Geographical Distribution, Pathotype Characterization, and Molecular Diversity of *Colletotrichum lindemuthianum* in Greece and Resistance of Greek Bean Cultivars

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ABSTRACT

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Thirty-five *Colletotrichum lindemuthianum* isolates were obtained from bean (*Phaseolus vulgaris*) fields in 23 prefectures of Greece during a survey conducted in 2003 and 2004. Race characterization based on the standardized set of 12 differential cultivars demonstrated the presence of races 2, 6, and 22, which are reported for first time in Greece, while race 22 is reported for the first time in the world. In addition, pathotype diversity showed significant correlation with the geographical origin of these isolates. All three races caused disease symptoms only on cultivars of Andean origin, suggesting that Greek isolates originated from South American countries. Virulence spectrum of the same isolates was also examined on a set of 30 Greek bean cultivars showing seven types of virulence. Based on their reactions to the pathogen isolates, Greek cultivars were divided into nine groups. Among these cultivars, two (Ithomi FS60 and Larisa) were resistant to all tested isolates, including the reference isolates. Molecular diversity was detected using random amplified polymorphic DNA (RAPD) primers and the restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA internal transcribed spacers, which revealed two main clusters of isolates. Thirty-two out of 35 isolates belonged to the same main cluster in both methods, indicating that Greek isolates have genotypic similarities. This study provides the information on population diversity of *C. lindemuthianum*, which can be useful in agricultural practices and in plant breeding programs.

Additional keywords: bean anthracnose, resistant cultivars

Colletotrichum lindemuthianum (Sacc. & Magnus) Briosi & Cavara is the causal agent of anthracnose, one of the most important diseases of common bean (*Phaseolus vulgaris*) throughout the world. The disease can cause yield losses exceeding 90% in subtropical and temperate regions, especially when contaminated seed is planted and prolonged conditions favorable to disease development occur during the crop season (22).

C. lindemuthianum is primarily spread by infected bean seed, in which the pathogen can remain as dormant mycelium within the seedcoat and cotyledon cells or as spores between cotyledons or elsewhere in the seed, but it can also survive in plant debris and by the formation of sclerotia (20,21,30). It causes black, sunken cankers in bean pods containing salmon-colored ooze centers consisting of millions of conidia in acervuli. The pathogen also affects

petioles, leaf veins, stems, and seeds, producing characteristic anthracnose sunken lesions filled with spores (20).

Anthracnose can be controlled by using disease-free or fungicide-treated seeds, foliar applications of fungicides, quarantine, and agricultural practices (10,28). These methods seem to be effective, but in the presence of the pathogen in the field, the cultivation of resistant cultivars encompasses economical and ecological benefits.

In order to make breeding programs more effective, knowledge of pathogen distribution is necessary. *C. lindemuthianum* shows continuous evolution through various means of selection pressure such as the cultivation of resistant bean cultivars, variable climatic conditions, or various cultivation practices (23,24). Pathogenic variability in this pathogen was first reported by Barrus in 1911 (6). Pastor-Corrales and Tu in 1989 (22) proposed a standard method for estimating the variability of anthracnose isolates, depending on their virulence on a set of 12 differential cultivars with nine identified resistance genes (1,15,20,32,34). Since the establishment of this method, different studies were carried out, and more than 100 pathotypes of *C. lindemuthianum* have been identified worldwide (4,14,16,24,27).

In Greece, areas cultivated with local or imported cultivars of dry and fresh beans are estimated at around 6,500 and 6,800 ha, respectively. The climate of the northern bean growing regions, which is characterized by medium temperatures and high relative humidity, favors anthracnose disease. The presence of *C. lindemuthianum* in Greece was first reported in Prefecture of Kavala by Demetriades and Zachos in 1962 (11). However, there have been no further studies on the anthracnose geographical distribution and race characterization of the pathogen in Greece.

