

Potential of *Pseudomonas stutzeri* strains isolated from rhizospheric soil endowed with antifungal activities against phytopathogenic fungus *Stemphylium botryosum*

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Manuscript received: 20 September 2019. Revision accepted: 21 October 2019.

Abstract. Mokrani S, Bejaoui B, Belabid L, Nabti E. 2019. Potential of *Pseudomonas stutzeri* strains isolated from rhizospheric soil endowed with antifungal activities against phytopathogenic fungus *Stemphylium botryosum*. *Asian J Agric* 3: 47-54. In this study, two *Pseudomonas* strains P4 and P5 were isolated from rhizospheric soil and characterized for PGP (Plant Growth Promoting) traits production like HCN (hydrogen cyanide), siderophores and IAA (Indole Acetic Acid). A phylogenetic tree based on 16S DNAr identification-related the two strains P4 and P5 to *Pseudomonas stutzeri* NR 116489 and NR 113652.1. One phytopathogenic fungus St-bt (*Stemphylium botryosum*) was isolated from *Phaseolus vulgaris* L. Macroscopic and microscopic identification attributed it to the genus *Stemphylium*. Antifungal activities of the two *Pseudomonas* strains P4 and P5 against fungus isolate St-bt had revealed very highly significant inhibition percentages of $38.46 \pm 3.85\%$ and $56.56 \pm 2.22\%$ for each strain, respectively.

Keywords: Antifungal activity, PGP traits, *Pseudomonas stutzeri*, *Stemphylium botryosum*

INTRODUCTION

Agriculture is the groundwork for food industries on a global scale. Sustainable agriculture strives to continue improving without compromising the environment (Gimenez et al. 2018). It is described as a system protecting manufacturing in the long conduct, except degrading of the natural properties, including the useful resources of the low-input applied sciences utilization (Altieri 1989; Saleh et al. 2017). On different sides, contemporary agriculture faces the extensive assignment of supplying a world population within continuous increase with food, at the same time as the natural sources augment to remain equal (González de Molina et al. 2017). Especially with abiotic stress prerequisites such as drought, heat, and salinity causing big losses in conformity with agricultural production worldwide. Also, the environment heterogenic conditions and world climatic changes are only a few of the challenges facing modern agriculture (Mittler and Blumwald 2010).

Furthermore, biotic stress is described as caused by plant living microorganisms that comprise fungi, bacteria, viruses, parasites, weeds, insects, and distinctive native and cultivated plants (Newton et al. 2011). Although fungal phytopathogens are considered causal agents on primary plant diseases, they can infect a range of plant components. This can include roots, stems, leaves, vegetation, and fruits; inducing characteristic visible symptoms as spots, blights, anthracnose, wilts, rots, etc. (Thilagam et al. 2018). In addition, microbial diseases caused about 16% of the loss of annual manufactured tonnage worldwide at the start of

the 21st century. 70-80% of these losses have been caused by fungi (Moore et al. 2011). To face all its constraints, chemical fungicides are extensively used for many plant disease treatments. Whereas these products can cause environmental pollution and disastrous effects on human health (Agrios 1988). The intensive uses of certain chemical compounds have influenced the improved resistance of the targeted organisms against certain chemicals (Goldman et al. 1994). However, biological control may remain a promising alternative in imitation of chemical elements to manage phytopathogenic fungi (Wallace et al. 2017; Tägele et al. 2018) and afford attractive dietary supplements among methods due to the limited environmental pollution (Compant et al. 2005; Nogórska et al. 2007).

The biological control entails the utilization of salutary organisms, their genes and/or gene products, such as metabolites that lessen the negative effect of plant pathogens and promote high-quality plant products (Vinale et al. 2008). Several microorganisms such as bacteria, fungi, viruses, algae, and protozoa have been recommended as individual or combined effective biopesticides (Chauhan et al. 2018).

The microbe can control a variety of pests (Nobutaka 2008), particularly a wide range of bacterial genera that can be used as biocontrol agents PGPR (Plant Growth Promoting Rhizobacteria) such as *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacteria*, *Pseudomonas*, *Rhizobium* and *Serratia* (Goswami et al. 2016). Among these bacteria,

Pseudomonas spp. are aerobic, gram-negative bacteria, ubiquitous in agricultural soils, and adapted in broad range growing area in the rhizosphere (Weller 2007). *Pseudomonas* possesses many traits to make them suitable for biocontrol and growth-promoting agents (Wei et al. 1996; Morrissey et al. 2004a; Stockwell and Stack 2007; Mishra and Arora 2017). The current study aimed to characterize the antifungal activities of two *Pseudomonas* strains endowed with PGPR characteristics against some phytopathogenic fungus isolate St-bt.

MATERIALS AND METHODS

Origin of *Pseudomonas* strains

In a previous study (Mokrani et al. 2019) two *Pseudomonas* strains P4 and P5 were isolated in 2010 from rhizospheric soil of *Phaseolus vulgaris* L from Tighanif Mascara (35°24' N 0°19'E). The strains were stored in TSB (Tryptic Soy Broth) broth supplemented with 25% glycerol by sub-culturing every 12-18 months.

