Identification and Characterization of Benomyl-Resistant and -Sensitive Populations of *Colletotrichum gloeosporioides* from Statice (*Limonium* spp.)

Marcel Maymon, Aida Zveibil, Shimon Pivonia, Dror Minz, and Stanley Freeman

First, second, and fifth authors: Department of Plant Pathology, and fourth author: Institute of Soil, Water, and Environmental Sciences, ARO, The Volcani Center, Bet Dagan 50250, Israel; and third author: Arava R&D, Sapir, 868252, Israel.
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ABSTRACT

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Sixty-four isolates of *Colletotrichum gloeosporioides* were isolated from infected *Limonium* spp. cultivated in 12 different locations in Israel. All isolates were identified as belonging to the *C. gloeosporioides* complex by species-specific primers. Of these isolates, 46 were resistant to benomyl at 10 μg/ml and 18 were sensitive to this concentration of fungicide. Based on arbitrarily primed polymerase chain reaction of all isolates and internal transcribed spacer-1 sequence analyses of 12 selected isolates, the benomyl-resistant and -sensitive populations belong to two distinct genotypes. Sequence analyses of the β-tubulin genes, *TUB1* and *TUB2*, of five sensitive and five resistant representative isolates of *C. gloeosporioides* from *Limonium* spp. revealed that the benomyl-resistant isolates had an alanine substitute instead of a glutamic acid at position 198 in *TUB2*. All data suggest that the resistant and sensitive genotypes are two independent and separate populations. Because all *Limonium* plant propagation material is imported from various geographic regions worldwide, and benomyl is not applied to this crop or for the control of *Colletotrichum* spp. in Israel, it is presumed that plants are bearing quiescent infections from the points of origin prior to arrival.

Additional keyword: anthracnose.

Many species of the herbaceous ornamental perennial genus *Limonium* (Plumbaginaceae Juss.) are native to the Mediterranean region. However, due to breeders' propriety rights, plants for commercial propagation and production are imported to Israel from Europe, North America, or Australasia without a documented history of cultivation treatments. During the last decade, two main hybrids have been cultivated in commercial production in the Arava and Negev desert regions: *Limonium latifolium* × *L. caspium* (cv. Beltlaard) and *L. latifolium* × *L. bellidifolium* (cvs. Supreme, Misty, and Sunglow), that are grown primarily for export to Europe as cut flowers for floral bouquets.

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. in Penz. is one of the most destructive diseases of *Limonium* spp. (12,25, 44), causing typical anthracnose symptoms (leaf, stem, and flower spot) and a crown rot that may result in 100% yield loss in the field (13). Since 1995, the crop has declined in Israel due to a wilt and death caused by *C. gloeosporioides* (4,39). Propagation material may be the source of the infection because the pathogen can occur in nurseries, on young seedlings in seedbeds, and in other seedling production areas (12).

In the genus *Colletotrichum*, it is common to find multiple hosts affected by a single species and multiple species infecting a single host (15). For example, *C. gloeosporioides*, known to be a complex species, has been reported on many hosts besides *Limonium* spp., such as apple, avocado, citrus, mango, passion fruit, strawberry, and others (1,2,16,23,38). Likewise, it has been reported that several *Colletotrichum* spp. or biotypes can be associated with a single host, such as avocado, citrus, coffee, cucurbits, mango, pepper, strawberry, and tomato (1,2,5,10,14–16,19,21). Although *C. gloeosporioides* is known to affect species of *Limonium* grown in different regions worldwide (e.g., Florida [12,44], Japan [25,41], and South Korea [34]), due to the heterogeneity and wide host range of this species, as mentioned above, various genotypes and even different *Colletotrichum* spp. may be responsible for the disease in this crop.

It usually is difficult to control anthracnose in established *Limonium* plantings in the field (12); however, effective control of *C. gloeosporioides* in *Limonium* spp. has been achieved with a benomyl foliar spray (44). Benomyl resistance, however, is common in fungi, especially after multiple applications (3,9,27, 30,47). This fungicide, currently and in the past, has not been applied in field cultivation of *Limonium* spp. in Israel. To evaluate the in vitro sensitivity of selected isolates from Israel against benomyl, to support a possible registration of this fungicide, a preliminary screening for benomyl sensitivity was conducted (*unpublished data*). Surprisingly, it was found that the majority (approximately 75%) of isolates were resistant to benomyl. Fungal resistance to benomyl typically is associated with point mutations in a β-tubulin gene that changes the amino acid sequence at the benzimidazole binding site (27). Codon changes reported are at positions 50, as in *Cladobotryum dendroides* (32); 198 and 200, as in *Tapesia* spp. (3) (e.g., in *Venturia inaequalis* [27], *Botrytis cinerea* [28,47], and *Penicillium* spp. [27]); or at positions 240, as in *Tapesia* spp. (3). Two β-tubulin genes have been described for some *Colletotrichum* spp. (6,23,37,38). In *C. graminicola* infecting maize and in *C. gloeosporioides* f. sp. *aeschynomene* affecting Northern jointvetch (*Aeschynomene virginica* (L.)), the two genes, *TUB1* and *TUB2*, have only 73 and 69% sequence identity, respectively (6,37). A point mutation in *TUB2* that results in a substitution of a glutamic acid for a lysine at amino acid 198 confers benomyl resistance in both species (6,37).