C. lindemuthianum is an increasing problem for bean production in Greece, but it has not been well investigated. It is important to document the pathogen population variability and pathogenicity. The objectives of this study were to (i) determine the geographical distribution of the pathogen in Greece, (ii) characterize races, (iii) evaluate the virulence spectra of the Greek isolates on a set of 30 selected Greek bean cultivars, and (iv) determine molecular diversity among the Greek *C. lindemuthianum* isolates.

MATERIALS AND METHODS

Disease survey. Twenty-three bean growing areas of Greece were surveyed for symptomatic bean plants during 2003 and 2004 (Fig. 1). *C. lindemuthianum* isolates were collected from symptomatic stems and pods. Individual isolates were obtained from different fields (Table 1). Fungal isolates were obtained using the dilution technique. Isolations of the pathogen were carried out on potato dextrose agar (PDA) (Oxoid, Basingstoke, UK) and maintained on the same medium. Additionally, three known races, 46, 47, and 49, were used as references, corresponding to isolates UPS2 (African origin), UPS9 (European origin), and UPS36S (Mesoamerican origin), respectively, in previous reports (12,27). These isolates were provided by R. Lauge (Laboratoire de Phytopathologie Moléculaire, Institut de Biotechnologie des Plantes, Université Paris – Sud Orsay).

Plant materials. Race characterization experiments were conducted on the differential set of bean cultivars proposed by Pastor-Corrales and Tu 1989 (22). Seeds of these cultivars were provided by G. Ma-

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huku (Centro Internacional de Agricultura Tropical [CIAT], Cali, Colombia). Virulence spectrum of Greek *C. lindemuthianum* isolates in Greek bean germplasms was tested on a set of 30 cultivars with desirable agronomical traits, which were obtained from local stations of the National Agricultural Research Foundation (NAGREF) (Table 2). In all tests, plants

were grown in a mixture of peat moss and perlite at a rate of 3:1 and maintained in a growth chamber at 22°C, 90% humidity, and 12-h photoperiod.

Race characterization. Race characterization was based on the virulence patterns of 35 isolates on the 12 differential cultivars (22). For inoculum production, *C. lindemuthianum* isolates were grown on

PDA for 10 days at 22°C in darkness. Spores were harvested by scraping the surface of fungal colonies flooded with 20 ml of sterile distilled water using a sterile glass rod. Suspensions for inoculation of bean seedlings were filtered through double-layer cheesecloth, and the concentration was adjusted to 10⁶ conidia/ml using a hemacytometer. Inoculations were made by spraying the conidial suspensions until runoff on the stem and both surfaces of the unifoliate leaves of plants at the primary leaf stage (10 days old). The assessment of disease severity on the differential cultivars was carried out 7 days after inoculation, using a slightly modified disease index according to the CIAT scale (29), where 1 to 2 (no visible symptoms, nonsporulating lesions on the main veins) were considered incompatible (resistant) and 3 to 6 (lesions on the secondary veins, lesions on the stem, extended necrotic symptoms, and dead plants, respectively) were considered compatible interactions (susceptible). There were three replications of six plants for each cultivar.

Greek bean cultivars disease reaction.

A set of Greek bean cultivars, mentioned above, was used for evaluation of disease reaction and definition of the virulence spectrum of the Greek *C. lindemuthianum* isolates. The methods for inoculation of the plants and assessment of disease severity have been described previously in the race characterization section. Six plants per cultivar and replication were used, and the experiments were replicated three times.

DNA isolation. Fungal isolates were grown in Erlenmeyer flasks, each containing 50 ml of potato dextrose broth medium (PDB) (Sigma-Aldrich, St. Louis, MO) supplemented with 0.1% yeast extract (Sigma-Aldrich) and 0.1% casamino acids (Sigma-Aldrich). Flasks were inoculated with 1 ml of conidial suspension (10⁶ conidia/ml) and incubated in darkness at 23°C for 7 days. Total genomic DNA was isolated from 50 to 100 mg of air-dried

Fig. 1. Geographical distribution of *Colletotrichum lindemuthianum* in Greece. Anthracnose was present in three (black colored) prefectures: Prefecture of Kavala (Municipality of Hrisoupolis, BH isolates), Prefecture of Serres (Municipality of Vrodou, BV isolates), and Prefecture of Drama (Municipality of Nevrokopi, BN isolates). Dark gray prefectures were anthracnose free, and light gray prefectures were not surveyed because of their unfavorable climatic conditions for anthracnose development.

