

# Morphological, Molecular, and Pathogenic Characterization of *Rhizoctonia solani* Isolates Associated with Bean Drying in Northern Sinaloa, Mexico

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## ABSTRACT

**Objective:** The objective of this study was to characterize, using morphology, DNA sequence analysis, and pathogenicity, *R. solani* isolates associated with bean plants with root rot symptoms in commercial plots in northern Sinaloa.

**Design/methodology/approach:** During the 2020-2021 cycle, diseased plants infected by *Rhizoctonia* were collected in the municipalities of Ahome, El Fuerte, and Guasave. Pure fungal isolates were obtained in specific media; which were morphologically characterized in PDA medium and preserved. Subsequently, the pathogenicity of the isolates was evaluated and they were molecularly identified. Genomic DNA was extracted from the isolates, part of the RPB2 gene was amplified by PCR, and the amplified products were sequenced.

**Results:** Phylogenetic analysis with RPB2 sequence data confirmed the identification of 63 isolates as *R. solani* and allowed them to be assigned to the anastomosis group (AG): AG-4. Of the total isolates analyzed, 86% correspond to the AG-4 HGI anastomosis subgroup and 14% to the AG-4 HGIII subgroup. In pathogenicity, the percentage of germination and severity of the isolates were evaluated, showing different levels of pathogenicity.

**Limitations of the study/implications:** None.

**Findings/conclusions:** *Rhizoctonia solani* AG-4 anastomosis subgroups HGI and HGIII are associated with bean drying in northern Sinaloa. Therefore, this study will serve as a basis for other studies that generate control strategies for this pathogen.

**Keywords:** pathogenicity, sclerotia, RPB2, phylogenetic analysis, anastomosis.

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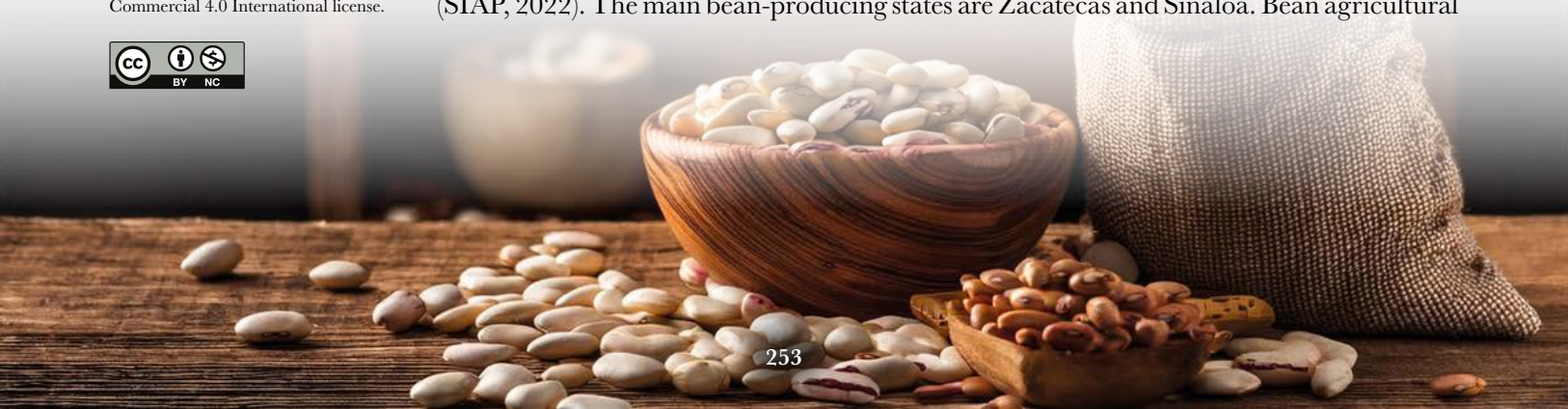
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## INTRODUCTION