Corresponding author: S. Freeman; E-mail address: freeman@volcani.agri.gov.il

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In light of our findings that the majority of *C. gloeosporioides* isolates from *Limonium* spp. were resistant to benomyl, the objectives of this study were to (i) characterize the benomyl-resistant and -sensitive populations and determine whether multiple genotypes of *C. gloeosporioides* are associated with propagation material and (ii) describe the molecular genetic basis for benomyl resistance among isolates of *C. gloeosporioides* from diseased *Limonium* spp. in Israel.

MATERIALS AND METHODS

Fungal strains, growth conditions, and plant material. We used previously characterized reference isolates of *C. acutatum* from strawberry (CA-TUT-149) and anemone (CA-ANE-HV83) and of *C. gloeosporioides* from avocado (CG-AVO-37-4B) (16,17), and 64 monoconidial isolates of *C. gloeosporioides* from *Limonium* spp. (Table 1). The cultures of *C. gloeosporioides* were isolated arbitrarily from wilted plants of four susceptible cultivars (Misty, Beltlaard, Sunglow, and Supreme) at 12 farm locations in the Arava and Negev desert regions of southern Israel from 1997 to 2003. Cvs. Beltlaard, Misty, and Supreme originated from various distributors in Japan, India, South Korea, and the Netherlands, whereas cv. Sunglow was propagated by Ben Zur Nurseries and Laboratory Ltd., Tirat Yehuda, Israel.

Infected crowns and stems of *Limonium* spp. were surface disinfested for 3 min (30% of dilute 0.03% household bleach) and plated on modified Mathur's medium (MS; 0.1% yeast extract, 0.1% bactopeptone, 1% sucrose, 0.25% $MgSO_4 \cdot 7H_2O$, 0.27% KH_2PO_4, 2% agar) supplemented with iprodione (Rovral 50WP; Rhone Poulenc, Lyon, France) at 2.5 µg/ml and acidified with 0.1% lactic acid to select for *Colletotrichum* and to suppress growth of fungal and bacterial contaminants (14). Cultures were grown on MS medium and maintained at 25°C in the dark. All cultures of *Colletotrichum* spp. from *Limonium* spp. were screened for sensitivity to benomyl (Benlate 50WP; DuPont, Wilmington, DE) at 1.0 and 10 µg/ml. Benomyl-resistant strains were grown on MS medium supplemented with the fungicide at 10 µg/ml by placing a freshly cultured disk of the mycelium of each isolate in the center of 9-cm plates containing amended medium. Subsequently, resistant isolates were maintained on MS medium supplemented with benomyl at 10 µg/ml for three to four consecutive transfers to verify resistance stability. Selected *C. gloeosporioides* isolates from *Limonium* spp. resistant (L11, L50, and LP3) and sensitive (L38, L46, and P1) to benomyl were thoroughly characterized morphologically in this study. For determination of size, 20 conidia of each isolate were selected per microscopic field on MS medium, and their average measurements were calculated.

Isolation and purification of fungal DNA. Fungal isolates were grown in 100 ml of potato dextrose broth (PDB) at 24°C for 7 days. The mycelium was collected by vacuum filtration, lyophilized, and extracted and purified as previously described (18). Purified DNA was dissolved in 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to an approximate concentration of 200 to 500 µg/ml and diluted to a final concentration of 10 to 100 ng/µl for polymerase chain reaction (PCR).

PCR amplification. For arbitrarily primed (ap)-PCR, the following primers that were derived from microsatellite or repeat sequences were used: $(CAG)_5$ (40), $(AGG)_5$ (22), and $(GACA)_4$ (45). PCR primers for the *C. gloeosporioides*-specific diagnostic amplification were the universal internal transcribed spacer (ITS)-4 primer (5′-TCCTCCGCTTATTGATATGC-3′) coupled with specific primer *Cg*Int (5′-GGCCTCCCGCCTCCGGGCGG-3′) (5). For amplification of the ITS-1 and ITS-2 regions between the small and large nuclear rDNA of seven selected benomyl-resistant (L11, L32, L50, L60, CG2, CG-L-P2, and CG-L-P3) and five benomyl -sensitive isolates (L28, L38, L46, L66, and P1), the universal primers ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS-4 were used (16,17). PCR reactions were performed in a total volume of 20 µl, containing 10 to 100 ng of DNA, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 1.5 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (Promega Corp., Madison, WI), and 1 µM each primer. PCR reactions were incubated in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA) starting with 5 min of denaturation at 95°C. For ap-PCR, this was followed by 30 cycles consisting of 30 s at 95°C, 30 s at either 60°C (for $(CAG)_5$ and $(AGG)_5$) or 48°C (for $(GACA)_4$), and 90 s at 72°C. *C. gloeosporioides*-specific PCR amplifications were performed under reaction conditions for primer $(CAG)_5$, with 0.5 µM primer ITS-4 and 0.5 µM primer *Cg*Int. For rDNA amplification, denaturation was followed by 40 cycles consisting of 30 s at 95°C, 30 s at 50°C, and 90 s at 72°C.