Table 1. Isolates of *Colletotrichum lindemuthianum*, year of collection, and bean cultivars from which the isolates were collected and their growing areas and origins

Greek <i>C. lindemuthianum</i> isolates ^x	Year	Bean cultivar	Cultivation area/origin	Fields ^y	
				Inspected	Diseased
BV1 – BV5	2003	Magirus	Vrodou, Serres (Northern Greece)	37	17
BV6 – BV9	2003	Unknown	Vrodou, Serres (Northern Greece)		
BV10 – BV17	2003	Zargana Kavalas	Vrodou, Serres (Northern Greece)		
BN1	2004	Mantra	Nevrokopi, Drama (Northern Greece)	25	16
BN2 – BN4	2004	Magirus	Nevrokopi, Drama (Northern Greece)		
BN5 – BN7	2004	Aquila	Nevrokopi, Drama (Northern Greece)		
BN8 – BN11	2004	Barbouni	Nevrokopi, Drama (Northern Greece)		
BN12 – BN16	2004	Borlotto lingua	Nevrokopi, Drama (Northern Greece)		
BH1 – BH2	2003	Zargana Hrisoupolis	Hrisoupolis, Kavala (Northern Greece)	29	2
UPS2 ^z	...	...	Middle America		
UPS9 ^z	...	...	Europe		
UPS36S ^z	...	...	Africa		

^x Each isolate was obtained from one plant per field with severe symptoms.

^y All plants of each field were scouted for symptoms.

^z Reference isolates used in this study.

frozen (−80°C) mycelium using a slightly modified CTAB method (25). DNA concentration was estimated by agarose gel stained with ethidium bromide.

Random amplified polymorphic DNA (RAPD) analysis. For the RAPD analysis, 55 random 10-mer primers from kits A, D, F, G, R, S, T, V, AB, AC, AG, AH, AK, AL,

AM, AP, AR, AT, and AW (Operon Technologies Inc., Alameda, CA) were tested for their ability to reveal polymorphism among the 38 *C. lindemuthianum* isolates. Amplification was carried out in 25 µl reaction mix according to Williams et al. (32), with minor modifications. In more detail, the reaction mix contained about 25 ng genomic DNA, 0.2 mM each of dNTP, 0.2 µM of primer, 2 mM MgCl₂, and 1 U of *Taq* DNA polymerase (Fermentas) with supplied buffer. DNA amplification was performed in a PTC 200 thermal cycler (MJ Research) according to the following thermal profile: initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 35 s, annealing at 35°C for 1 min, and extension at 72°C for 1 min. Amplification products were separated in 2% agarose gel and detected by staining with ethidium bromide (26). The gels were photographed under UV light. Each PCR reaction was run in duplicate, and only well-defined and reproducible bands were analyzed.

Restriction fragment length polymorphism (RFLP) analysis. PCR amplification of the ribosomal DNA spacers (ITS1 and ITS2) and the 5.8S subunit of the ribosomal gene was conducted according to Sicard et al. (28) using the primers PN3 (5'-CCGTTGGTGAACCGAGCGAGG ATC-3') and PN10 (5'-TCCGCTTATTGA TATGCTTAAG-3'), which were located in the 18S and 28S DNA regions, respectively

Table 2. Dry bean cultivars used in this study and their market types and cultivation areas

