Virulence and molecular characterization of Costa Rican isolates of *Rhizoctonia solani* from common bean

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ABSTRACT

Web blight is one of the main diseases that affects bean (*Phaseolus vulgaris*) cultivation. It infects diverse organs at any growth stage of the plant and can be present at different altitudes in a humid tropical climate. The causal agent of this disease is *Thanatephorus cucumeris* in its sexual stage and *Rhizoctonia solani* in the anamorph. The objective of this investigation was to characterize molecular isolates of *R. solani* obtained from bean plants from diverse production regions in Costa Rica and determine their virulence. Fifty-one samples of symptomatic bean plants were collected using a global positioning system. Virulence was evaluated using the detached leaf technique. Isolates were identified using AG 1-IA, AG 1-IB, AG 1-IC, AG 1-ID, AG 2-2, AG 2-IIIB, AG 2-2IV and AG 4 molecular markers. ITS sequences were obtained and analyzed with BLAST, aligned, and a phylogenetic tree was constructed. A high degree of virulence and genetic variability between isolates was identified and the anastomosis subgroups of isolates were independent of their geographical origin.

Key words: *Phaseolus vulgaris*, anastomosis group (AG), genetic variability, molecular characterization, sclerotium size, virulence.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is one of the basic components of Costa Rican and Latin-American diets. It is a valuable legume that supplies a significant amount of nutrients in developing countries (FAO, 2004; Godoy-Lutz et al., 2008). Web blight (WB) is a common and destructive bean disease in tropical and subtropical regions of the American continent. The causal agent is the fungus *Thanatephorus cucumeris* (Frank) Donk, the sexual stage of *Rhizoctonia solani* Kühn, a basidiomycete. It survives from one season to the next mostly via sclerotia or mycelium in crop residues in the field (Godoy-Lutz et al., 2000; Polanco et al., 1996). It is endemic to Central America and the Caribbean, and causes defoliation and severe losses in seed quality and production (Godoy-Lutz et al., 2003). Crop losses above 20% have been reported in more than 200 cultivars around the world (Beaver et al., 2002). Chemical and cultural control are the most common management techniques (Beebe et al., 1991; Godoy-Lutz et al., 1996a; Polanco et al., 1996), but during the last decades the

biocontrol using different bacterial genus has been adopted as a sustainable approach for crop health management. However, they are not sufficient to avoid production losses (Solanki et al., 2013; Kanini et al., 2013).

A common bean line with high tolerance to WB in different geographical regions has not been obtained or identified (Beaver et al., 2002). Breeding efforts to create a WB resistant common bean have been limited by fungal variability in varietal reactions and the lack of knowledge of pathogen variation, mating systems and disease resistance mechanisms (Godoy-Lutz et al., 2003; Godoy-Lutz et al., 2008).

R. solani is a complex, heterogeneous species of several anastomosis groups (AG), which are defined as isolates with somatic or vegetative compatibility (Matsumoto, 2005). Worldwide, 14 AGs and numerous subgroups have been identified based on physical, chemical, pathogenic and molecular characteristics. Isolates can exist as multinucleate homokaryons or heterokaryons and present diverse mating strategies (homothallic or heterothallic) (González et al., 2012). *Phaseolus vulgaris* isolates belong mainly to AG 1 (6 subgroups), AG 2 (4 subgroups) and AG 4 (Carling, 1996; Schneider et al., 1997; Carling et al., 1999; Carling et al., 2002; Godoy-Lutz et al., 2003; Godoy-Lutz et al., 2008; González et al., 2006).

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When evaluated on the same plant species, virulence between AGs varies from highly virulent to non-virulent. Godoy-Lutz et al. (1996b) observed discrepancies in the field since virulence patterns of isolates from different geographical regions may vary within the same subgroup, for example AG 4 or AG 1-IE (Godoy-Lutz et al., 2000). Awareness of the pathogenicity and genetic diversity of isolates helps to explain the variation in the cultivar reaction in pathogen-host interactions (Godoy-Lutz et al., 2003).

The development and progress of the disease is favored by high temperature and relative humidity. The pathogen can be spread as rain splashed sclerotia or mycelia, often in association with plant debris, airborne basidiospores, infested soil debris and asymptomatic infected seeds (Echandi, 1965; Godoy-Lutz et al., 1996a; Godoy-Lutz et al., 2003).

