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Could *Alternaria solani* IA300 be a plant growth-promoting fungus?

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Abstract In recent years the search for beneficial microorganisms that could induce plant growth has increased. Most have been found on crop seeds, soil and on the rhizosphere. In this work, we collected mature seeds of *Phaseolus vulgaris* var. Pinto Saltillo in Zacatecas, Mexico; and isolated endophytic fungi from these samples. Three different fungal strains were obtained. Sequencing analysis showed that one of the analyzed fungi, *Alternaria solani* IA300, showed the capacity to promote plant growth. Chili plants (*Capsicum annuum* cv. Mirasol) were inoculated with *Alternaria solani* IA300. After 15, 30, 45, and 60 days post-interaction, nine vegetative variables were analyzed (length of the root and aerial parts; weight of the root and aerial parts; number of leaves, flowers and fruits; as well as the dry weight from the root and aerial parts). Results showed that *Alternaria solani* IA300 can promote growth in chili pepper plants at later times of interaction (45 and 60 days post interaction), where most of the vegetative variables analyzed showed an increase in relation to control plants. To our knowledge, this is the first report where *Alternaria solani* IA300 induces growth and development in plants.

Keywords *Alternaria solani* IA300 · *Capsicum annuum* cv. Mirasol · Plant growth-promotion · Beneficial microorganisms · Symbiosis

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Introduction

Although the presence of endophyte microorganisms on seeds has been reported for years (Rijavec et al. 2007; López-López et al. 2010; Herrera et al. 2016), little is known about how these contribute to crop development or even if these can be applied to different crops and induce a beneficial effect. *Phaseolus vulgaris* has been ranked as the third most consumed and important legume worldwide, due to its high content of carbohydrates, minerals and fiber (Singh 1999; Lara-Flores 2015). The state of Zacatecas is the main producer of beans and chili pepper in Mexico (SIAP 2018). The production of these two crops are significantly influenced by biotic and abiotic stresses. Therefore, it is of vital importance to increase the production and reduce the biotic and abiotic stress factors that affect it. It has been reported that inoculation of crops with beneficial

microorganisms can help to lessen different biotic and abiotic stresses (Mendes et al. 2013).

Previously, the isolation of 21 fungal strains from *Phaseolus vulgaris* germinated seeds of Colombia cultivars was reported (Abdel-Hafez et al. 2016; Parsa et al. 2016) one of these reported fungi was *Alternaria* spp. This fungal genus is known for being a plant pathogen, specifically for the *Solanaceae* family. This pathogen can cause early blight disease, which has a significant impact on plant production and quality (Song et al. 2011). To date, few *Alternaria* strains have been recognized to have beneficial characteristics (Zhou et al. 2018). In this research we isolated three fungal strains from *Phaseolus vulgaris* germinated seeds, from Zacatecas (Mexico) cultivars. The analysis of the 16S ribosomal RNA gene of these three fungal isolates showed that one fungus is *Alternaria solani* IA300, which has been reported as a non-pathogenic fungus strain (Abdel-Hafez et al. 2016). Therefore, the principal aim of this research was to analyze if *Alternaria solani* IA300, isolated from *Phaseolus vulgaris* germinated seeds, improve the growth and development of chili pepper plants.

Materials & methods

Isolation of beneficial microorganisms

In 2018, seeds of *Phaseolus vulgaris* cv Pinto Villa were analyzed for the presence of endophytic fungus. The bean seeds were surface sterilized with 20% sodium hypochlorite for 5 min and rinsed five times with sterile distilled water. After surface disinfection, each seed were transferred into Murashige Skoog medium (MS) plates. The plates were kept in a plant growth chamber at 22 °C with a photoperiod of 16-h light / 8 h dark for 7 days. Later, the germinated seeds were transferred to potato dextrose agar medium (PDA); any fungal growth was isolated and cultured separately in PDA medium.