Amplification and subsequent sequencing of the DNA fragment of the β-tubulin gene *TUB2* known to harbor mutation sites conferring benomyl resistance, from representatives of the benomyl-resistant isolates (L11, L32, L50, L60, and CG2), was performed with primer pair GENC (5′-GAGGAATTCCCAGACCGTATGATG-3′) (27) and TUB2 (5′-GACATCCTTCATAGCG-3′) (27)

TABLE 1. GenBank accession numbers of the complete rDNA internal transcribed spacers (ITS) 1-2 fragment and partially sequenced *TUB1* and *TUB2* β-tubulin genes of benomyl-resistant (R) and benomyl-sensitive (S) *Colletotrichum gloeosporioides* isolates from *Limonium* spp. and other reference strains, and arbitrarily primed polymerase chain reaction (ap-PCR) groupings of isolates used in this study[a]

Isolate	ITS 1-2	*TUB1*	*TUB2*	ap-PCR group
L11 (R)[b]	DQ185438	DQ084499	DQ084509	1
L32 (R)[b]	DQ185441	DQ084500	DQ084510	1
L50 (R)[b]	DQ084495	DQ084501	DQ084511	1
L60 (R)[b]	DQ084496	DQ084502	DQ084512	1
CG2 (R)[b]	DQ185439	DQ084503	DQ084513	1
P1 (S)[c]	DQ084498	DQ084504	DQ084514	2
L28 (S)[c]	DQ185440	DQ084505	DQ084515	2
L38 (S)[c]	DQ084497	DQ084506	DQ084516	2
L46 (S)[c]	DQ185442	DQ084507	DQ084517	2
L66 (S)[c]	DQ185443	DQ084508	DQ084518	2
CG-L-P2 (R)	DQ185444	nd	nd	3
CG-L-P3 (R)[d]	DQ185445	nd	nd	4
C. graminicola (37)	nd	M34491	M34492	nd
C. gloeosporioides f. sp. *aeschynomene* (6)	nd	M90977	U14138	nd

[a] S and R = fungal sensitivity or resistance, respectively. Growth of isolates exhibiting resistance was not inhibited on media containing benomyl at 50 µg/ml; nd = not determined.

[b] Thirty-eight additional R isolates from the same ap-PCR group: L12, L13, L15, L16, L17, L18, L19, L21, L22, L23, L24, L25, L26, L27, L29, L30, L31, L33, L34, L35, L36, L41, L42, L43, L48, L49, L51, L52, L53, L54, L55, L56, L57, L58, L59, L61, L62, and L63.

[c] Eighteen additional S isolates from the same ap-PCR group: L14, L20, L37, L39, L40, L44, L47, L64, L65, L67, L68, L69, and L70.

[d] One additional R isolate in the same ap-PCR group: CG2.

and from representatives of benomyl-sensitive isolates (L28, L38, L46, L66, and P1) using primer pair GENC and TUB2B (5′-GAC(A/G)TCCTTCAT(A/G)GCGA-3′) based on specific sequences aligned with the intron of this gene from *C. gloeosporioides* f. sp. *aeschynomene* (*unpublished data*) and *C. graminicola* (37). A portion of the *TUB1* gene was amplified by PCR with primers GENC and TUB 1C (5′-TCAATCTGCTTGGTCGACACCTT-3′) (*unpublished data*). PCR reactions were performed in a total volume of 20 µl, containing 10 to 100 ng of DNA, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 1.5 mM $MgCl_2$, 1 unit Deep Vent DNA polymerase (New England Biolabs, Beverly, MA), and 0.5 µM each primer pair. The reactions were incubated in a T-Gradient Biometra thermocycler (Göttingen, Germany) starting with 60 s of denaturation at 94°C; followed by 35 cycles consisting of 30 s at 94°C, 30 s at 50°C, and 60 s at 72°C; and a final extension period of 10 min at 72°C. Amplification products were separated in 1.5% agarose gels in Tris-acetate-EDTA buffer (42) at 8 V/cm for 2 h. All ap-PCR experiments were repeated at least four times. Variation in the ap-PCR banding patterns was interpreted visually.

