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Article

Evaluation of resistance to neck rot by *Sclerotium rolfsii* Sacc. in chickpea genotypes

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Abstract

They were collected from plants with symptoms of yellowing and wilting in commercial chickpea fields in five municipalities in southern Guanajuato, Mexico. From these, the pathogens present in the stem and root were isolated and identified. Of the isolates obtained, those with morphological characteristics of *Sclerotium rolfsii* had their identity confirmed by sequencing the ITS fragments obtained with the oligonucleotides: NsiF and Nlb4R; the sequences obtained were compared with the NCBI database. Of the two isolates identified as *S. rolfsii* by both methodologies, the most virulent was identified, which was used to inoculate 181 chickpea genotypes of the kabuli and desi types. The results indicate that, according to the mortality percentage, the genotypes of the desi type ICC3287, ICC 4874, ICC1882, ICC 10259 and WR-315, and kabuli type CUGA 08-3160 presented moderate resistance to the pathogen. Of these resistant genotypes, ICC 10259 stands out, which has genetic resistance to four races of *Fusarium oxysporum* f. sp. *ciceris* and *M. phaseolina*.

Keywords:

Anthelia rolfsii, Cicer arietinum L., kabuli type, desi type.

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Introduction

Neck rot in chickpea (*Cicer arietinum* L.) is caused by *Sclerotium rolfsii* Sacc., a soil-dwelling pathogen that has a wide host range, around 500 species (Chen *et al*., 2011; Pande *et al*., 2012), which causes root and neck rots. The disease is known by different names such as basal stem and root rot, sclerotial disease, or southern blight. The importance of this disease lies in the fact that the pathogen can attack the plant throughout its life cycle and survive in residues from previous sowings (Red SICTA-IICA, 2008).

The symptoms it causes are yellowing of the lower leaves, sinking, softening and discoloration of the bark below the soil line, the disease progresses as the days go by, and leaves and upper branches wilt until the plant dies (Chitale *et al*., 1990). The formation of sclerotia usually appears in large quantities at the base of plant stems, and the development of this disease requires temperatures of 15 to 21 °C and high relative humidity (Chitale *et al*., 1990).

Under favorable conditions, this fungus can produce large amounts of mycelium in infected tissues, in addition to being transmitted by the seed and therefore affecting its emergence or the death of the surviving seedlings (Pande *et al*., 2012). These symptoms are often difficult to distinguish from those caused by other phytopathogenic fungi such as *Fusarium solani* (Mart.) and *Rhizoctonia solani* (Kuhn.).

The main characteristics of *S. rolfsii*, in the potato dextrose agar (PDA) culture medium, are the rapid growth of the mycelium, which covers the 9 cm diameter of the Petri dish in 3 to 4 days, abundant whitish mycelium, and the formation of sclerotia as a resistance structure for survival. Hyphae are hyaline, septate, highly branched, with thin cell walls. Sclerotia are the main source of inoculum in most epidemics, although on a smaller scale, basidiospores may have the same function (Punja, 1985; Almeida *et al*., 2001).

This phytopathogen has the ability to thrive in a wide range of environmental conditions. In optimal environments it can cause a mortality of 55-95% of chickpea seedlings; in addition, it can survive in the form of mycelium in plant debris and/or as sclerotic structures in soil, causing it to persist in infested soils for long periods of time (Sharma and Ghosh, 2017).

The chickpea is a legume that is grown mainly in two regions of Mexico, northwest (Sonora, Sinaloa, and Baja California) and in 'El Bajío' (Guanajuato, Michoacán, and Jalisco). In the Bajío region, located in central Mexico, root rot is a major biotic stress in the crop, both for the 'desi' (brown and small in size) and the 'kabuli' (white and large in size) types.

In this region, the first report of the genus *Sclerotium* as a pathogen of chickpea crops was made by Huitrón and Campos (1966). Globally, Ghosh *et al*. (2013), when sampling in central and southern India, indicate the presence of various pathogens causing wilt and root rot in chickpeas, reporting that *Sclerotium rolfsii* was widely distributed in the region studied and was considered a major problem for the production of this crop. However, not enough information has been collected about *S. rolfsii* in Mexico in the Bajío region and how to control it.

Neck rot caused by *S. rolfsii* is a prevalent disease and can cause considerable losses to the plant when soil moisture is high and temperatures are warm (30 °C) at sowing (Pande *et al*., 2012). Hussain *et al*. (2006), when studying the effect of different inoculum levels, seedling age, and soil type on the development of neck rot in chickpeas, found that *S. rolfsii* caused higher seedling mortality in soils with high clay textures, and noted that susceptibility decreased with increasing plant age, with the youngest seedlings being the most susceptible.