PCR amplification and analysis of sequence data

Fungal DNA was extracted according to Raeder and Broda (1985). The molecular identification of the isolated fungi was done with the primers: ITS-1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS-2 5′-GCTGCGTTCTTCATCGATGC-3′ (amplification of the 16S rRNA gene, 500 pb); ITS4 5′-TCCTCCGC TTATTGATATGC-3′ and ITS5 5′-GGAAGTAA AAGTCGTAACAAGG-3′ (amplification of the 5.8 S gene, 500 pb); and HEF1 5′-GACTCTGGCAAGTC GACCAC-3’ and HEF1R 5′-CTGGCCATCCTTG GAGATAC-3′ (amplification of elongation factor 1α, 742 pb). The PCR was performed in a programmable thermocycler (Applied Biosystems) with the following parameters: 94 °C for 5 min, 35 cycles of 94°C, for 30 s, 58 °C for 30 s and 72 °C for 1 min, and a final extension cycle of 72 °C for 5 min. The PCR products were cloned into a pGEM-T® Easy Vector System (Promega, Madison, WI, USA). The clones obtained from the fungal DNA samples were completely sequenced and the sequences obtained were compared using the BLASTn (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) algorithm to determine the taxonomy of the fungal strains.

Plant material and growth conditions

A random selection of *Capsicum annuum* cv. Mirasol seeds were collected. Later, the seeds were surface-sterilized for 10 min with a 40% (*v*/v) chlorine solution and rinsed six times in sterile distilled water. Aseptic seeds were transferred to Petri dishes (150 × 15 mm) containing 0.2X MS medium [0.2X MS salts (Phytotechnology), 1% (*w*/*v*) agar, 0.75% (w/v) sucrose (pH 7.0)] and placed at 4 °C for 2 days for vernalization. Plates were incubated at 22 ± 1 °C in a 16-h light/8-h dark cycle for 7 days. Subsequently, the seedlings were transferred each one to a pot containing a mixture of Peat Most, perlite and vermiculite substrate in a 3:1:1 ratio, respectively. At this time, the inoculation of chili plants was carried out. Later, each pot was maintained in a growth chamber (22 ± 1 °C) under a 16 h light/8 h dark photoperiod.

Fungal growth conditions and seedling inoculation

The fungal strain was grown on potato dextrose agar (PDA) plates for 8 days at 28 °C. Fungal conidia were collected in 15 ml of sterile distilled water at room temperature. Total conidia were counted with a Neubauer chamber and a microscope at 40X magnification. The fungal spore suspension was adjusted to 1×10^6 spores per mL and used as inoculum. The spore suspension was applied to roots of 7-day-old *Capsicum annuum* seedlings; non-inoculated control plants were cultured under the same conditions as inoculated seedlings.

Analysis of the growth and development parameters of *Capsicum annuum* inoculated plants and statistical analysis

The variables analyzed were: root length (cm), aerial part length (cm), root fresh weight (g), aerial fresh weight (g), root dry weight (g), aerial dry weight (g), number of leaves, flowers, and buttons at 15, 30, 45, and 60 days post inoculation (dpi). Sample weights (mg) were obtained with an analytical balance and sample lengths (cm) were obtained with a graduated ruler. The values obtained were the average length with mean values done in triplicate. The asterisk indicates significant differences between different conditions ($p < 0.05$); determined by a Student's t test, using the GraphPad software (version 6.01).

Results

Several studies have reported the isolation of microorganisms from the rhizosphere of plants and how these isolates could be used as biofertilizer or inoculum to increase crop yield due to their beneficial microorganism activity (Vassilev et al. 2006; Avis et al. 2008; Robles-Yerena et al. 2010). Therefore, in recent years the need to know if microorganisms that have been reported as beneficial in one crop can be beneficial for others crops, has increased (Van Aken et al. 2004; Sreenivasa et al. 2010; Gopalakrishnan et al. 2011). The aim of this research was the isolation and molecular characterization of a fungus obtained from bean seeds, and the analysis of its capacity to induce growth and development in chili seedlings.