DNA sequencing and sequence analysis. PCR-amplified β-tubulin and rDNA gene products were extracted from agarose gels with a Jetsorb kit (Genomed GmbH, Löhne, Germany). The Big Dye Terminator DNA sequencing kit (Perkin-Elmer Inc., Branchburg, NJ) and an ABI prism 377 DNA sequencer (Applied Biosystem Inc., Foster City, CA) at the sequencing unit of the Hebrew University of Jerusalem were used to sequence the DNA fragments. Identity of the sequenced fragments with β-tubulin genes from *C. gloeosporioides* f. sp. *aeschynomene* and from *C. graminicola* was determined in a BLAST-X search and alignments were made with the CLUSTAL W program. The percent similarity between fragments of the different isolates was compared by Genedoc 2.5.

Analyses of ITS-1 sequences (from seven benomyl-resistant and five benomyl-sensitive isolates) were carried out using the ARB program package (29). Alignment of sequences was performed with the ARB automated alignment tool and alignments were refined manually. Phylogenetic analyses were performed by applying ARB parsimony, distance matrix, and maximum-likelihood methods. Accession numbers of the ITS-1 fragments and partial *TUB1* and *TUB2* genes of *C. gloeosporioides* isolates from *Limonium* spp. used in this study have been submitted to GenBank and are listed in Table 1. Additional ITS-1 sequences of *Colletotrichum* spp., retrieved from the GenBank, are included in this study for comparison (Table 1).

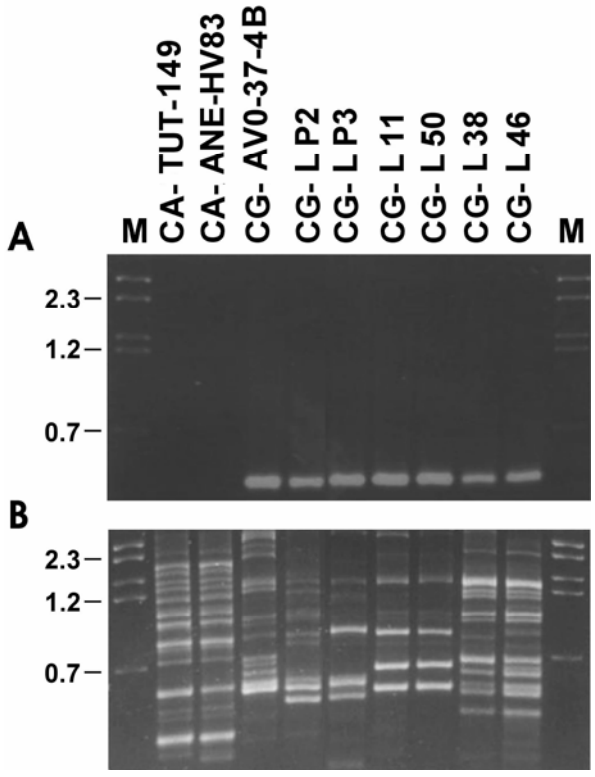

Fig. 1. *Colletotrichum gloeosporioides*-specific **A,** amplification and **B,** band patterns of arbitrarily primed polymerase chain reaction using primer $(AGG)_5$ of genomic DNA from representatives of *C. acutatum* isolates from strawberry (CA-TUT-149) and anemone (CA-ANE-HV83), *C. gloeosporioides* isolate from avocado (CG-AVO 37-4B), and selected *C. gloeosporioides* isolates from *Limonium* spp. resistant (CG-L-P2, CG-L-P3, CG-L11, and CG-L50) and sensitive (CG-L38 and CG-L46) to benomyl. Lane M: DNA markers with sizes in kilobases.

RESULTS

Detection of benomyl-resistance and morphological characterization of *Colletotrichum* isolates. Resistant isolates of *C. gloeosporioides* from *Limonium* spp. (46 in number) grew at a similar rate on benomyl-amended MS medium compared with sensitive isolates (18 in number) cultured on nonsupplemented medium, whereas sensitive isolates did not grow at benomyl concentrations of either 1.0 or 10 µg/ml. The selected isolates resistant (L11, L50, and LP3) and sensitive (L38, L46, and P1) to benomyl were identified and characterized within the *C. gloeosporioides* species aggregate by morphological criteria. Conidia from the resistant and sensitive populations were typical for *C. gloeosporioides* and could not be differentiated; they were all oblong, possessed cylindrical, rounded ends, and measured 11.0 to 18.0 by 5.0 to 7.0 µm. Setae, typical for *C. fragariae*, were not present in any of the colonies.

Species-specific primer amplification, ap-PCR, and ITS sequence analyses of *Colletotrichum* spp. isolates. *C. gloeosporioides*-specific amplification was conducted on all 64 isolates of *Colletotrichum* from *Limonium* spp., and 3 additional representative isolates (TUT-149, *C. acutatum* from strawberry; ANE-HV83, *C. acutatum* from anemone; and AVO-37-4B, *C. gloeosporioides* from avocado) to verify species identification (Table 1). The *C. gloeosporioides*-specific *Cg*Int primer in conjunction with the ITS-4 primer amplified a 545-bp fragment from DNA of the representative isolate of *C. gloeosporioides* from avocado (AVO-37-4B), and from all isolates of *C. gloeosporioides* from *Limonium* spp. used in this study; however, no reaction was evident with DNA of two *C. acutatum* isolates, TUT-149 from strawberry or ANE-HV83 from anemone (Fig. 1A).