The classical hyphal fusion for identification and classification of *Rhizoctonia* spp. into anastomosis groups has been widely used and is still valid. It has been genetically confirmed in recent years by the use of DNA-based molecular methods. One of the most useful tool is the analysis of sequences from rDNA ITS (Grosch et al. 2004, 2007). This has been used for identification of anastomosis groups. According to Pannecouque & Höffe (2009), ITS regions evolve faster and have been used often for molecular identification of closely related fungi. Using this advantage, Godoy-Lutz et al. (2008) developed specific primers for each AG that affected bean. Recently, Wibberg et al. (2013) and Zheng et al. (2013) published the genome of *R. solani* AG 1-IB and AG 1-IA, respectively. All this information can be used to learn about the structure and gene function and to develop phylogeny and diversity studies.

This investigation was conducted to determine the virulence of *R. solani* isolates infecting bean in different climate regions of Costa Rica, and the intraspecific diversity of WB isolates using molecular markers specific for each AG group and ITS sequences.

MATERIALS AND METHODS

Sampling, isolation and morphological fungus identification

Leaf samples of bean plants with symptoms of web blight were collected in six climate regions in Costa Rica (South Pacific, Central Valley, North Region, Southern Mountain Region, North Pacific and Caribbean Region) during 2007 and 2009. A global positioning system (GPS) was used to mark the sampling sites. The pathogen was isolated and identified considering the hyphal morphological traits as branching at right angles with constriction at the base and presence of a septum near the point of origin. Monilioid cells and presence of multinucleated cells were checked out and verified (Naito, 1996). Isolates were stored at 4°C in Petri dishes on potato-dextrose-agar medium (PDA). A photographic database was established, with pictures and a morphological description

(mycelium color, size and shape of the sclerotia) of each isolate.

Virulence

Virulence of the 51 isolates was evaluated on two bean cultivars (Bribri and Brunca) using the detached leaf method (Steadman et al., 1997). Severity was evaluated 24, 48, and 72 hours post inoculation (h.p.i) using the CIAT 1-9 severity scale, where 1= no visible symptoms of disease, 3= 5% of the foliar area infected, 5=10% of the area infected, 7=25% of the area infected and 9=>50% area infected (van Schoonhoven et al., 1987). In each evaluation, photographs were taken to determine the affected area with Scion Image alfa version 4.0.3.2.

Statistical analysis of data

Analysis of variance was used for isolate comparison, with time as a covariable, and isolate and variety as factors. The interaction between time and isolate was also included. Due to the high interaction between variables, the numerous samples and the low significance of cultivars, isolates were compared without considering cultivar. When differences were encountered, multiple Tukey comparisons were made.

Isolates were classified in three categories according to the virulence level: group A: isolates with average affected leaf areas less than 6.66 cm²; group B: isolates with average areas between 6.66 cm² and 12.5 cm²; group C: isolates with averages higher than 12.5 cm². Each evaluation was compared separately by time and group; however, only the 72 hour evaluations were reported because of their greater importance. Equality of variances and normality of residue, indispensable for the ANOVA, were determined. For the analysis a program was written in R language version 2.10.0.

DNA extraction and amplification

DNA was extracted from lyophilized mycelium of *R. solani*, previously cultivated on AC agar (Sigma) with 120 µL/L (50 mg/mL) of ampicillin, according to the modified protocol by Pascual et al. (2000). Extracted DNA was treated with 5 µL RNase A (Fermentas, 10 mg/mL) and quantified with a spectrophotometer (Thermo Spectronic Helios Y).

For PCR, a mixture of 25 µL was prepared with 0,2 units of Taq DNA polymerase (Dreamtaq, Fermentas), 2µL dNTP mix (25mM), 2,5 µL 10x buffer, 3µL MgCl₂ (25mM), 1,5 µL of specific primer for each AG (10 µM) and 50 ng/µL of the DNA sample. The amplification was made using the primers WB-A, WB-B, AG 1-IA, AG 1-IB, AG 1-IC, AG 1-ID, AG 4, AG 2-2, AG 2-2 IIIB and AG 2-2IV (Carling et al. 2002, Godoy-Lutz et al. 2008). Amplifications were performed a PTC-100 thermocycler (MJ Research), with a temperature profile of 95°C for 5 min for denaturing, 58-62°C, for 45s for annealing of the primer and 72°C for 1 min for extension. A second step consisted of 40 cycles with

a temperature profile of 95°C for 45s to denature the DNA, 45s at 58-62°C for annealing and 1 min at 72°C for extension. The third step consisted of 72°C for 10 min. The PCR products were separated by electrophoresis in a 1% agarose gel in TAE buffer for 90 min at 100V. The gel was dyed with ethidium bromide, observed under UV light and photographed with a Kodak EDAS 290 system. A molecular weight marker of 100 bp (50 ng/μL) (Fermentas) was used.

