa. *Mycologia* 93:277-285.
- Taylor, J. L. 1993. A simple, sensitive, and rapid method for detection of seed contaminated with highly virulent *Leptosphaeria*

Fig. 4. Sensitivity of the primer pair BDI and BDII. Polymerase chain reaction amplification of serial amounts of genomic DNA (50 fg to 5 ng) from the *Fusicoccum* sp. isolate FP497 collected from pistachio in California. M is a molecular weight marker (1-kb DNA ladder, Promega, Madison, WI).

Fig. 5. PCR amplification of genomic DNA of four pistachio isolates (FP498, FP579, FP624, and FP719) of *Fusicoccum* sp. extracted by **A**, boiling mycelia (1:10 or 1:100 supernatant dilutions), and **B**, FastDNA Kit (BIO 101, Vista, CA) (1 µl of DNA extraction per reaction). M is a molecular weight marker (1-kb DNA ladder, Promega, Madison, WI).

- maculans*. Appl. Environ. Microbiol. 59:3681-3685.
23. Wang, H., Meiqing, Q., and Cutler, A. J. 1993. A simple method of preparing plant samples for PCR. Nucleic Acids Res. 21:4153-4154.
 24. White, T. J., Bruns, T., Lee S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego.
 25. Zhang, A. W., Hartman, G. L., Ricconi, L., Chen, W. D., Ma, R. Z., and Pedersen, W. L. 1997. Using PCR to Distinguish *Diaporthe phaseolorum* and *Phomopsis longicolla* from other soybean fungal pathogens and to detect them in soybean tissues. Plant Dis. 81:1143-1149.