Isolation and molecular characterization of fungus endophytes from *Phaseolus vulgaris* seeds

Germinated seeds of *Phaseolus vulgaris* cv Pinto Villa were analyzed for the presence of endophyte fungi. Three different fungal strains were obtained. PCR analysis of genomic DNA were carried out in order to identify each one of these isolated fungi. Sequencing results reveal that these belong to different fungal genera (Table 1). Interestingly, one of these fungi belongs to the *Alternaria* fungal genus, specifically it was found to correspond to the *Alternaria solani* IA300 strain.

Table 1 Fungal strains isolated from *Phaseolus vulgaris* germinated seeds

Endophyte ID	GenBank
Alternaria solani	AY154716
Fusarium sp.	MK050646
Fusarium oxysporum	KR012886

Inoculation effect of *Alternaria solani* IA300 strain in *Capsicum annuum* cv. Mirasol seedlings

It has been reported that one of the most devastating and widespread distributed diseases in chili is early blight caused by the fungus *Alternaria solani* (Kumar et al. 2013). Considering the aforementioned and knowing that *Alternaria solani* strain IA300 promotes growth in bean plants (unpublished data), the aim of this research was to determine if the endophytic *Alternaria solani* IA300 strain, isolated from *Phaseolus vulgaris* germinated seeds, could have a beneficial effect on chili plants. An interaction model of *A. solani* – *C. annuum* was established on a bioclimatic chambers growth system. The parameters analyzed were: root length (cm), aerial part length (cm), root fresh weight (g), aerial fresh weight (g), root dry weight (g), aerial dry weight (g), number of leaves, flowers, and buttons at 15, 30, 45, and 60 dpi. At 15 and 30 dpi the *Capsicum annuum* plants did not show significant statistical differences versus control plants in the variables analyzed. At 45 dpi the control plants were 10% bigger (length of the plants) than those inoculated with *A. solani* IA300 (Fig. 1a and b), showing statistical differences in both sites analyzed (leaf and root area). There was also a statistical difference in number of flowers, and in the fresh and dry weight of roots (Fig. 1c, d, and e). Interestingly, at 60 dpi almost all the variables analyzed saw an increase in the inoculated plants versus control plants (Fig. 2). The only parameter where the inoculated plants showed a decrement was in the length, where the control plants showed a 12% increase versus inoculated plants (Fig. 2a and b). In number of leaves, buttons, and flowers the increase of the inoculated plants was of 23, 24, and 50%, respectively, versus control plants (Fig. 2a and c). Weight showed an increase in the inoculated plants of 16 and 22% in the fresh and dry specimens, respectively. In both cases the significant statistical difference was observed in the aerial part (Fig. 2d and e).