Greek bean cultivars	Market type	Cultivation areas
1 Zargana Hrisoupolis	Dark red kidney	Macedonia (Northern Greece)
2 Zargana Kavalas	Brown kidney	Macedonia (Northern Greece)
3 Aises	Black beans	Macedonia, Thrace (Northern Greece)
4 Rapsani	Navy	Thessaly (Central Greece)
5 FS62	White kidney	Thessaly (Central Greece)
6 Ithomi FS60	White kidney	Thessaly, Macedonia (Central–Northern Greece)
7 FS 64	White kidney	Thessaly (Central Greece)
8 Lida	Navy	Thessaly, Macedonia (Central–Northern Greece)
9 M-15418	White kidney	Thessaly (Central Greece)
10 M-9985	White kidney	Thessaly (Central Greece)
11 Aridaia M-4403	Navy	Macedonia, Thessaly (Central–Northern Greece)
12 FS66	White kidney	Thessaly (Central Greece)
13 M-14542	Navy	Thessaly (Central Greece)
14 Larisa	White kidney	Thessaly (Central Greece)
15 M-13070	White kidney	Thessaly (Central Greece)
16 FS72	White kidney	Thessaly (Central Greece)
17 Semeli FS51	White kidney	Thessaly, Macedonia (Central–Northern Greece)
18 Pirgetos FS46	White kidney	Thessaly (Central Greece)
19 M-14904	White kidney	Thessaly (Central Greece)
20 Iro FS52	White kidney	Thessaly (Central Greece)
21 M-10802	White kidney	Thessaly (Central Greece)
22 Mirsini FS49	Navy	Thessaly (Central Greece)
23 Barbouni I	White kidney	All Greek bean growing areas
24 Barbouni II	Pinto	All Greek bean growing areas
25 Barbouni III	Pinto	All Greek bean growing areas
26 Tsaoulia	Brown kidney	Peloponnesus (Southern Greece)
27 Lasithiou	Black beans	Peloponnesus, Crete (Southern Greece)
28 Handres	Pinto	Peloponnesus, Crete (Southern Greece)
29 Handrelia	Navy	Peloponnesus, Crete (Southern Greece)
30 Pastalia	Pinto	Peloponnesus, Crete (Southern Greece)

Table 3. Race characterization of 35 isolates of *Colletotrichum lindemuthianum* from Greece

Differential ^u cultivars	Gene pool	Binary number	Isolates																		
			BH1 ^v	BH2	BV1	BV2	BV3	BV4	BV5	BV6	BV7	BV8	BV9	BV10	BV11	BV12	BV13	BV14			
Michelite	MA ^w	1	R ^x	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
MDRK	A ^y	2	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Perry Marrow	A	4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cornell 49242	MA	8	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Widusa	A	16	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Kaboon	A	32	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Mexico 222	MA	64	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
PI 207262	MA	128	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
TO	MA	256	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
TU	MA	512	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
AB 136	MA	1024	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
G 2333	MA	2048	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Races			6	6	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22

Differential cultivars	Gene pool	Binary number	Isolates																Reference isolates ^z		
			BN1	BN2	BN3	BN4	BN5	BN6	BN7	BN8	BN9	BN10	BN11	BN12	BN13	BN14	BN15	BN16	UPS2	UPS9	UPS36S
Michelite	MA	1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S
MDRK	A	2	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
Perry Marrow	A	4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R
Cornell 49242	MA	8	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R
Widusa	A	16	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
Kaboon	A	32	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S
Mexico 222	MA	64	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
PI 207262	MA	128	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
TO	MA	256	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
TU	MA	512	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
AB 136	MA	1024	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
G 2333	MA	2048	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Races			2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	46	47	49

^u Differential cultivars as proposed by Pastor-Corrales and Tu (22).

^v BH, BV, BN: Greek *C. lindemuthianum* isolates.

^w MA: Mesoamerican gene pool.

^x R: resistant reaction, S: susceptible reaction.

^y A: Andean gene pool.

^z Reference isolates studied before (12,28).

(12,27). The amplification products were digested with restriction enzymes *MspI* and *HaeIII* and separated by electrophoresis in 3.5% agarose gel. Experiments were performed twice.