Genomic DNA from all isolates included in the species-specific analysis was amplified by ap-PCR using primers $(AGG)_5$ (Fig. 1B), $(CAG)_5$, and $(GACA)_4$. Ap-PCR using all three primers consistently showed that the 43 benomyl-resistant and 18 benomyl-sensitive isolates of *C. gloeosporioides* from *Limonium* spp. belonged to two distinct genotypes (Table 1). Three additional resistant isolates produced distinct banding patterns and were assigned to groups 3 (isolates CG-LP2 and CG2) and 4 (isolate CG-LP3) (Table 1). Ap-PCR also distinguished between representative isolates of the different genotypes of *C. acutatum* and *C. gloeosporioides* from avocado.

ITS-1 sequence analysis was performed on representative isolates of ap-PCR groups 1 to 4 of *C. gloeosporioides* from *Limonium* spp. and additional representatives of the genus (Fig. 2). Sequence was identical among each of the representatives of group 1 and group 2. Isolates of group 4 were identical to group 1, whereas isolate CG-LP2 of group 3 differed from groups 1 and 4 by one base pair change. Phylogenetic analyses based on parsimony, distance matrix, or maximum-likelihood methods produced similar tree topologies. Sequence analysis confirmed species identification for all isolates from *Limonium* spp. as *C. gloeosporioides*; however, resistant and sensitive isolates were

grouped within separate clusters (Fig. 2). Thus, the resistant population was placed within a cluster containing isolates of *Glomerella cingulata* and *C. gloeosporioides* f. sp. *aeschynomene*, whereas the sensitive population grouped with a cluster closely related to the species *C. fragariae* and *G. cingulata* that was closely linked to another cluster containing isolates of *C. musae* (Fig. 2). Sequence analysis indicated an identity of 96.9% between the benomyl-resistant and -sensitive populations. However, 100% identity was evident among specific reference isolates of *Colletotrichum* spp. and the two clusters with *Limonium* spp.

Characterization of the *TUB2* β-tubulin gene from benomyl-resistant and -sensitive isolates of *C. gloeosporioides*. Partial *TUB2* gene sequences from representatives of resistant and sensitive isolates of *C. gloeosporioides* from *Limonium* spp. were 563 bp in length. The deduced amino acid sequence of *TUB2* from the five sensitive *C. gloeosporioides* isolates from *Limonium* spp. was identical to that of the sensitive *C. gloeosporioides* f. sp. *aeschynomene* from *A. virginica* (L.) (Northern jointvetch) and *C. graminicola* isolates (*data not shown*). The common single-base-pair mutation that changes codon 198 from GAG (glutamic acid) to GCG (alanine), rendering benomyl resistance, was observed in all resistant isolates of *C. gloeosporioides* from *Limonium* spp. (Fig. 3). Significant differences in similarity for the sequenced *TUB2* fragment also were observed among the different populations (Table 2). The resistant isolates from *Limonium* spp. were more similar in sequence to the sensitive *C. gloeosporioides* f. sp. *aeschynomene* (98.6% similarity) than to the sensitive population from *Limonium* spp. (93.1% similarity), indicating that this may be a different species (Table 2).

Characterization of the *TUB1* β-tubulin gene from benomyl-resistant and -sensitive isolates of *C. gloeosporioides*. Amplified fragments of the *TUB1* gene from both resistant and sensitive isolates of *C. gloeosporioides* from *Limonium* spp. were 506 bp in size, based on sequence analysis. Sequences were compared with *TUB1* β-tubulin genes from other isolates within each population and between isolates of *C. gloeosporioides* f. sp. *aeschynomene* and *C. graminicola* (Table 2). Within the resistant genotype, all *TUB1* sequences were identical, as were those within the sensitive isolates. Similarity of *TUB1* fragments between benomyl-resistant and -sensitive isolates of *C. gloeosporioides* from *Limonium* spp. or *C. gloeosporioides* f. sp. *aeschynomene* isolates was 92.5 and 92.7%, respectively (Table 2).

DISCUSSION

Some species of *Colletotrichum* can be identified reliably using specific PCR primers (1,16,17). In this study, amplifications using species-specific primers indicated that all isolates of *Colletotrichum* affecting *Limonium* spp. in Israel belonged to *C. gloeosporioides*, although *C. fragariae* seems to be difficult to separate from *C. gloeosporioides* based only on this criterion. However, because this is a complex species including host-specific isolates that may be classified to the level of forma specialis, additional methods, such as morphological characters, are required to distinguish between genotypes within *C. gloeosporioides* (1,2,8,15). According to morphological criteria, conidia of selected isolates from both benomyl-resistant and -sensitive populations were not borne on setae and possessed dimensions typical for *C. gloeosporioides.* In contrast, *C. fragariae* conidia are obovate, tapered to a truncate base, and are borne on setae (1,21).