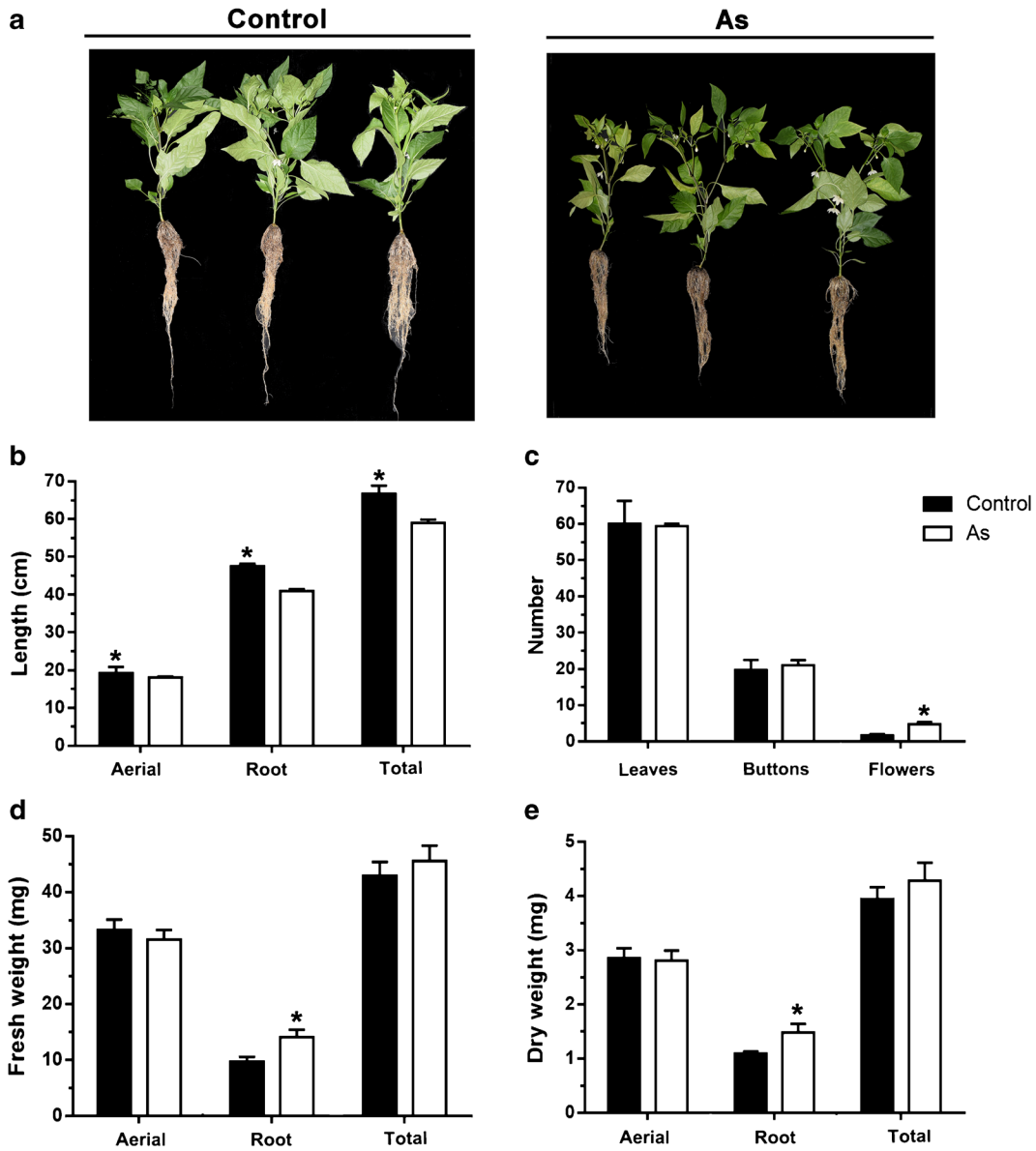

Fig. 1 Phenotype of *Capsicum annuum* cv. Mirasol plants during interaction with *Alternaria solani* at 45 days after inoculation (dpi) Representative photographs of *C. annuum* cv. Mirasol plants. **a** Control *Capsicum annuum* cv. Mirasol plants were grown in each pot without fungus. Also, the *A. solani* IA300 strain was grown in direct contact with *C. annuum* cv. Mirasol plants in each pot, with a 1×10^6 spores per ml density. **b** Length of the aerial, root and total parts of the plant, **c** Number of leaves, buttons and flowers. **d** Fresh weight of the aerial, root and total parts of the plant. **e** Dry weight of the aerial, root and total parts of the plant. Estimation of the physiological parameters was determined by measuring three plants grown in three independent experiments. The asterisk indicates significant differences between different conditions ($p < 0.05$); determined by a Student's t test, using the GraphPad software (version 6.01)