Data analysis. Data of disease severity revealed from the three replications of race characterization were combined and subjected to analysis of variance (ANOVA) and compared using Fisher's least significant difference (LSD) procedure at $P = 0.05$. Similarly, data of disease severity revealed from the three replications of the experiments carried out to determine the susceptibility of Greek bean cultivars were analyzed. Statistical analysis was supported by SPSS 14.0 (SPSS, Chicago, IL). For RAPD analysis, each polymorphic band in every sample was scored as a binary character (1 for presence and 0 for absence), and a data matrix was created. DNA bands of identical gel migration were assumed to represent the same allele at a locus. Only reproducible and clearly visible bands were scored. The bands detected in all samples at the same position (monomorphic) and bands not directly comparable between individuals because of very similar migrations were excluded from the analysis. Genetic similarities between individual isolates were calculated using the Jaccard coefficient, and pairwise similarity matrix was produced. The similarity matrix was used to construct

a dendrogram based on the unweighted pair group method and arithmetic average (UPGMA) of the software NTSYS pc version 2.1 (NTSYS, NY). The co-phenetic correlation for the dendrogram was computed as a measure of "goodness of fit" for the method of cluster analysis used for the data set. A number of clusters closest to the square root of the number of individuals were accepted. The clustering was validated by bootstrap analysis using 1,000 replicates using the software WINBOOT (33).

RESULTS

Geographical distribution. Anthracnose infections were found in samples of the surveyed areas of three prefectures, namely Hrisoupolis (Prefecture of Kavala, BH isolates), Vrodou (Prefecture of Serres, BV isolates), and Nevrokopi (Prefecture of Drama, BN isolates) (Fig. 1, Table 1). In particular, 2 isolates were obtained from Hrisoupolis, 17 isolates were obtained from Vrodou, and 16 isolates were obtained from Nevrokopi (Table 1).

Race characterization. Isolates from different geographical areas showed various interactions with the differential cultivars, but there were no different reactions among isolates from the same area. BH isolates produced compatible interactions with the MDRK and Perry Marrow cultivars, BV isolates produced compatible

interactions with MDRK, Perry Marrow, and Widusa cultivars, and BN isolates were virulent only on cultivar MDRK. Table 3 shows the reactions of the 12 differential cultivars to the 35 isolates of *C. lindemuthianum*. Those results demonstrated the presence of races 2, 6, and 22 in isolates from municipalities of Nevrokopi, Hrisoupolis, and Vrodou, respectively.

Disease reactions of Greek bean cultivars. The evaluation of resistance in 30 Greek bean cultivars when inoculated with the Greek isolates of *C. lindemuthianum* revealed nine groups of disease reactions (Table 4). Group 1 included 13 cultivars (Zargana Hrisoupolis, Zargana Kavalas, Aises, Rapsani, FS62, FS 64, M-9985, Aridaia M-4403, FS66, Semeli FS51, Barbouni I, Barbouni II, and Lasithiou) showing compatible interactions to all tested isolates and produced typical disease symptoms. In group 2, two cultivars (Ithomi FS60 and Larisa) were resistant to all tested isolates, including the reference isolates. Group 3, composed of cultivars Lida and M-15418, was resistant to three isolates (BN12, BN13, and BN14). Cultivars M-14542, FS 72, Pirgetos FS46, M-14904, and Iro FS52 belonged to group 4 and showed resistance to all BN isolates. Group 5 (cultivar M-13070) showed resistance to isolates BV6 to BV8 and all BN isolates. Group 6 (M-10802 and Tsaoulia) was resistant to 12 isolates (BV1 to BV8

Table 4. Virulence spectra of 35 Greek *Colletotrichum lindemuthianum* isolates and reactions of 30 Greek bean cultivars