To confirm the relationships among the isolates, 35 isolates were selected at random and amplified using universal primers ITS4 and ITS5. A 25 μL PCR mixture was prepared using 0.5 μL of Taq DNA polymerase (Dreamtaq, Fermentas), 2.5 μL dNTPs mix (25mM), 2.5 μL master mix buffer (10x) Fermentas, 0.4 μL of each primer (10 μM) and 50 ng/μL of the DNA sample. DNA was amplified with the oligonucleotides ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) (White et al., 1990) using a PTC-100 thermocycler (MJ Research) with a temperature profile of 94°C for 1 min, followed by 30 cycles of 94°C for 40 s, 55°C for 1 min, and 72°C for 1 min, and a final step at 72°C for 5 min. PCR products of 740 bp were visualized as indicated before. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced using both primer strands by using the BigDyeTerminator v.3.1 cycle sequencing kit (Applied Biosystems) and an ABI Prism 3130 (Applied Biosystems). With the identification of each anastomosis subgroup and the GPS data, a map was constructed using Arcview GIS 3.2.

Sequence data for the complete ITS-5.8S rDNA of isolates of AG 1 and AG 2 and AG 4 was manually checked and edited using the Multiple Sequence Alignment of BioEdit software (Hall, 2007). Sequences of the complete ITS-5.8S rDNA region were compared with nine reference sequences (NCBI GenBank): AJ868444 (AG 1), AF308624 (AG 2-2), AF308623 (AG 2 WB), JF519829 (AG P), AB195928 (AG 1-IA), AB122139 (AG 1-IB), AB122141 (AG 1-IC), EF197798 (AG 1-ID), JF946728 (AG 1-IE) and JF946731 (AG 1-IF), JQ669932 (AG4).

All nucleotide substitutions were equally weighted and unordered. Alignment gaps were treated as missing data. The sequences were analyzed with BLAST for confirmation of the AG, aligned with the MAFFT algorithm in the GUIDANCE server and analyzed with MrBayes3.2 using 1,000,000 Markov chains (Ronquist et al., 2011) for phylogenetic tree construction with a GTR model. The tree was visualized in MEGA5.2 (Tamura et al., 2011). To ensure the robustness of the analysis additional trees were constructed using Neighbor joining (NJ) and Maximum likelihood (ML) methods with 5,000 bootstrap replications using MEGA 5.2.

RESULTS

Sampling, isolation and identification

During 2007-2009, foliar bean tissue infected with *R. solani* was collected throughout the national

territory. Anastomosis subgroup, sclerotium size, GenBank accession, host, altitude, climate subregions and collecting year for each isolate are presented in Table 1. Fifty-one isolates of *Rhizoctonia solani* were obtained. The mycelium coloration varied in intensity from a dark brown to beige and/or white. Sclerotia size varied from 5 to 20 mm, for macrosclerotia single or aggregated; and 1 mm for isolates with microsclerotia. Also all those mycelium presented multinucleated cells and near-right-angle branching pattern.

Virulence

The CIAT scale scores were compared with measured affected areas for each isolate and a linear correlation of 0.46 was obtained. Although significant, the value was lower than expected. Since the CIAT scale is subjective, these data were not used and conclusions were based on average measured affected leaf areas.

Group A was characterized by a number of heterogeneous isolates ($p < 0.0001$, 27, 350) (Figure 1). Tukey comparisons showed differences among isolates when analyzed by pairs. In group B, significant differences in average areas were observed between isolates ($p < 0.0001$, 16, 264) however a great homogeneity was observed. This group included 17 isolates and the Tukey comparisons showed differences between isolate 3 and those with lower averages.

Group C was homogenous at every evaluation time, as observed in the Figure 2. The ANOVA confirmed that there was no significant difference between average areas of different isolates at the 72 hour evaluations ($p = 0.13$, 7, 119). Isolates 3843, 3636, 3233, 3801, 3242, 3841 and 3804 are the members of this group. These seven isolates are AG 1, but belong to different subgroups and climate regions. Only isolate 3979 belongs to AG 2-2WB.