The pathogen is favored by the presence of non-decomposed organic matter on the soil surface and excessive moisture at the time of sowing and at the seedling stage, a situation that can occur in the residual moisture cultivation system in the Bajío region when chickpeas are sown in clay soils (Vertisols) at the end of the rainy season, after a short-lived crop such as beans or oats (Hussain *et al*., 2006).

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Among the alternatives for managing the disease, chemical and biological control have been considered as viable alternatives; however, there are reports that mention that there is great variability in the behavior of the fungus when evaluations of chemicals and antagonists for the control of S. rolfsii (Bracho *et al.*, 1995; Hagan and Olive, 1999; Pérez-Moreno *et al.*, 2009; Guédez *et al.*, 2012; Martínez-Martínez *et al*., 2020).

The best alternative to control this disease in chickpeas is the development of resistant varieties; however, there are few reliable sources of resistance in the kabuli and desi types worldwide (Gurha and Dubey, 1983; Tripathi, 2006; Pande *et al.*, 2012; Singh et al., 2012; Amule *et al.,* 2014; Shirsole *et al*., 2018; Sager and Kumar, 2021) and no sources of resistance have been detected in Mexico.

Therefore, this research aimed to evaluate resistance to S. rolfsii in a group of elite genotypes of the chickpea improvement program of the National Institute of Forestry, Agricultural and Livestock Research (INIFAP, for its acronym in Spanish), in order to identify possible sources of resistance that can be used as progenitors of new varieties.

Materials and methods

Pathogen collections in the field

During the 2012-2013 autumn-winter season, a collection was made in five commercial kabuli chickpea fields located in municipalities in southern Guanajuato, including Celaya (20.584242 north latitude, 100.823528 west longitude), Salvatierra (20.305005 north latitude, 100.847980 west longitude), Cuerámaro (20.621625 north latitude, 101.671082 west longitude), Valle de Santiago (20.417354 north latitude, 101.101113 west longitude) and Pénjamo (20.438667 north latitude, 101.665407 west longitude). Ten plants per field, with symptoms of wilting and yellowing, were collected in plastic bags and transported in coolers to the phytopathology laboratory of the Bajío Experimental Field of INIFAP. They were kept at 4 °C until pathogen isolation.

Isolation and morphological characterization of pathogens

To isolate the fungi from diseased plants, 3 mm fragments were cut from the stems showing the typical damage of neck rot, they were disinfected by immersing them in a 5% sodium hypochlorite solution, for 1 min, rinsed in sterile distilled water for 1 min, and dried by placing them on sterile absorbent paper. Five fragments of each collected plant were placed in the Petri dish with acidified PDA culture medium (200 µl L-1 of 85% lactic acid), incubated at room temperature (22 to 25 °C) for three days.

The presence of isolated phytopathogenic fungi that occurred in the sampled plots was recorded, S. rolfsii was selected, and purification was performed through single-basidiosporic cultures, using the methodology reported by Punja and Grogan (1983). Permanent preparations were made from the purified colonies and their morphological characteristics were observed under the microscope (Leica model DME®, Germany). Isolates were identified based on color and sclerotia formation (Ainsworth, 1995; Kendrik and Carmichael, 1973; Pande *et al.*, 2012; Mahadevakumar *et al.*, 2016).

Determination of virulence

To determine the differences in virulence between the two morphologically identified isolates of S. rolfsii, inoculum of two isolates was prepared by growing mycelium for ten days in Petri dishes with PDA. Subsequently, under a completely randomized design with three repetitions, fifteen seeds of the desi chickpea genotypes were sown: ICC 1882, ICC 3287, ICC 4874, ICC 10926, and WR 315.

Five seeds per repetition were placed in pots containing 6 kg of soil inoculated with 20 fragments of 1 cm2 of mycelium and 164 ±16 sclerotia from four Petri dishes; the control treatment was one pot per genotype with five seeds. They were irrigated with purified water to field capacity, maintaining this level of humidity during the 25 days that the experiment lasted; the percentage of mortality was calculated every third day and was converted to the scale of 1-5 published by the Indian Institute of Pulses Research (IIPR, 1999).