The production of beans (*Phaseolus vulgaris* L.) is one of the most significant agricultural activities in Mexico's economy. The annual income generated by bean cultivation in the country ranges around 17 million pesos, with a production exceeding 965 thousand tons (SIAP, 2022). The main bean-producing states are Zacatecas and Sinaloa. Bean agricultural



production in Sinaloa takes place during the autumn-winter cycle and covers a planted area of 63,262 hectares, of which 70% is established in northern Sinaloa, in the municipalities of Ahome, El Fuerte, and Guasave (SIAP, 2024). Local bean production is characterized by poor organic matter soils, highly fragmented without crop rotation schemes, and very variable environmental conditions, which favor the incidence of pests and diseases that jeopardize and limit this activity. A recurring problem in northern Sinaloa is bean drying, and associated with this, phytopathogenic fungi have been found, among which the species *Rhizoctonia solani* occurs most frequently.

The basidiomycete *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris*) is considered a heterogeneous genus of fungi that inhabit the soil, with a global distribution. It is a phytopathogen that infects a wide variety of agriculturally important crops (Chavarro *et al.*, 2015; Gonzalez, 2006; Kanetis *et al.*, 2016). The genetic, morphological, and pathogenic variability of *R. solani* strains has classified it into 14 anastomosis groups (AG) that are reproductively incompatible with each other (Carling *et al.*, 2008). The anastomosis groups are numbered from AG1 to AG13 and AG BI (Cubeta and Vilgalys, 1997; Hane *et al.*, 2014). The complexity of the species within the anastomosis groups has led to the subdivision of many AG into anastomosis subgroups that differ in one or more biochemical, genetic, or pathogenic characteristics (Cubeta and Vilgalys, 1997).

Traditionally, AGs were assigned by observing the fusion of hyphae with known tester isolates. However, molecular analysis is now the most efficient method for the classification of *Rhizoctonia* (Sharon *et al.*, 2006). This allows for the determination of the species, the anastomosis group, and the corresponding anastomosis subgroup. In beans, the reported anastomosis groups are AG 1, AG 2, and AG 4. Individually, they have been associated with different types of diseases. For example, AG 1 and its various registered anastomosis subgroups in beans include AG-IA, AG 1-IB, AG 1-IE, and AG 1-IF, which cause damage to foliage, resulting in blighting, necrotic and watery lesions on the leaves (Gonzalez, 2008; Valentin *et al.*, 2016). In contrast, AG 2 and AG 4 cause root lesions, such as dark cankers, corky lesions, and plant drying (Woodhall *et al.*, 2008). Host specificity among anastomosis groups is variable (Carling *et al.*, 2002; Fiers *et al.*, 2011). AG 4 was originally thought to infect only potatoes; however, it has been reported that isolates from this group also infect soybean, corn, tobacco, tomato, sugar beet, beans, among others.

In bean plants, the AG-4 anastomosis group of *R. solani* is recognized for causing seed rot, post-emergence wilting, tip rot, and root rot in important crops such as beans.

In bean plants, the AG-4 anastomosis group of *R. solani* is recognized for causing seed rot, post-emergence wilting, tip rot, and root rot in important crops such as beans. Currently, Mexico lacks research on this species; previous studies have only focused on *Rhizoctonia solani* in vegetables like potato, as it has had the most significant problems with this pathogen. Regarding bean cultivation in Sinaloa, there are no studies related to *Rhizoctonia solani*, despite the ongoing and aggressive presentation of Rhizoctoniasis. In this regard, the objective of the research is to morphologically, molecularly, and pathologically identify the anastomosis groups and subgroups of *Rhizoctonia* present in bean cultivation, which will help determine control strategies that should focus on seeking sustainable alternatives.

## MATERIALS AND METHODS

During the period from 2020 to 2021, 50 bean lots were inspected in the municipalities of El Fuerte, Ahome, and Guasave in northern Sinaloa. Each lot was sampled using the “five of gold” technique (Martínez, 2012), with 2 samples taken per sampling site, resulting in 10 samples of infected tissue from each lot. These samples were placed in plastic bags labeled with the location and date, geographical coordinates, variety, and symptoms, and then stored in a cooler at approximately 8-10 °C (Agrios, 2005) for transportation to the laboratory for processing. For the isolation of *Rhizoctonia solani*, 0.5×0.5 cm cuts were made from the margins of the lesions and were planted in 8.5 cm diameter Petri dishes containing acidified PDA (potato dextrose agar) with lactic acid (Yang *et al.*, 2014; Muzhinji *et al.*, 2015).