Groups of Greek bean cultivars	Types of <i>C. lindemuthianum</i> isolates virulence spectrum						
	Type I BV1 ^a -BV5, BV14-BV17	Type II BV6-BV8	Type III BV9-BV13, reference isolates ^w	Type IV BN1, BN2, BN5, BN6, BN10, BN11, BN15, BN16	Type V BN3, BN4, BN7-BN9	Type VI BN12-BN14	Type VII BH1-BH2
Group 1 Zargana Hrisoupolis, Zargana Kavalas, Aises, Rapsani, FS62, FS 64, M-9985, Aridaia M-4403, FS66, Semeli FS51, Barbouni I, Barbouni II, and Lasithiou	S ^x (4-5 ^y b ^z)	S (4-5 b)	S (4-5 b)	S (4-5 b)	S (4-5 b)	S (4-5 b)	S (4-5 b)
Group 2 Ithomi FS60 and Larisa	R (1-2 a)	R (1-2 a)	R (1-2 a)	R (1-2 a)	R (1-2 a)	R (1-2 a)	R (1-2 a)
Group 3 Lida and M-15418	S (4-5 b)	S (4-5 b)	S (4-5 b)	S (4-5 b)	S (4-5 b)	R (1-2 a)	S (4-5 b)
Group 4 M-14542, FS 72, Pirgetos FS46, M-14904, and Iro FS52	R (1-2 a)	S (4-5 b)	S (4-5 b)	R (1-2 a)	R (1-2 a)	R (1-2 a)	S (4-5 b)
Group 5 M-13070	S (4-5 b)	R (1-2 a)	S (4-5 b)	R (1-2 a)	R (1-2 a)	R (1-2 a)	S (4-5 b)
Group 6 M-10802 and Tsaoulia	S (4-5 b)	R (1-2 a)	S (4-5 b)	S (4-5 b)	S (4-5 b)	S (4-5 b)	S (4-5 b)
Group 7 Mirsini FS49 and Barbouni III	S (4-5 b)	S (4-5 b)	S (4-5 b)	R (1-2 a)	R (1-2 a)	R (1-2 a)	R (1-2 a)
Group 8 Handres and Handrelia	S (4-5 b)	S (4-5 b)	S (4-5 b)	R (1-2 a)	S (4-5 b)	S (4-5 b)	S (4-5 b)
Group 9 Pastalia	S (4-5 b)	S (4-5 b)	S (4-5 b)	S (4-5 b)	S (4-5 b)	S (4-5 b)	R (1-2 a)

^a BV, BN, BH: Greek *C. lindemuthianum* isolates.

^w Reference isolates: UPS2, UPS9, UPS36S.

^x S: susceptible reaction, R: resistant reaction.

^y Disease index values.

^z Different letters for statistically significant differences according to ANOVA LSD test for $P = 0.05$.

and BV14 to BV17). Group 7 (Mirsini FS49 and Barbouni III) was resistant to all BN and BH isolates. Group 8 (cultivars Handres and Handrelia) showed resistance to eight BN isolates (BN1, BN2, BN5, BN6, BN10, BN11, BN15, and BN16). Group 9 (Pastalia beans) was resistant to all BH isolates.

The 35 Greek *C. lindemuthianum* isolates showed seven virulence patterns (Table 4). Isolates BV1 to BV5 and BV14 to BV17 had compatible interactions with the majority of the tested cultivars, except those that belonged to groups 2 and 4. Isolates BV6 to BV8 did not produce disease symptoms on the cultivars of group 2, 5, and 6. Isolates BV9 to BV13 were the most virulent among Greek isolates, producing compatible interactions on all Greek bean cultivars except those of group 2; these isolates showed the same virulence spectrum as the reference isolates UPS2, UPS9, and UPS36S. Isolates BN1, BN2, BN5, BN6, BN10, BN11, BN15, and BN16 did not produce compatible interactions on the cultivars of groups 2, 4, 5, 7, and 8, while isolates BN3, BN4, and BN7

to BN9 were avirulent to the cultivars of groups 2, 4, 5, and 7. Isolates BN12 to BN14 produced disease symptoms in the cultivars of groups 1, 6, 8, and 9, while BH isolates were highly virulent on all cultivars but avirulent on those of groups 2, 7, and 9.