Molecular identification of the AGs

A high degree of variability between isolates was determined using specific primers for each AG and subgroup. AG groups AG 1, AG 2 and AG 4 were identified in different climate regions of Costa Rica using specific primers for each AG. Subgroups AG 1-IA, AG 1-IB, AG 1-ID, AG 1-IE, AG 1-IF, AG 2WB and AG 4 were identified. The AG was independent of geographical origin and climate region (Table 1).

A map with the distribution of the anastomosis subgroup was constructed. In the Figure 3 there is possible to observe that AG 1-IE subgroup was found with the greatest frequency in the different climate subregions, which are characterized by differences in altitude and precipitation. A single isolate of AG 1-1A was identified in a sample from the South Pacific region. AG 1-1B was found in the South Pacific, Central Valley and Northern regions.

ITS sequences confirm previous results obtained with specific primers. Also, Costa Rican isolates 3977, 3625 and 3629 corresponded to AG P, which has not

TABLE 1 - Code, anastomosis subgroup (AG), sclerotium size, GenBank accession, host, altitude (meters above sea level), climate subregions and collecting year of 51 isolates of *Rhizoctonia solani* collected in different geographic regions of Costa Rica and reference accessions used for phylogenetic tree construction obtained from GenBank

Code	Anastomosis subgroup	Sclerotium size	GenBank accession number	Host	Altitude	Climate subregions	Year
3979	AG 1-IE	Macrosclerotia	JX294346	<i>P. vulgaris</i> cv. Mantequilla	949	North Pacific	2008
3977	AG P	Microsclerotia	JX294345	<i>P. vulgaris</i> cv. unknown	1000	North Pacific	2008
G14	AG 4	Microsclerotia	JX294349	<i>P. vulgaris</i> cv. unknown	1233	North Pacific	2009
4161	AG 2 WB	Microsclerotia	JX294348	<i>P. vulgaris</i> cv. Don Pedro	1249	North Pacific	2008
4160	AG 2 WB	Macrosclerotia	JX294347	<i>P. vulgaris</i> cv. Chimbolo	1203	North Pacific	2009
9 D	AG 2 WB	Macrosclerotia		<i>P. vulgaris</i> cv. unknown	1229	North Pacific	2009
11 D	AG 2 WB	Macrosclerotia	JX294316	<i>P. vulgaris</i> cv. unknown	1203	North Pacific	2009
3812	AG 1-IA	Microsclerotia	JX294340	<i>P. vulgaris</i> cv. unknown	1647	South Pacific	2008
3307	AG 2WB	Macrosclerotia		<i>P. vulgaris</i> cv. unknown	1600	South Pacific	2007
3796	AG 1-IE	Macrosclerotia	JX294331	<i>P. vulgaris</i> cv. unknown	1600	South Pacific	2008
3791	AG 1-IE	Macrosclerotia	JX294328	<i>P. vulgaris</i> cv. unknown	1600	South Pacific	2008
3800	AG 1-IE	Macrosclerotia	JX294333	<i>P. vulgaris</i> cv. unknown	1645	South Pacific	2008
3810	AG 1-IA	Macrosclerotia	JX294339	<i>P. vulgaris</i> cv. unknown	1642	South Pacific	2008
3798	AG 1-IF	Microsclerotia	JX294332	<i>P. vulgaris</i> cv. unknown	1600	South Pacific	2008
3794	AG 1-IE	Macrosclerotia	JX294329	<i>P. vulgaris</i> cv. Kiura Colorado	1600	South Pacific	2008
3240	AG 1-IE	Macrosclerotia	JX294318	<i>P. vulgaris</i> cv. unknown	1600	South Pacific	2007
3233	AG 1-IE	Macrosclerotia		<i>P. vulgaris</i> cv. unknown	1600	South Pacific	2007
3801	AG 1-IE	Macrosclerotia	JX294334	<i>P. vulgaris</i> var. Brunca	1645	South Pacific	2008
3803	AG 1-IF	Microsclerotia	JX294335	<i>P. vulgaris</i> cv. Green bean	1640	South Pacific	2008
3792	AG 1-IF	Microsclerotia		<i>P. vulgaris</i> cv. unknown	1600	South Pacific	2008
3811	AG 2 WB	Microsclerotia		<i>P. vulgaris</i> cv. Poroto amarillo	1640	South Pacific	2008
3838	AG 2 WB	Microsclerotia	JX294341	<i>P. vulgaris</i> cv. unknown	1600	South Pacific	2007
3708	AG 2 WB	Microsclerotia		<i>P. vulgaris</i> cv. unknown	1642	South Pacific	2008
3249	AG4	Microsclerotia	JX294320	<i>P. vulgaris</i> cv. unknown	1600	South Pacific	2007
3845	AG 1-ID	Microsclerotia	JX294344	<i>P. vulgaris</i> cv. Cuba	1138	Caribbean Region	2008
3236	AG 1-IE	Macrosclerotia		<i>P. vulgaris</i> var. Cabecar	600	Caribbean Region	2007
3847	AG 2 WB	Macrosclerotia		<i>P. vulgaris</i> cv. unknown	1149	Caribbean Region	2007
3628	AG 1-IE	Macrosclerotia	JX294324	<i>P. vulgaris</i> cv. unknown	1000	Southern Mountain Region	2008