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Molecular characterization of the pathogen

In order to corroborate the identity of the most virulent isolate, it was molecularly characterized by extracting DNA using the method of Doyle and Doyle (1990). The region of the internal transcribed spacers (ITS) of the ribosomal genes was amplified by PCR using the sense oligonucleotide NsiF 5'GATTGAATGGCTTAGTGAGG3' and the antisense oligonucleotide Nlb4R 3'GGATTCTCACCCTCTATGAC5'.

The reaction mixture used for DNA amplification was in a 25 µl reaction volume: 3 µl of template DNA (45 ng μl^{-1}), 2.5 mM of dNTPs, 25 mM MgCl2, 5 µM of each oligonucleotide, and 1 U of Taq polymerase (Palmerín *et al*., 2011). The conditions for the reaction were six minutes of initial denaturation at 95 °C, followed by 35 cycles with a denaturation program at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 7 min.

Electrophoresis of the amplified fragments was performed in agarose gels with 1.5% TBE 1X buffer at a voltage of 120 v; they were stained with GelRed® (Biotium Catalog No. 41002) and observed with ultraviolet light. The amplified 800 bp fragment was cut and purified by following the protocol of the purification package (QIAquick PCR Purification Kit, Catalog No. 28104); the obtained product was bound to the pGem T-easy vector (pGEM-T® easy vector systems, Promega Catalog No. A1360), and competent cells of *Escherichia coli* DH5α (Invitrogen™ Catalog No. 18265017) were transformed.

The separation of the plasmid DNA was performed by the alkaline lysis method (Birnboim and Doly, 1979), to test the presence of the insertion, the fragment was released by cutting it with the enzyme Eco R1. Two clones were sequenced with the inserted fragment and their sequence was compared with the National Center for Biotechnology Information (NCBI) database.

Chickpea genotypes

One hundred and eighty-one chickpea genotypes from the Chickpea Improvement Program of the National Institute of Forestry, Agriculture and Livestock Research (INIFAP, for its acronym in Spanish) were evaluated for their reaction to *S. rolfsii*. In terms of type, 147 were kabuli and 34 were desi (Table 1). Several of these genotypes were developed by the Improvement Program itself at its headquarters in Bajío, Culiacán and Hermosillo, and others corresponded to introduced accessions.

Table 1. Response to inoculation with *S. rolfsii* to kabuli and desi chickpea genotypes.

Reaction	Percentage of mortality	No. of genotypes, Kabuli type	No. of genotypes, Desi type
Resistant	< 10	0	0
Moderately resistant	10-20	1	5
Moderately susceptible	21-30	8	11
Susceptible	31-40	63	16
Highly susceptible	> 40	75	2

The isolate that showed the highest virulence in the previous experiment was used to inoculate chickpea genotypes. The inoculum was made as described above. In the greenhouse, 6 kg capacity pots were filled with sterile soil, irrigated to field capacity with purified water, and each pot was inoculated with 20 fragments of 1 cm^2 of mycelium and 160 ±12 sclerotia obtained from the culture of the pathogen in four Petri dishes. Subsequently, 15 seeds of each of the 181 chickpea genotypes were sown in duplicate in each pot.

Fifteen plants of each genotype without inoculation were considered as a control treatment; all genotypes were sown under the same conditions in the greenhouse. The experiment was established in April 2015 under a completely randomized design in natural light conditions and with average day/night temperatures of 28/17 ±3 °C. The count of seedlings that emerged and the

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calculation of the mortality percentage were carried out every third day after sowing, during the 25 days that the experiment lasted. The mortality percentage was converted to the scale of 1-5 published by the Indian Institute of Pulses Research (IIPR, 1999).

Results and discussion

Pathogen identification

In all fields sampled, there was at least one pathogen associated with wilt symptoms of chickpea plants. *Fusarium oxysporum* sp. *ciceris* was the fungus present in all municipalities, while *F. solani* and *R. solani* were found in three of the five sites sampled. The pathogen that causes neck rot, *Sclerotium rolfsii*, was only found in fields in the municipalities of Valle de Santiago and Salvatierra, Guanajuato.