RAPD analysis. Among the 55 tested RAPD primers, eight (OPA9, APAP16, OPAR9, OPD2, OPD7, OPF13, OPG2, and OPD7) revealed DNA polymorphism and resulted in a total of 43 polymorphic and 19 monomorphic bands among the fungal isolates tested. The number of bands produced per primer ranged from 3 to 9, and band sizes varied from 0.2 to 3 kb. Pairwise genetic similarity estimates for 38 isolates based on RAPD analysis ranged from 0.14 to 0.89 (data not shown). The dendrogram derived from the UPGMA analysis is shown in Figure 2. The 38 *C. lindemuthianum* isolates were grouped into two main clusters. The first cluster consisted of 33 isolates belonging to races 2, 6, 22, and 47 and was further divided into five subclusters (I: BV1 to BV5, BV7, BV9, BV12, and BV14 to BV17; II: BN3 to

BN8, BN10, BN11, BN16, BH1, BH2, and UPS9; III: BN12, BN14, and BN15; IV: BV6, BV8, BV10, BV11, and BV13; and V: BN1). The second main cluster consisted of five isolates, belonging to races 2, 46, and 49, and was divided into two subclusters (VI: BN2, BN9, BN13, and UPS2; and VII: UPS36S).

The clustering of 38 *C. lindemuthianum* isolates revealed by RAPD analysis showed that isolates with identical profiles of pathogenicity were included in different clusters or subclusters. For example, the BN isolates were present in four clusters belonging to both main clustering groups, and therefore showed the greatest molecular diversity. In contrast, the BV isolates were present in two subclusters belonging to the same main cluster, and all isolates of race 6 (BH isolates) were grouped together in the same subcluster.

Genetic similarity between the majority of the Greek isolates (32 out of 35) and reference strain 47 (UPS9 isolate) was revealed by RAPD analysis. On the other hand, three Greek BN isolates (BN2, BN9, and BN13) were similar to the other two

Fig. 2. Dendrogram showing the clusters of 38 *Colletotrichum lindemuthianum* isolates (35 Greek isolates, 3 reference isolates, 6 races) based on random amplified polymorphic DNA (RAPD) analysis (cophenetic value $r = 0.87$). Percentages from 1,000 bootstrap replications (33) are shown near the two main branches.

reference strains (races 46 and 49 belonging to the isolates UPS2 and UPS36S, respectively).

RFLP analysis. A fragment of 580 bp was produced by the amplification of the internal transcribed spacer (ITS) region in all Greek and tested reference isolates. Digestion of the fragment with restriction enzymes *Msp*I and *Hae*III revealed two groups (I and II). Only two out of 35 Greek isolates (BN9 and BN13) together with the reference isolates UPS2 and UPS36S belonged to group I. Group II consisted of the majority of Greek *C. lindemuthianum* isolates (BV1 to BV17, BN1 to BN8, BN10 to BN12, BN14 to BN16, BH1, and BH2) and the reference isolate UPS9. Restriction digestion profiles of the amplification product of the isolates BV1, BV2, BN1, BN2, BH1, BH2, BN9, BN13, UPS2, UPS9, and UPS36S with the enzymes are shown in Figure 3. The amplification product of group II isolates contained a *Hae*III recognition site, while isolates of group I had an additional restriction site. Similarly, *Msp*I revealed another recognition site for group I isolates, since the fragment of 130 bp in group II isolates was replaced by two fragments of 100 and 30 bp.

According to results of ITS analysis, there was no differentiation among all Greek isolates, which showed the same pattern with the reference strain UPS9, except BN9 and BN13.

DISCUSSION

This study was undertaken to understand the variability of Greek *C. lindemuthianum* isolates by means of geographical distribution, race characterization, virulence spectrum in Greek cultivars, and molecular diversity. Knowing the variability of these isolates, we have a key factor for the efficient management through dis-

ease prediction and development of resistant bean cultivars.

Anthraxnose is a major fungal disease in some bean growing areas of Greece. Among the surveyed areas, anthracnose was present in three locations of northern Greece. Those regions, especially Vrodou and Nevrokopi, are characterized by having favorable climate conditions for the pathogen, such as moderate temperatures and high relative humidity, which result in anthracnose outbreaks every year. A possible reason for *C. lindemuthianum* entrance into these areas is the use of imported seed of dry bean cultivars. This hypothesis is enhanced by the fact that the majority of the dry bean cultivars used in Greece is imported from American or European countries. The fact that race 22 is reported for the first time in the world can be explained by continuous evolution through various selection pressures by growing several bean cultivars in various climatic conditions or with different cultivation practices (23,24).