Discussion

For more than 50 years it has been reported that some microorganisms can interact in a beneficial way with plants. Increasing the promotion of growth, either by the synthesis of phytohormones, nitrogen fixation, mineral solubilization, increased root volume, siderophore production; or as biocontrol agents when producing antimicrobial compounds (Vassilev et al. 2006, Avis et al. 2008, Robles- Yerena et al. 2010). Due to these

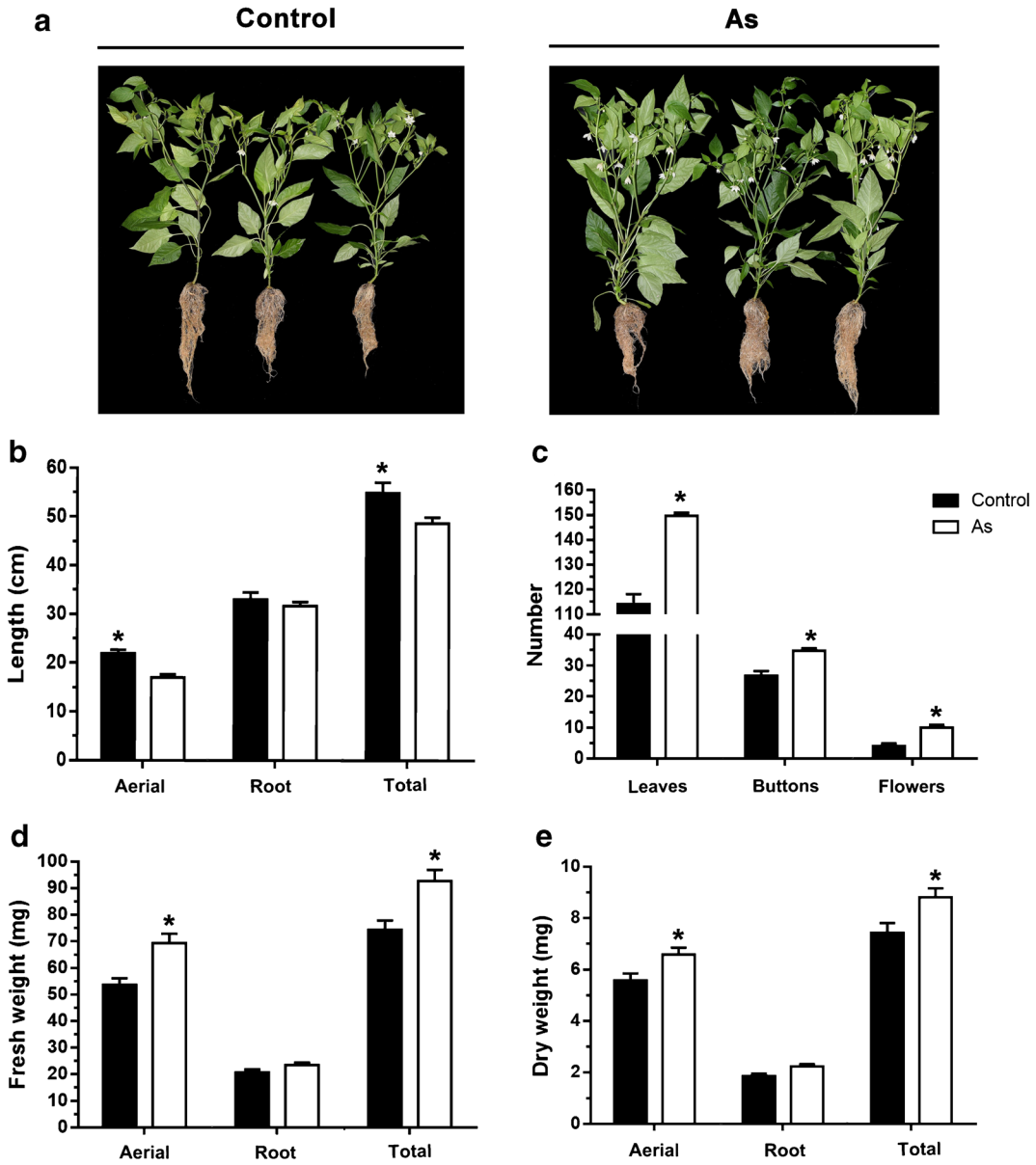

Fig. 2 Phenotype of *Capsicum annuum* cv. Mirasol plants during interaction with *Alternaria solani* at 60 days after inoculation (dpi) Representative photographs of *C. annuum* cv. Mirasol plants. **a** Control *C. annuum* cv. Mirasol plants were grown in each pot without fungus. Also, the *A. solani* IA300 strain was grown in direct contact with *C. annuum* cv. Mirasol plants in each pot with 1×10^6 spores per ml density. **b** Length of the aerial, root and total parts of the plants, **c** Number of leaves, buttons and flowers. **d** Fresh weight of the aerial, root and total parts of the plants. **e** Dry weight of the aerial, root and total parts of the plants. Estimation of the physiological parameters was determined by measuring three plants grown in three independent experiments. The asterisk indicates significant differences between different conditions ($p < 0.05$); determined by a Student's t test, using the GraphPad software (version 6.01)

advantages, interest in using them as biofertilizers or microbial inoculants has increased recently. The main reason for this is the search for a microbiological balance in the soil, in order to increase crop production and protection. This will create a more sustainable agriculture and environment (Vessey et al. 2003). In our study we performed the isolation and molecular characterization of fungi isolated from bean seeds. It was found that two of the fungal isolates belong to the *Fusarium* genus and the other strain belongs to the