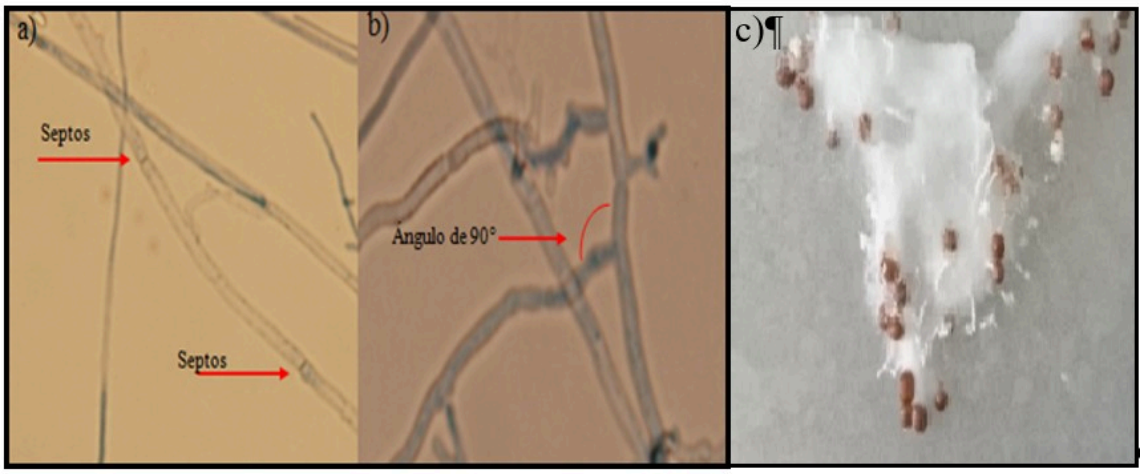
This is in line with what was reported by Fierros *et al*. (2019), in the study of the distribution of fungi associated with chickpea root rots in northwestern Mexico, they point out that *Fusarium oxysporum* f. sp. *ciceris*, *Fusarium solani*, *Macrophomina phaseolina* and *Rhizoctonia solani* are the most abundant pathogens.

In a similar study conducted in the state of Sinaloa, it was detected that the main genus was *Fusarium* sp. (Ramírez *et al*., 2012). Ghosh *et al*. (2013) concluded that *Sclerotium rolfsii* and *Macrophomina phaseolina* were widely distributed in central and southern India causing wilt and root rots in chickpeas. Likewise, in a sampling carried out in southern Spain (Trapero and Jiménez, 1985), they found the presence of *F. oxysporum* f. sp. *ciceris*, *F. solani* and *M. phaseolina*.

Morphological characterization

The morphological characteristics observed under the microscope of *S. rolfsii* strains grown in PDA were similar to those described by Stevens, (1925); Ainsworth *et al*. (1973); Kendrik *et al*. (1973). The fungus had rapid mycelial growth, white, cottony, septate and hyaline, and branched with clamp connections. Five days after plate seeding, rounded sclerotia began to develop, initially as small whitish pimples and eight days later they turned brown (Figure 1). Based on these characteristics, the isolated fungus was identified as *S. rolfsii*.

Figure 1. Structures of *S. rolfsii*. a) septate hyphae; b) mycelium branching angle; and c) formation of mycelium and sclerotia in PDA culture medium.



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Molecular characterization

The region of the internal transcribed spacers (ITS) of the ribosomal genes of the fungi morphologically identified as *Sclerotium rolfsii*, when amplified by PCR, generated an 850 bp fragment, which agrees with that expected for the species. The nucleotide base sequences of these fragments, when compared with the database, showed 99% similarity with the ITS region of *Athelia rolfsii S. rolfsii* (Curzi) Tu & Kimbr (accession KJ677121 of GenBank), teleomorph of *S. rolfsii*. This result agrees with that obtained by the morphological characterization.

Determination of virulence

The two isolates of *S. rolfsii* inoculated in five chickpea genotypes produced similar reactions, although with differences between genotypes and between strains. It was found that the *S. rolfsii* isolate from Salvatierra was more virulent than that from Valle de Santiago, so it was chosen for the inoculation of the 181 chickpea genotypes.

These results coincide with those obtained by Kumari and Ghatak (2018), who, when analyzing *S. rolfsii* isolates, found differences in their virulence. This difference in virulence was to be expected since it is considered a fungus with a wide genetic diversity despite the fact that its teleomorph, *A. rolfsii*, has not been found naturally, although it has mycelial compatibility groups that limit its genetic exchange (Harlton *et al*., 1995; Nalim *et al*., 1995; Flores-Moctezuma *et al*., 2008).

Genotype evaluation

Of the 181 chickpea genotypes inoculated with *S. rolfsii*, 77 did not emerge in the inoculated treatment, but all emerged in the pots of the control plants, indicating that the seed was not contaminated by another pathogen that would prevent its germination. Seventy-five non-emerged genotypes are kabuli-type and two are desi-type (Table 1).