Race characterization of the 35 Greek isolates resulted in the identification of races 2, 6, and 22 of *C. lindemuthianum*. All three races attack only differentials with Andean origin, suggesting that European isolates originated from South American countries (2,13). Introduction of American germplasms of common bean seems to be a promising strategy for breeding programs in Greece. The revealed positive correlation between virulence patterns and geographical origins of the tested isolates agreed with previous reports (17). This observation may result from the same selection pressure for evolution of the pathogen in a given area through the use of a limited number of bean cultivars.

Resistance assessment of the Greek cultivars to the anthracnose pathogen isolates showed the existence of nine different

groups of interactions. One of the groups consisted of two cultivars (Ithomi FS60 and Larisa) resistant to all tested isolates, including the reference isolates 46, 47, and 49. This finding is of great importance. However, additional research is required for identifying and characterizing the resistance genes. Conversely, group I, consisting of 13 cultivars, was found to be susceptible to all tested isolates. Additionally, virulence evaluation of the *C. lindemuthianum* isolates on Greek dry bean cultivars revealed seven types of virulence. Although there were differences in pathogenicity among isolates obtained from the same regions, all seven types contained only isolates from the same geographical origin, in agreement with the results of the race characterization experiment. There are several examples in which the use of different bean germplasms resulted in different virulence characterization of the same isolates (20,27). This assessment is needed to determine the potential economic impact of the pathogen on the cultivation of dry bean in Greece and to provide breeders useful information for choosing genotypes to develop resistant cultivars.

Molecular techniques, including RFLP and RAPD analysis, have been widely used to differentiate fungal pathogens at inter- or intra-race level (3,7,8,18,19). In this study, molecular diversity through RAPD and RFLP analyses were in agreement and revealed the existence of two main groups of *C. lindemuthianum* isolates. These results were similar to previous studies (4,5,12). Thus, in both molecular analysis methods, main clusters contained the same isolates with one exception of BN2 isolate.

Comparison between molecular and pathogenic diversity of isolates showed that they did not correlate well to each other, agreeing with previous findings

Fig. 3. Restriction analysis of the amplification spacers of isolates BV1, BV2, BN1, BN2, BH1, BH2, BN9, BN13, UPS2, and UPS9 using endonucleases *Hae*III and *Msp*I. V1 stands for isolate BV1, V2 for BV2, N1 for BN1, N2 for BN2, H1 for BH1, H2 for BH2, N9 for BN9, N13 for BN13, U2 for UPS2, and U9 for UPS9. UPS36S does not appear in the agarose gel because of its identical profile with UPS2 isolate.

reported for *C. lindemuthianum* (2,24) and other pathogens (9). In RAPD analysis, there were isolates of the same race present in several clusters (i.e., pathotypes 2 and 6). In contrast, there were clusters, especially the larger one, that contained more than one race (i.e., races 2, 6, and 47). However, in the same dendrogram, some clusters contained only one race. Additionally, concerning the polymorphism in the ITS regions, there was no evidence of correlation between genotype and pathotype diversity. In this case, the two groups contained more than one race. The use of virulences may be important to access genetic variability in pathogen populations because it provides direct information concerning the effects of host selection. However, it may represent only a small proportion of the genetic variation in the population. Combining data from virulence and molecular analysis can lead to a better understanding of the variability present in Greek populations of the pathogen.

Molecular and geographical diversity, at a country level, can be correlated using the fact that the majority of the Greek isolates belonged to the same main group as determined by either RAPD or RFLP analysis. Also, in RAPD analysis, there were five subclusters that consisted of isolates originating from the same region. The results of this study are in agreement with those of previous studies (2,4,28) indicating that isolates from the same geographic region are, in some cases, genotypically more closely related to one another than isolates from different geographic origins.

Further analysis of *C. lindemuthianum* isolates using different molecular tools is necessary for better understanding of the diversity of the pathogen populations. Characterization of resistance genes of Greek bean cultivars should be useful in plant breeding programs in Greece and also worldwide.

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