Cont.

Cont.

Code	Anastomosis subgroup	Sclerotium size	GenBank accession number	Host	Altitude	Climate subregions	Year
3628	AG 1-IE	Macrosclerotia	JX294324	<i>P. vulgaris</i> cv. unknown	1000	Southern Mountain Region	2008
3626	AG 1-IE	Macrosclerotia	JX294323	<i>P. vulgaris</i> cv. Landrace	1125	Southern Mountain Region	2008
3636	AG 1-IE	Macrosclerotia		<i>P. vulgaris</i> cv. unknown	1000	Southern Mountain Region	2008
3242	AG 1-IF	Microsclerotia	JX294319	<i>P. vulgaris</i> cv. unknown	1600	Southern Mountain Region	2007
3625	AG P	Macrosclerotia	JX294322	<i>P. vulgaris</i> cv. Mantequilla	1000	Southern Mountain Region	2008
3629	AG P	Microsclerotia	JX294325	<i>P. vulgaris</i> var. Mexico 80	1125	Southern Mountain Region	2008
3668	AG 1-IB	Macrosclerotia		<i>P. vulgaris</i> cv. unknown	543	Southern Mountain Region	2008
3620	AG 2 WB	Microsclerotia	JX294321	<i>P. vulgaris</i> cv. unknown	1097	Southern Mountain Region	2008
3806	AG 1-IA	Macrosclerotia	JX294336	<i>P. vulgaris</i> cv. unknown	1107	Northern Region	2008
3804	AG 1-IB	Microsclerotia		<i>P. vulgaris</i> cv. unknown	1214	Northern Region	2008
3808	AG 1-IE	Macrosclerotia	JX294338	<i>P. vulgaris</i> cv. unknown	1202	Northern Region	2008
3077	AG 1-IE	Macrosclerotia		<i>P. vulgaris</i> cv. unknown	1000	Northern Region	2007
3093	AG 1-IE	Macrosclerotia	JX294317	<i>P. vulgaris</i> cv. unknown	1000	Northern Region	2007
3840	AG 1-IF	Microsclerotia		<i>P. vulgaris</i> cv. unknown	1236	Northern Region	2008
3841	AG 1-IB	Microsclerotia	JX294342	<i>P. vulgaris</i> cv. unknown	1123	Central Valley	2008
3843	AG 1-IE	Macrosclerotia	JX294343	<i>P. vulgaris</i> cv. unknown	600	Central Valley	2008
3973	AG 1-IE	Macrosclerotia		<i>P. vulgaris</i> cv. unknown	1042	Central Valley	2008
Cach	AG 2 WB	Microsclerotia		<i>P. vulgaris</i> cv. unknown	1042	Central Valley	2008
3677	AG 1-IE	Macrosclerotia	JX294326	<i>P. vulgaris</i> cv. unknown	543	Central Valley	2008
3667	AG 1-IE	Macrosclerotia		<i>P. vulgaris</i> cv. Landrace	700	Central Valley	2008
3793	AG 1-IF	Microsclerotia		<i>P. vulgaris</i> cv. unknown	700	Central Valley	2008
3795	AG 1-IF	Microsclerotia	JX294330	<i>P. vulgaris</i> cv. unknown	1470	Central Valley	2008

Cont.

Cont.