The cultures were incubated at 25 °C for 48 hours and observed under a biological microscope to purify the colonies of *Rhizoctonia solani*. The isolates were transferred to 1.6% water agar (WA) and incubated at 25 °C. After 24 hours, the plates were examined under the microscope to select a hyphal tip, which was then transferred to PDA medium (Sneh, 1996) and incubated at 25 °C for 7 days. Subsequently, the isolates were preserved in 30 ml Falcon tubes with sterile substrate and sterile distilled water.

From the pure strains obtained from *Rhizoctonia solani*, morphological characterization was performed by taking macro and microscopic variables such as colony color, sclerotium formation, hyphal diameter, and number of nuclei per hypha (Sneh *et al.*, 1991). The isolates were grown on PDA and incubated at 25 °C for 30 days, with daily inspections to measure the time of sclerotium formation and colony color. To measure the diameter of hyphae and nuclei per hyphal cell, 7-day-old colonies were used. The mycelium from each isolate of *Rhizoctonia solani* was transferred to a glass slide and stained with Safranin-O (Bandoni, 1979). Twenty hyphal cells were examined under a biological microscope with an Olympus Lx micrometer.

To evaluate the anastomosis reaction among the isolates, Petri dishes containing 10 ml of agar water were used, with a sterile slide placed inside. On one end of the slide, a 0.5 cm<sup>2</sup> piece of mycelium agar from one isolate was placed, and on the other end, a different isolate was added for confrontation. The setup was incubated at room temperature for 24 hours, and then observed under a microscope to check if the hyphae from the two isolates made contact. If contact occurred, the contact zone was stained with 0.05% trypan blue, and the type of anastomosis reaction was determined according to Carling (1998).

For molecular identification, genomic DNA was extracted from each of the two isolates. The DNA extraction was performed using the CTAB method (Murray and Thompson, 1980; Doyle and Doyle, 1990; Porebski *et al.*, 1997) from 50 to 100 mg of mycelium. Partial fragments of the second largest subunit of the RNA polymerase II gene (*rpb2*) were amplified and sequenced using the primer pairs RBP2-980F (5'-TGYCCIGCIGARACICCHGARGG-3') and RPB2-7Cr (5'-CCCATRGCCTTGYTTTRCCCAT-3') (Liu *et al.*, 1999; Reeb *et al.*, 2004), respectively.

Subsequently, the sample was sequenced. The sequence data obtained were compared using a BLAST search in the National Center for Biotechnology Information (NCBI) database to determine the anastomosis group (AG) of the individual isolates. The sequences

were aligned using the Clustal W algorithm integrated into the MEGA 6.0 software package (Tamura *et al.* 2013), and the phylogenetic relationship among isolates was calculated using the Neighbor-Joining (NJ) method (Saitou and Nei 1987) under the Kimura two-parameter model (Kimura, 1980) as the substitution model, omitting all sites with gaps. For comparison purposes, ITS rDNA sequences from other known AG isolates were obtained from GenBank and used for phylogeny. The Bootstrap analysis was performed using 1000 pseudoreplicates of the dataset. The sequence of *Botryobasidium simile* (GenBank accession number GEL2348) was used as the outgroup for rooting.

Pathogenicity tests for each isolate were conducted by inoculating 5 seeds of common bean, variety Azufrado Higuera, planted in 2 kg pots with agricultural soil. A total of 50 ml of a mycelial suspension adjusted to a concentration of  $1 \times 10^5$  mycelial fragments/ml was placed directly onto the seeds.