By using ap-PCR, two distinct genotypes corresponding to the benomyl-resistant and -sensitive populations from *Limonium* spp. were identified. Heterogeneity is common among populations of *C. gloeosporioides*, especially those possessing the teleomorphic stage (1,8,15,34). Therefore, the discovery of the perfect stage of *C. gloeosporioides*, *G. cingulata*, on naturally infected *Limonium* spp. in Israel (4), Japan (25), and Korea (34) may be an additional factor contributing to genetic variability between the resistant and sensitive populations.

The benomyl-resistant and -sensitive populations of *C. gloeosporioides* from *Limonium* spp. were further characterized by their ITS-1 sequence diversity. Our data indicated that the resistant and sensitive genotypes shared 96.9% similarity. In previous studies, it was shown that an ITS-1 sequence similarity between 98.9 to 100% existed among isolates of *C. gloeosporioides* from different hosts, such as avocado, apple, mango, and strawberry (1,13,14). This indicates that the two *Limonium* populations are as diverse as genotypes from various different hosts and suggests that these populations may be distinct species, even though these findings were based on the relatively conserved ITS regions. ITS-1 sequences from the resistant population were identical to that of an isolate of *C. gloeosporioides* f. sp. *aeschynomene*, a species specific to the Northern jointvetch (*A. virginica*), whereas sequences of the sensitive population were identical to two isolates of *C. fragariae*, a pathogen specific to strawberry. This further indicates that the *C. gloeosporioides* populations affecting *Limonium* spp. are diverse, although conidial and setae morphology did not support these findings. In addition, analysis of partial sequences of the two β-tubulin genes corroborated that these popu-

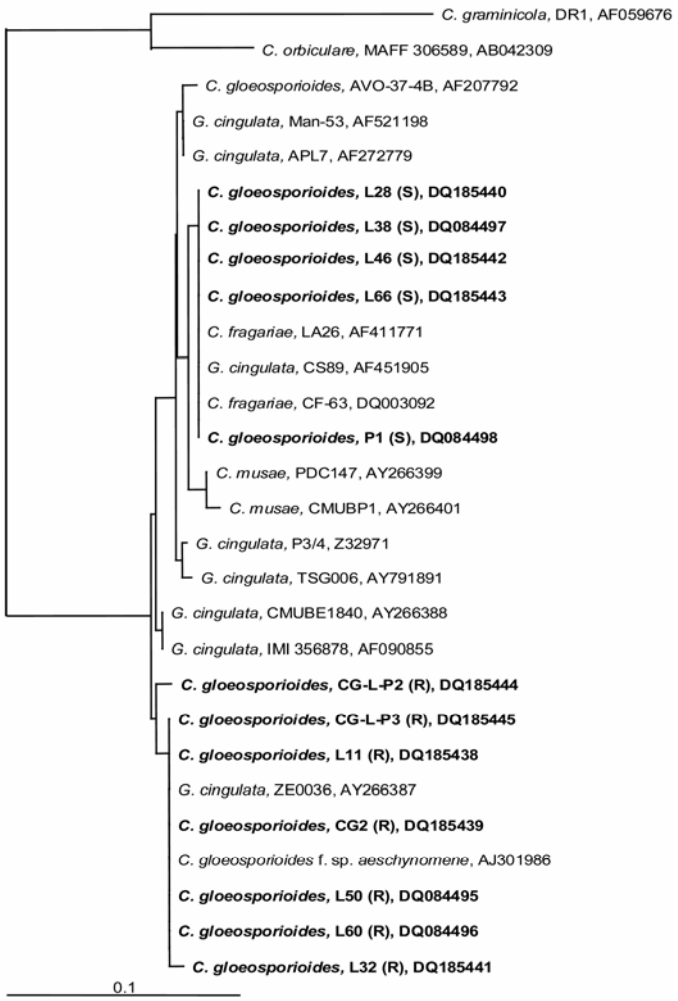

Fig. 2. Internal transcribed spacer-1-based phylogenetic tree of *Colletotrichum* isolates used in this study. Benomyl-resistant (R) and -sensitive (S) isolates of *Colletotrichum gloeosporioides* from *Limonium* spp., including respective accession numbers, appear in bold print. Accession numbers of additional *Colletotrichum* sequences retrieved from the GenBank that are closely related to those of *C. gloeosporioides* isolates from *Limonium* spp. are included for comparison. The tree was produced using the neighbor-joining algorithm (29). The order of branching was similar in all tree construction approaches used. Scale bar indicates estimated 10% sequence divergence.