Code	Anastomosis subgroup	Sclerotium size	GenBank accession number	Host	Altitude	Climate subregions	Year
3807	AG 2 WB	Macrosclerotia	JX294337	<i>P. vulgaris</i> cv. unknown	646	Central Valley	2008
3809	AG 2 WB	Macrosclerotia		<i>P. vulgaris</i> cv. unknown	646	Central Valley	2008
	AG 1-IB		AJ868444				
	AG 2-2		AF308624				
	AG 2 WB		AF308623				
	AG P		JF519829				
	AG 1-IA		AB195928				
	AG 1-IB		AB122139				
	AG 1-IC		AB122141				
	AG 1-ID		EF197798				
	AG 1-IE		JF946728				
	AG 1-IF		JF946731				
	AG 4		JQ669932

FIGURE 1 - Degree of virulence (average affected area) of *Rhizoctonia solani* isolates inoculated *in vivo* on detached bean leaves, evaluated at 72 hours post inoculation (h.p.i) according to virulence groups: A, low; B, medium; C, high.

FIGURE 2 - Average affected area (cm²) by the most virulent isolates (group C) at 24, 48 and 72 hours.

FIGURE 3 - Costa Rica map and anastomosis group distribution by altitude (meters above sea level).

FIGURE 4 - Phylogenetic analysis using Bayesian inference based on ITS sequences, using MrBayes 3.2 with 1,000,000 Markov chains, with a multiple sequence alignment with the MAFFT algorithm on the GUIDANCE server.

previously been reported in *P. vulgaris*. The phylogenetic tree (Figure 4) confirmed the grouping of isolates in two clades. The first clade contained accessions of AG 4 and AG 2 WB from diverse climate regions. Bootstrap values from 60-100% allowed subgroup separation. Accessions of the AG 1 subgroup were included in second clade. This was a heterogeneous group that included AG 1-ID, AG 1-IE, AG 1-IF and AG P from different geographical regions of the country. NJ and MP trees were constructed (data not shown) and similar results were found.

DISCUSSION

Fifty-one isolates of *R. solani* were obtained from symptomatic leaves of common bean from different climate regions of Costa Rica. Isolates were characterized by size of sclerotia (macro or micro). Godoy-Lutz et al. (2003) point out the importance of mycelium and sclerotia in the epidemiology of the disease, as they constitute the main source of primary inoculum. AG 1-IF and AG 1-IE differ in sclerotia size and culture pigmentation. These

characteristics could be a key for distinguishing between isolates (Godoy-Lutz et al., 2003). The different phenotypes found in this study is indicative of the high variation that this fungi presents in our study.

Of the 51 isolates, 18 were from the South Pacific Region, and seven from the Northern Pacific Region (Table 1), both areas of major bean production in Costa Rica. The lowest number of isolates was found in the Caribbean, which is not a bean producing region and it is located near the Atlantic shore. The isolates with the highest virulence according to the CIAT scale and the measured area were placed in group C. Isolate 3843 was considered the most virulent at 24 h.p.i, because it caused the highest amount of affected foliar tissue in the shortest time period. At 48 hours there was no more healthy foliar tissue to infect, so at 72 hours, the affected area was similar to the other isolates. This isolate is characterized by macroesclerotia. Godoy-Lutz et al. (2003) mention that AG 1 macroesclerotia producing isolates were the most virulent, and AG 4 no if you read well is AG 2-2 the least virulent. Similar results have been observed in Latin America and the Caribbean, where virulence patterns vary between regions and genotypes (González-Vera et al., 2010).

Diverse levels of virulence between and within the identified groups were observed. The most virulent isolate (3843) was identified as AG 1-IE, and the least aggressive isolate was AG 2 WB. Isolate 3843 was from the Central Valley region, an area with precipitation from 1500 to 2500 mm³ and average temperatures between 19 and 22°C. With these data one might infer that these are favorable conditions for the disease development. Beaver et al. (2002) report differences in virulence on common bean using isolates of *R. solani* from Central America and the Caribbean. Lee et al. (2006) mention that the virulence of *R. solani* can be affected by environmental conditions present at the beginning of the infection.

No relation was observed between virulence, AG and geographical origin. Different AGs and subgroups were present in all of the regions sampled, regardless of edaphic and climatic conditions. This demonstrates the great adaptability and survival capacity of this pathogen, an important consideration in breeding *P. vulgaris* for resistance. The variable genetic composition of the fungus can affect the dynamics of the pathogen-host relationship, as pathogens are able to adapt to specific ecological niches in diverse agrosystems (Araya, 2003).