As a control, five seeds of common bean, variety Azufrado Higuera, were used without inoculation. All plants were maintained in a greenhouse for 15 days at temperatures ranging from 22 to 32 °C. Four repetitions were made for each treatment. Germination percentages were evaluated 10 days after planting, and symptoms of root rot, stem canker in the seedlings, and wilting were assessed 30 days after planting. The pathogenicity test was conducted twice with similar results. The fungi were re-isolated from the infected roots and found to be morphologically identical to the isolates used for inoculation, thereby fulfilling Koch's postulates. To assess the severity in the roots and hypocotyls of each plant, an ordinal scale from 0 to 5 was used, developed by Cardoso and Echandi (1997). ANOVA was performed with a  $p > 0.05$ .

## RESULTS AND DISCUSSION

During field surveys, the symptoms observed in common bean crops in the municipalities of Fuerte, Ahome, and Guasave included plant wilting, corky lesions at the base of the stem, dark cankers, and root rot (Figure 1), as described by Woodhall and colleagues (2008) for *Rhizoctonia* spp.



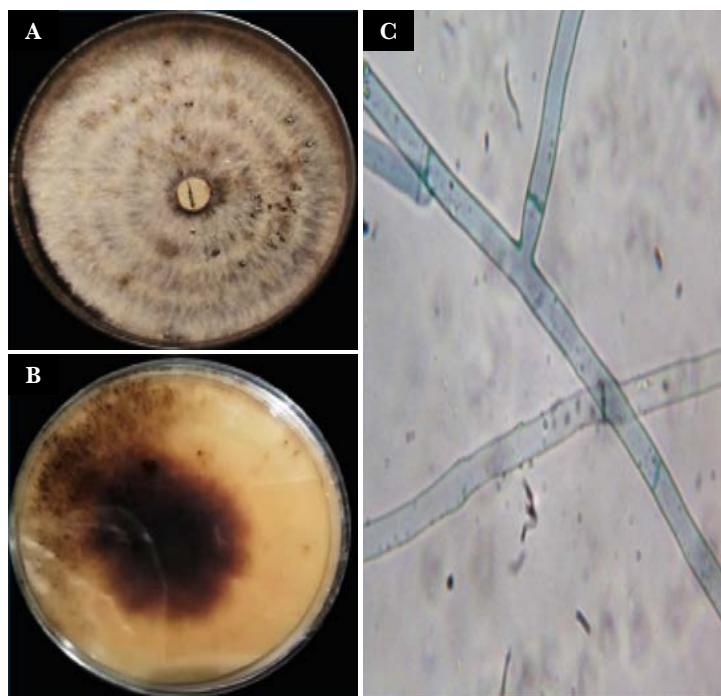
**Figure 1.** Symptoms in field bean plants. A. Bean plant wilt. B. Dark cankers at the base of the stem and root. C. Corky lesions.



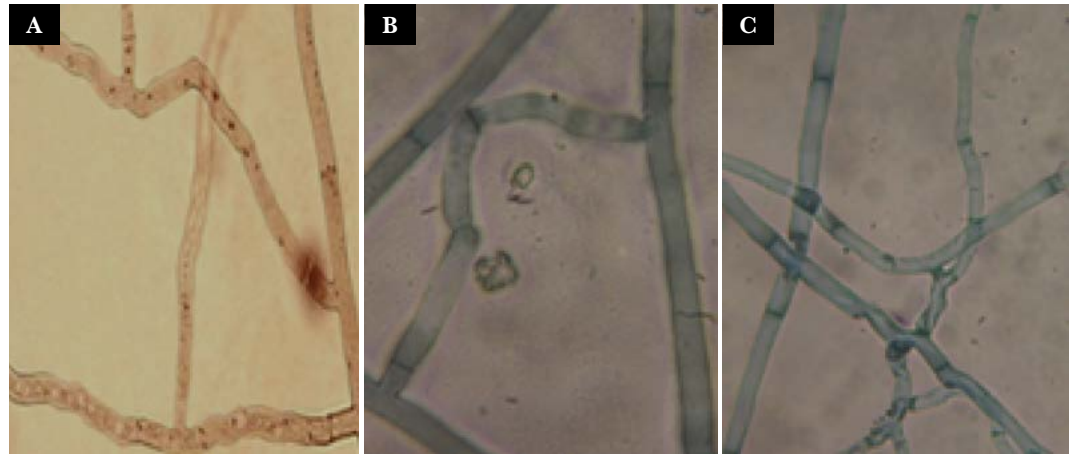
A total of 71 isolates were obtained, named from FAVF400 to FAVF470. The colonies of isolates from bean plants exhibiting wilt symptoms showed a cottony mycelial growth ranging from white to brown, turning the medium amber. The mycelium of the isolates is septate, hyaline, with constriction at the basal cell, forming branches at right angles, with hyphae measuring 5-8  $\mu\text{m}$  in width and sclerotia 1-3 mm in diameter, with no presence of spores (Figure 2), typical characteristics of *R. solani* (Sneh *et al.*, 1991; Wantanabe, 2002; Zitter *et al.*, 2004). Staining of nuclei revealed the presence of only multinucleate isolates, and in the anastomosis test, all isolates anastomosed with each other, classifying into categories C3 and C2 (Figure 3), according to Carling *et al.*, 1988 and MacNish *et al.*, 1993.