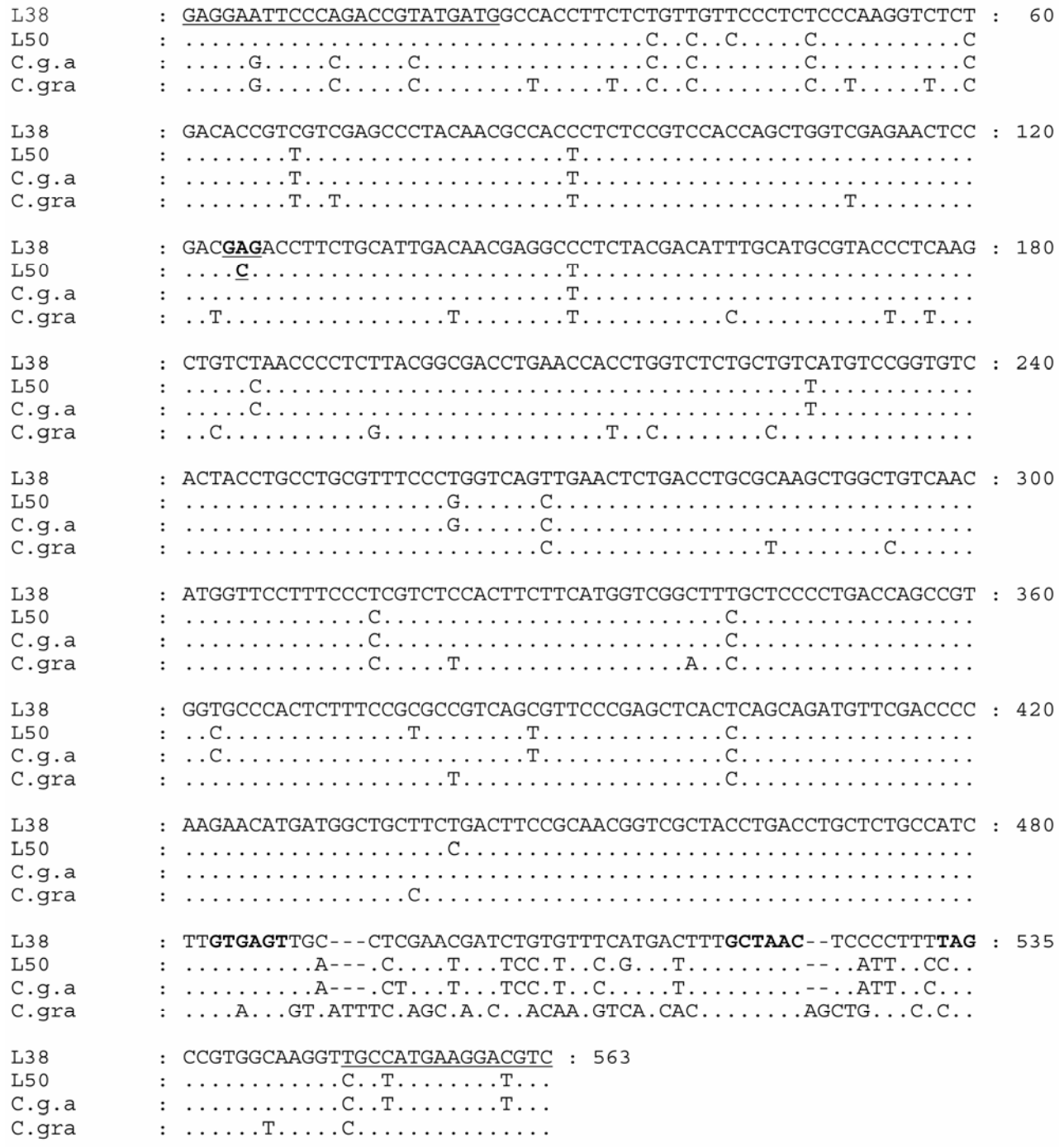

Fig. 3. Partial nucleotide sequence of the β-tubulin *TUB2* gene from different isolates of *Colletotrichum*: L38 of *Colletotrichum gloeosporioides* from *Limonium* spp., sensitive to benomyl; L50 of *C. gloeosporioides* from *Limonium* spp., naturally resistant to benomyl; *C. gloeosporioides* f. sp. *aeschynomene* (C.g.a) from *Aeschynomene virginica*, sensitive to benomyl (6); and *C. graminicola* (C.gra) from corn, sensitive to benomyl (37). The respective conversion in codon 198, from GAG (glutamic acid) to GCG (alanine), denoting the sensitive (L38) as opposed to resistant (L50) isolates of *C. gloeosporioides* from *Limonium* spp., is underlined and in bold. Intron sequences are in bold and primer sequences (GEN C, forward and TUB 2B, reverse) are underlined.

lations are uniquely different; namely, that the resistant population is more closely related to *C. gloeosporioides* f. sp. *aeschynomene* than to the sensitive population. Furthermore, in a preliminary study, it was shown that the resistant and sensitive populations belong to two distinct vegetative compatibility groups (4).

Cross-infection potential exists between certain groups of *C. gloeosporioides* on a variety of plants, and different species can infect a single host (1,2,19). This indicates that the potential exists for different genotypes of *C. gloeosporioides* to infect a single host, such as *Limonium* spp.; however, natural cross-infections in the field need to be assessed.