Results of this study show that WB in common bean in Costa Rica is caused at least by four AGs, AG 1, AG 2, AG 4 and AG P, and diverse subgroups, AG 1-IA, AG 1-IB, AG 1-IC, AG 1-ID, AG 1-IE, AG 1-IF, AG 2-WB and AG 4. The dominant groups in this study were AG 1 (65%) and the AG 1-IE subgroup (37%). AG 1-IE and AG 2 WB subgroups were found in every climate region sampled. AG 1-IE was the most frequent (Table 1). These results are consistent with reports in the literature that show a prevalence of this subgroup in field populations causing WB

in common bean in several Latin American and Caribbean countries (Echandi, 1965; Godoy-Lutz et al., 1996b).

The presence of different AGs found in this study in different geographical regions suggests that bean varieties are susceptible to a broad range of AG subgroups (Godoy-Lutz et al., 2008). The presence of AG P, not previously reported in bean, in the North Pacific and Southern Mountain regions, suggests that new AGs may be appearing. AG P is a heterogeneous group, as shown by the percent similarity between isolates of this AG using MP and NJ methods. For some isolates, similarity ranged from 93% to 100%, but for others it was lower, from 90% to 94%. This AG may include several subgroups, but additional work with more isolates is needed to confirm the existence of AG P subgroups (Sharon et al., 2008).

Identification with specific primers aids in determining the prevalence of an AG or subgroup in a certain geographic area. Population genetics of this pathogenic fungus may be affected by diverse mechanisms not yet elucidated. Since in this study AGs were not related to place of origin, it appears that many mechanisms, such as mating systems, reproduction strategies and gene flow, could be contributing to pathogen dispersal (González et al., 2006). Bean programs in Costa Rica in collaboration with farmers associations have been done during the last years to provide certified seeds. However, the presence of anastomosis subgroups between regions could be an indicative that contaminated seed movement still spreads in the country. Godoy-Lutz et al. (2003) affirm that gene flow may be occurring through seed movement as the pathogen can be spread on asymptomatic seeds (Godoy-Lutz, 2003).

One of the most important results of this research was to confirm the presence of different anastomosis subgroups in the country and their virulence. Systematical sampling of open field crops is necessary to generate recommendations for disease management such as use of crop rotations. According to Okubara et al. (2008) there are voids in the knowledge of pathogen dispersal, crop loss and the role of environmental conditions such as rain and temperature on pathogen populations.

The variable genetic composition of *R. solani* can be affected by the dynamics of the host-pathogen relationship, genetic flexibility and the degree of adaptation to the ecological niche in diverse agroecosystems (Godoy-Lutz et al., 2008). Our phylogenetic tree shows that some anastomosis subgroups are not closely related to the controls. This may be an indication of the mentioned plasticity of the genetic composition of the *R. solani* AG subgroups since they present higher sequence variability than other isolates of *R. solani*. AG P is closer to AG 1-IC. AG P clusters may split into several clusters. Some AG P and AG S, R and Fa clusters have appeared between AG 2-1, AG 2-2 and AG 12. Other AG P isolates cluster together with AGs Fb and E and are between AG 6, AG 1-IC and AG 7 subgroups in some cases (Sharon et al., 2008).

The support percentages of the Bayesian analysis ranged from 60% to 100%, most of the results were supported by a high value. This supports the tree structure for these isolates and their identification within an AG group or subgroup. Previous studies have shown that AG 4 and AG 2-WB have a close phylogenetical relationship, supporting our hypothesis presented on the tree. In this case, the sequences used were 5.8S rDNA with flanking sequences of ITS1 and ITS2. Sharon et al. (2008) affirm that there is a difference between ITS 1 and ITS2 in terms of variability. ITS 1 sequences are more variable than ITS2 among fungal isolates, but the combined 3 sequences ITS1 + 5.8S + ITS2 used in this study give more information than either sequence separately.

Proper deployment of molecular methods allows the identification and over time monitoring of subgroups associated with the disease in high epidemic risk areas and provides an important tool for analysis of isolate variation in the field and the possible generation of more pathogenic AG subgroups. Knowledge of virulence variation and distribution of the AGs in the country is useful in the assessment of management and breeding strategies in Costa Rica.

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