The molecular identification of 63 isolates, based on partial fragments of the second largest subunit of RNA polymerase II gene (*rpb2*), using the primer pairs RBP2-980F and RPB2-7Cr, revealed that the 63 sequences had 99-100% similarity to *Rhizoctonia solani* with homologous sequences from NCBI. Phylogenetic analysis was performed with the sequences of the isolates of *Rhizoctonia solani*, *Rhizoctonia oryzae-sativa*, and *Botryobasidium simile*. A matrix was processed comprising the RPB2 regions of 84 isolates, including those from the present study and 21 reference strains. This analysis established the identity of a single anastomosis group for *Rhizoctonia solani* AG-4, and two subgroups of this, where 54 isolates were found to belong to AG-4 HGI and 9 isolates to the AG-4 HGIII subgroup (Figure 4).

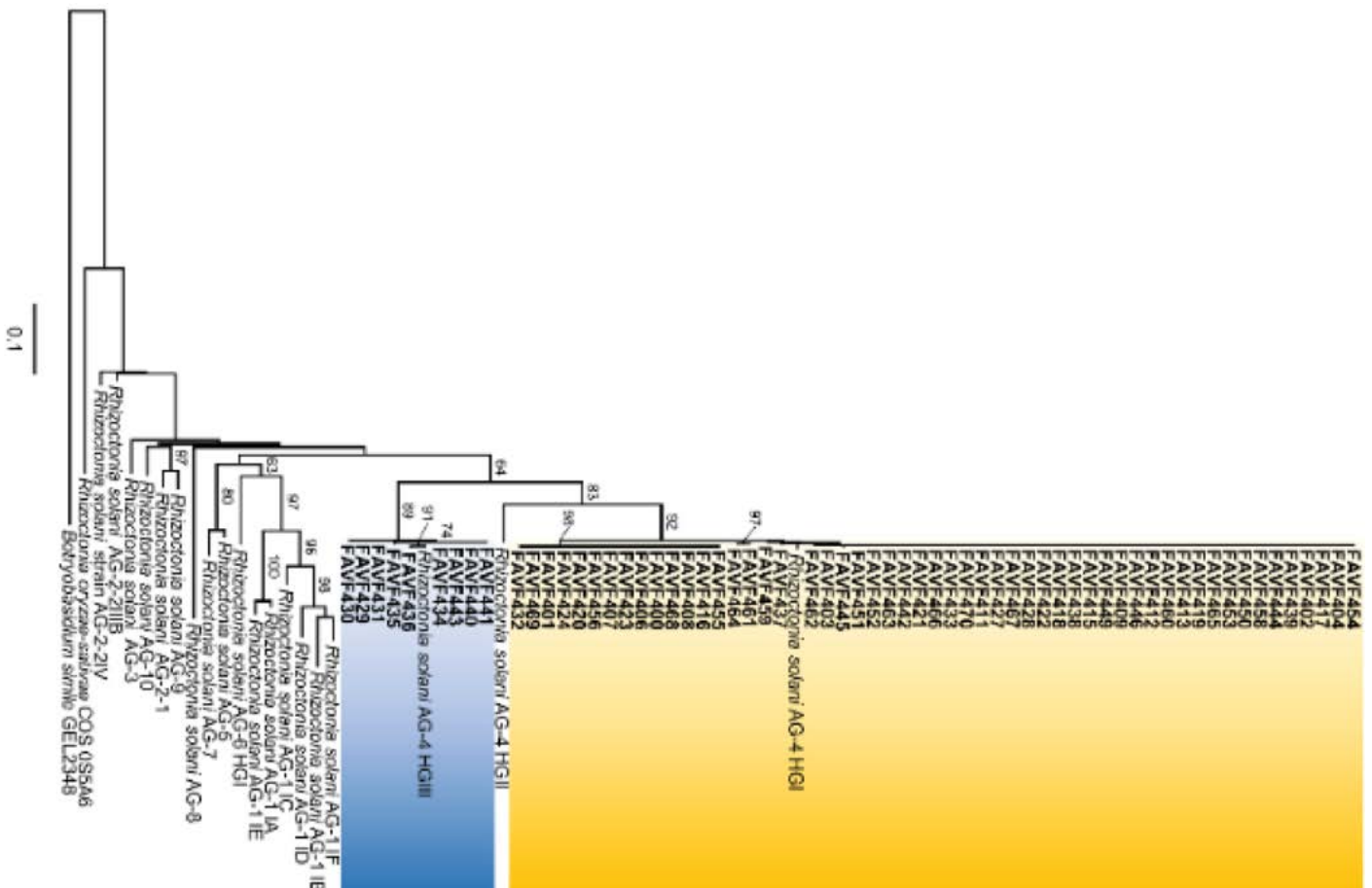
In Mexico, these anastomosis subgroups have been reported in cultivated plants such as hibiscus, potato, chili, and tomato (Moreno *et al.*, 2013; Ortega *et al.*, 2022), but not in