It was considered that the lack of emergence is due to the damage to the seeds caused by the underground invasion of *S. rolfsii*, it was observed that both the mycelium and the sclerotia were growing on the surface of the seeds preventing their germination. Ten random samples of the pathogen growing underground on the damaged seeds were taken and morphologically checked for the presence of *S. rolfsii*. In addition, Koch's postulates were performed, confirming again that they corresponded to the pathogen. These genotypes were classified as highly susceptible.

According to these results, much of the damage caused by this pathogen goes unnoticed by farmers as it occurs underground and could be attributed to poor seed quality or other factors. Of the 104 inoculated genotypes that emerged, most had symptoms of neck rot caused by *S. rolfsii*.

In most of them, at 15 days after sowing (DAS), susceptible emerged seedlings showed an initial elongated light brown lesion on the stem, followed by rot and necrosis at the base of the stem and formation of sclerotia around the base and taproot, a characteristic symptom caused by *S. rolfsii* in chickpeas (Chen *et al*., 2011; Pande *et al*., 2012).

According to the percentage of mortality, only the Cuga 08-3160 genotype of the kabuli type was moderately resistant, with an average of 18% mortality; among the notable genotypes of the desi type, ICC 3287, ICC 4874, ICC 1882, ICC 10259, and WR-315 stood out with 20% mortality, so they were considered moderately resistant.

These results coincide with the findings of Singh *et al*. (2012), who report having found, in fifty chickpea genotypes artificially inoculated with *Sclerotium rolfsii*, only four moderately resistant: KG-1226, KG-8, B-321 and B-311. Tripathi (2006) obtained similar findings, he artificially inoculated 90 genotypes and found that they were not completely free of the disease; however, he identified three lines as moderately resistant, PG 9414-7, BG-371, and H-00-256.

Other studies reported promising chickpea germplasm accessions such as ICC 1696, ICC 4709 and ICC 14391, RSG 130, 132 and 191 (Chitale *et al*., 1990), the SAKI 9516 cultivar (Dua *et al*., 2001). Amule et al. (2014) report the cultivars GNG 1958 of the desi type and GNG 1969, BG 2086 of the kabuli type as resistant.

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In more recent work, it has been reported that the genotypes JG-13-14-16, KAK-2, CSJ-515 and PBG-5 are considered resistant with 0% mortality both in artificial inoculations and in field conditions (Sager and Kumar, 2021). These genotypes along with those identified in this research provide an opportunity to pyramid different sources of resistance against neck rot (Choudhary *et al*., 2013).

It is important to consider that all genotypes reported as resistant must be challenged with different isolates of the pathogen to verify the stability and usefulness of resistance in order to be able to identify, in the desi and kabuli types, possible progenitors with possibilities of being used in breeding schemes with the aim of generating new chickpea varieties and segregating populations of wide genetic diversity with resistance to *Sclerotium rolfsii*.

As for the genotypes found in this study as moderately resistant, the ICC 10259 genotype of the desi type stands out, which, in previous studies, had been confirmed to be resistant to *Macrophomina phaseolina* and four races of *Fusarium oxysporum* f. sp. *ciceri* (Foc 0, Foc 1 B/C, and Foc 6) existing in the Bajío region, specifically in Guanajuato, and the Foc 5 race present in Sinaloa (Guerrero-Aguilar *et al*., 2015).

In the case of the Cuga 08-3160 genotype of the kabuli type, which is a large seed material, it is important to start using it as a progenitor of new varieties since most commercial cultivars and improved lines from improvement programs in Mexico are susceptible to neck rot.

As observed in the results, neck rot leads to low plant survival by affecting seed germination underground, a fact that is not easily observed by farmers, so incorporating resistance to this disease into new cultivars should be a priority objective in improvement programs.

Conclusions

Sclerotium rolfsii is present and is affecting chickpea cultivation in central Mexico. INIFAP's chickpea improvement program does not have resistant genotypes and the vast majority are highly susceptible or susceptible to this pathogen. Five genotypes of the desi type: ICC 10259, ICC 3287, ICC 4874, WR-315, and ICC 1882, and an improved line of large seeds of the kabuli type, CUGA 08-3160, were found to be moderately resistant to *S. rolfsii*.

Of these resistant genotypes, ICC 10259 stands out, which has genetic resistance to four races of *Fusarium oxysporum* f. sp. *ciceri* and *M. phaseolina*. These genotypes are currently protected by the Chickpea Improvement Program of the National Institute of Forestry, Agriculture and Livestock Research (INIFAP, for its acronym in Spanish) at its headquarters in 'Bajío', Culiacán, and Hermosillo.

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