The majority (≈75%) of isolates from diseased statice in Israel were resistant to the fungicide benomyl. Resistance to benzimidazole fungicides has been reported in many filamentous fungi, whereby the fungicide is unable to bind to a mutated β-tubulin protein containing an altered amino acid sequence (11). In the majority of cases studied, a single β-tubulin gene exists within the fungal genome; however, in others, an additional gene may be present that is divergent in sequence. Single copies of the gene, responsible for benomyl resistance when mutated, have been reported in *Saccharomyces cerevisiae* (35), *Septoria nodorum* (9), *Monilinia fructicola* (30,43), *Gibberella pulicaris* (26), *Neuro-*

TABLE 2. Matrix of similarity (%) between isolates of *Colletotrichum* spp. based on sequence comparisons of fragments of the *TUB2* and *TUB1* β-tubulin genes[a]

Isolate	*TUB2/TUB1* genes			
	L38 (S)	L50 (R)	*C. gloeosporioides* f. sp. *aeschynomene* (S)	*C. graminicola* (S)
L38 (S)	…	…	…	…
L50 (R)	93.1/92.5	…	…	…
C. gloeosporioides f. sp. *aeschynomene* (S)	94.0/90.5	98.6/92.7	…	…
C. graminicola (S)	87.3/74.5	86.8/74.5	87.3/73.7	…

[a] Isolates shown: L38, *Colletotrichum gloeosporioides* from *Limonium* spp., sensitive to benomyl (S); L50, *C. gloeosporioides* from *Limonium* spp., resistant to benomyl (R); *C. gloeosporioides* f. sp. *aeschynomene* from *Aeschynomene virginica* (S); and *C. graminicola* from corn (S).

spora crassa (36), *Venturia inaequalis* (27), *Botrytis cinerea* (47), *Tapesia* spp. (3), and other fungi (7). The presence of two such genes has been reported in only a few fungi: *Aspergillus nidulans* (24,31), *Trichoderma* spp. (20,33), and *Colletotrichum* spp. (6,37). In *C. graminicola* (37) and *C. gloeosporioides* f. sp. *aeschynomene* (6), the two β-tubulin genes, *TUB1* and *TUB2*, are highly divergent. Similar results were obtained in this study when comparing the two partial gene sequences from benomyl-resistant and -sensitive isolates of *C. gloeosporioides* from *Limonium* spp., further indicating that these two populations may be different species. Of the two β-tubulin genes in *Colletotrichum*, the *TUB2* fragment was more closely related in sequence to *B. cinerea benA* and β-tubulin genes from other fungi than to that of *TUB1* (6,37,46).

Analysis of the β-tubulin *TUB2* region that is responsible for benomyl resistance showed that the typical single-base-pair mutation converting codon 198 from glutamic acid to alanine (27,47) was observed in the resistant but not the sensitive isolates of *C. gloeosporioides* from *Limonium* spp. (Fig. 3). In general, various levels of resistance to benomyl have been reported for certain fungi (e.g., "very high resistance" to the fungicide was reported for the pathogens *Venturia inaequalis*, *V. pirina*, *Penicillium puberulum*, *P. expansum*, and *P. aurantiogriseum*, all possessing the same mutation) (27). With the stone fruit pathogen *Monilinia fructicola*, "high levels of resistance" were associated with the same conversion at codon 198 (30). Furthermore, "highly resistant" isolates of the gray mold pathogen, *B. cinerea*, had the identical mutation (28,47). Although no fungicide spray regime documentation is available from the overseas suppliers of the *Limonium* plantlets to Israeli growers, it is plausible to assume that the mutation leading to benomyl resistance was not derived in Israel. This is further substantiated by the fact that farmers in Israel do not apply benomyl to their *Limonium* crop under cultivation.

The presence of two different *C. gloeosporioides* genotypes from *Limonium* spp. in Israel may indicate that two separate introductions of infected propagation material occurred. Additional genotypes (groups 3 and 4) were identified that may represent additional introductions. Furthermore, cross-infection of *Limonium* spp. by *C. gloeosporioides* may be occurring due to cultivation of this crop in close proximity to mango, citrus, avocado, ornamentals, and other indigenous hosts that are infected with *C. gloeosporioides* that may provide inoculum for infection of *Limonium* spp. in Israel.

In summary, the pathogen *C. gloeosporioides* that is responsible for wilting of *Limonium* spp. in Israel is composed of both benomyl-resistant (characterized with a typical mutation in the *TUB2* β-tubulin gene at codon 198) and benomyl-sensitive populations that are genetically distinct. Because benomyl is not applied to this crop in Israel, the pathogen populations may be (i) originating as quiescent infections on imported plantlets, (ii) dispersed locally from plot to plot among the different growers by workers in the fields, or (iii) cross-infecting *Limonium* spp. from alternate hosts cultivated adjacent to the affected crops.

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