**Figure 2.** Morphology of the colonies and coloration of the medium in PDA of *Rhizoctonia* spp. A. Cottony mycelium and microsclerotia on the surface in PDA. B. Color change of the culture medium. C. Septate mycelium, constriction at the basal cell, and formation of right angles.



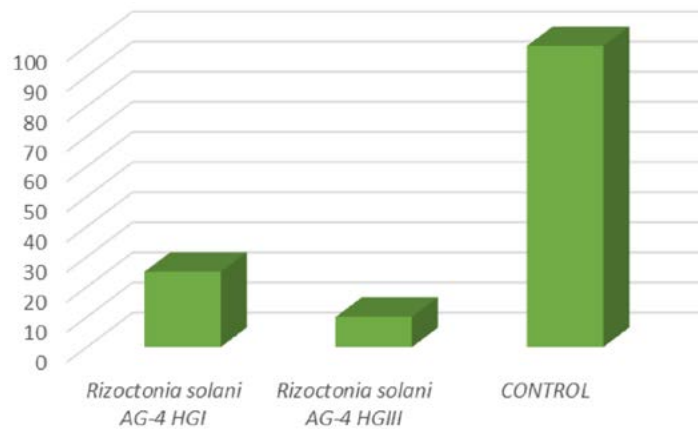
**Figure 3.** Staining of nuclei and anastomosis reaction. A. Multinucleated hyphae. B. Wall fusion, fused membranes; the anastomosis point is not very frequent. The diameter of the anastomosis is almost always equal to that of the hypha. Anastomosis reaction C3, positive, indicates that the confronted isolates belong to the same AG. C. Obvious wall connection; it is unknown whether there is membrane exchange. Adjacent cells die, and the fusion diameter is very narrow. Anastomosis reaction C2, positive, indicates that the confronted isolates belong to the same AG but a different subgroup.