Alternaria genus. This last fungal genus has been reported as the cause of the disease known as early blight, this disease has a worldwide presence, and affects mainly *Solanaceae* family plants and other horticultural crops (Spletzer and Enyedi 1999; Chaerani and Voorrips 2006; Li et al. 2011). Our results were similar to those reported by Abdel-Hafez et al. (2016), who carried out the isolation of fungus from germinated *Phaseolus vulgaris* Colombia cultivars seeds; finding the presence of fungi such as *Alternaria solani, Colletotrichum lindemuthianum, Fusarium solani and Macrophomina phaseolina.*

The *A. solani* IA300 (AY154716) strain that we isolated has a 100% of sequence identity with *Alternaria solani* F10 (KT721914) (a 623 base pair sequence DNA fragment corresponding to the ITS1–5.8S-ITS2 region). This last *A. solani* strain has been reported to be non-pathogenic and a silver nanoparticles producer (Abdel-Hafez et al. 2016). Previously it was observed that inoculation with *Alternaria solani IA300* induces the growth and development of bean seeds (data not shown). Based on this, it was decided to determine if this fungal strain has the capacity to induce or promote the growth in *Capsicum annuum* cv. Mirasol (a crop of economic importance in Zacatecas state). The results show that at 15 and 30 dpi there is no significant difference with respect to the control plants (supplementary Figs. 1 and 2). Interestingly, at 45 and 60 dpi an increase in almost all the parameters analyzed was observed in the inoculated plants versus the control plants (Figs. 1 and 2). Our results are similar to those reported by Zhou et al. (2018), who reported that *Alternaria sp*. A13 markedly enhances *Salvia miltiorrhiza* root growth. Based on these results, it can be established that some *Alternaria* strains can be considered to promote plant growth. Moreover, to our knowledge, this is the first report where *A. solani* IA300 induces plant growth in chili plants.

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Author contributions S.S.M., J.A.M.C., L.R.R.T., M.A.S.L. and F.B.S.B. conceived the experimental design. S.S.M., L.R.R.T. and F.B.S.B. contributed with reagents, materials and analysis tools. S.S.M., J.A.M.C., L.R.R.T., M.A.S.L. and F.B.S.B. conducted the experiments and analyzed the data. S.S.M., J.A.M.C., L.R.R.T., M.A.S.L. and F.B.S.B. contributed to data interpretation and manuscript preparation. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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