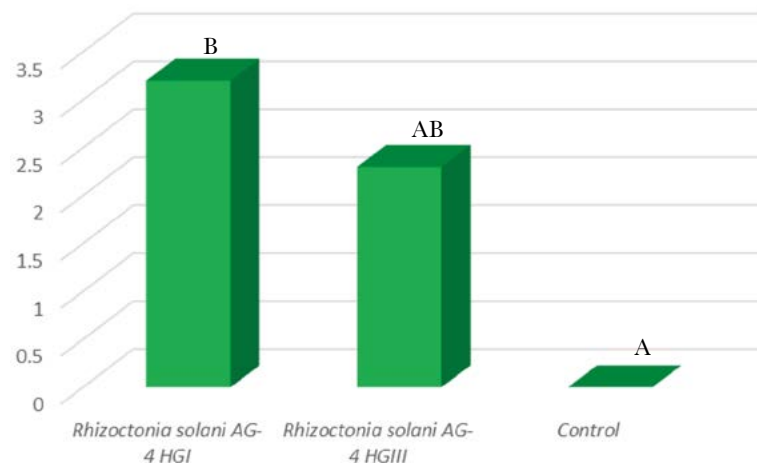
**Figure 4.** Phylogeny by ML of the concatenated RPB2 gene sequences of 63 *Rhizoctonia* spp. isolates representative of 70 obtained from bean plants exhibiting wilting and root rot symptoms. Bootstrap of 1000 replicates; outgroup: *Botryobasidium simile*. AG=Anastomosis group.

common bean. However, studies in other bean-producing countries report the presence of anastomosis groups AG-1, AG-2, AG-3, AG-4, AG-5, AG-6, AG-9, AG-10, and AG-11. In the northern region of Sinaloa, Mexico, the results showed that all the *R. solani* isolates obtained from common bean crops in the municipalities of El Fuerte, Ahome, and Guasave belong to the anastomosis group AG-4. This aligns with previous reports indicating that *R. solani* AG-4 is the predominant AG in bean production areas, causing root rot and plant wilting worldwide, followed by AG-1 and AG-2 (Gülsüm *et al.*, 2024). The HGI and HGIII subgroups were the most prevalent.

In the pathogenicity test, all isolates were found to be pathogenic, directly affecting the germination process of common bean seeds by up to 100% compared to the controls (Figure 5). Symptoms associated with rhizoctoniasis appeared 10 days after inoculation, with infected plants showing root rot, dark cankers on the root and hypocotyl, corky lesions, and wilting. The treatments applied showed significant differences compared to the control (Figure 6), with the HGI subgroup being the most severe.



**Figure 5.** Percentage of bean seed germination.



**Figure 6.** Severity of anastomosis subgroups. Means with a common letter are not significantly different ( $p > 0.05$ ).

This demonstrates that the fungi isolated from *Rhizoctonia solani* AG-4 HGI and HGIII are causal agents of root rot and wilting in beans in northern Sinaloa. The results of the seed germination percentage and the severity of the isolates showed that these pathogens are capable of limiting crop development, leading to decreased yield and production of this grain, resulting in economic losses due to root diseases in the studied regions.

## CONCLUSIONS

*Rhizoctonia solani* was found naturally causing corky lesions, black cankers, root rot, and plant wilt in common bean crops in northern Sinaloa, particularly in the municipalities of Ahome, El Fuerte, and Guasave. The anastomosis group present in the bean crop in northern Sinaloa is AG-4, along with its subgroups HGI and HGIII. Pathogenicity tests indicated that both anastomosis groups cause disease in beans, being extremely severe, preventing seed germination, and leading to the manifestation of symptoms such as plant wilt.

Future studies should focus on the search for common bean varieties resistant to rhizoctoniasis, as well as on disease control and pathogenicity in other crops, to develop a management strategy against the different groups and subgroups of anastomosis of *Rhizoctonia solani*. The pathogenicity tests indicated that both anastomosis groups cause disease in beans, being extremely severe, preventing seed germination, and leading to the manifestation of symptoms such as plant wilt. Future studies should focus on the search for common bean varieties resistant to rhizoctoniasis, as well as on disease control and pathogenicity in other crops, to develop a management strategy against the different groups and subgroups of anastomosis of *Rhizoctonia solani*.

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