Phylogenetic identification of *Pseudomonas* strains

DNA extraction and PCR amplification

Genomic DNA of the two strains, P4 and P5, were extracted using the technique of phenol/chloroform/isoamyl alcohol according to (William et al. 2012). PCR amplifications were performed using the following primers: Amorced Forward 16S Forward (5'-AGA GTT TGA TCC TGG CTC AG-3') and Amorced reverse 16S Reverse (5'-CTA CGG CTA CCT TGT TAC GA-3') for 16S rRNA gene. 35 cycles were realized with the following conditions: preheating at 94°C for 3 min; denaturation at 94°C for 45 sec; hybridization at 55°C for 1 min; elongation at 72°C for 2 min; final elongation at 72°C for 7 min and final cooling at 4°C for 7 min (Mokrani et al. 2018a).

16S DNAr sequencing

Partial 16S DNAr was compared with the nucleotide sequences from international databases using BLAST (Basic Local Alignment Search Tool Program). The alignments of the nucleotide sequences were conducted out by using the Clustal W algorithm. The phylogenetic tree was obtained by MEGA 7 software program and the neighbor-joining algorithm. Bootstrap values have been determined from 100 replicates.

PGPR analysis

Production of HCN

Hydrogen cyanide (HCN) production was carried out as described by Lorck (1948). Bacterial strains were streaked on nutrient agar supplemented with 0.44% L-glycine. The change of color from yellow to light brown or red-brown indicated a moderate or high production of HCN, respectively (Trivedi et al. 2008).

Siderophores synthesis

Siderophores determination was carried out by qualitative detection on a Chrome Azurol-S (CAS) agar

medium. *Pseudomonas* strains were streaked on Petri dish plates containing CAS medium and incubated at 30°C/48 h. Formation of orange halos surrounding colonies indicated siderophore production (Yeole et al. 2001).

IAA production

Qualitative IAA production. Bacterial strains were examined to produce Indole Acetic Acid (IAA) by the technique described by Bric et al. (1991). Bacterial strains were inoculated in Petri dish plates containing Luria Bertani (LB) medium supplemented with 5 mM L-tryptophan. Production of IAA and/or its analogs in the medium manifested by leading to a red-pink color. For other indoles, color produced was yellow or brown-yellow.

IAA chromatography. To confirm the IAA chromatography of IAA extracts was done according to the technique reported by (Kuang-Ren et al. 2003, modified). *Pseudomonas* strains were cultured on TSB (Tryptic Soy Broth) and incubated at 30°C/24 h. After incubation, centrifugation was carried out and the supernatants were recuperated and mixed with ethyl acetate (1:2). After vigorous stirring, the partial extracts of the solvent layer were allowed to rest for 10 min. The procedure was repeated 3 to-4 times. Then, IAA chromatography was executed using dissolvent system propanol: water (8:2). Chromatogram of two IAA extracted samples and control (10 mg/100 mL about IAA) were developed with Salkowski's reagent.

Fungus isolation and identification

The phytopathogenic fungal isolate St-bt was isolated from *P. vulgaris*, and planted under a greenhouse in Tighanif Mascara (35°24' N 0°19'E). Small slices of leaves of an infected plant showing characteristic symptoms were cut sterilely. The slices were then placed on Petri dishes containing a PDA (Potato Dextrose Agar) medium followed by incubation at 25°C/5-7 days (Grewal and Jhooty 1984). The purification of the fungal isolate was performed by successive culturing 2-3 times of mycelium on a new PDA medium. The St-bt isolate was identified by observation of the macroscopic and microscopic aspects (Terbeche 2011). Colonies of the isolate St-bt obtained on PDA medium after incubation at 25 °C/7 days were examined for their macroscopic aspects like perception, colonies border, and color (Lecellier 2013).

The microscopic observation was carried out for the appearance of the mycelium and the prospective presence of specific forms of reproduction using the technique reported by Su et al (2012). The method consisted of observation of a part of the mycelium taken from a PDA agar culture at 25°C/7days under an optical microscope (10x and 40x). The activation of sporulation of the St-bt isolate was stimulated by the application of UV light for 16 h at room temperature followed by incubation in the darkness at 25 °C from 3 to 5 days. The identification of the isolate St-bt was then performed using the keys proposed by Pitt and Hocking (2009); Hernandez-Perez and du Toit (2000) for the identification of species belonging to the genus *Stemphylium*.

Pathogenicity test

The pathogenicity test was reproduced on *Phaseolus vulgaris* L plants by the modified method reported by Ben Hassena (2009). 50 mL of distilled water was added to an old fungal culture of 10 to 15 days on PDA medium. Then, the surface was thoroughly scraped and mixed to release the sporulated forms. The suspension containing (spores + mycelium) was used for contamination by pouring into a pot containing a *P. vulgaris* plant for 2-3 weeks. The onset of symptoms was taken within one week after contamination.

Antifungal activity

Antagonist activity of the *Pseudomonas* strains against the isolate St-bt was carried out by the method of dual culture described by Landa et al (1997). Evaluation of the antifungal activity was estimated by calculation of the percent inhibition of the mycelium growth according to the following formula:

$$PI = \frac{(r \text{ control} - r \text{ test})}{r \text{ control}} \times 100$$

Where: PI: percent inhibition;

r test: maximum radial distance of fungus on a line towards the antagonist bacteria in dual culture;

r control: maximum radial distance of fungus inoculated in the center of petri plate).

Statistical analysis

Results of antifungal activity were represented as the percent inhibition means±SD (standard deviation). Those percentages were then analyzed using one-way variance analysis with a significance level of $p < 0.05$. Similarly, when significant differences were found, multiple comparisons were performed using Dennett's test.

RESULTS AND DISCUSSION

Microorganisms growing in the rhizosphere, particularly *Pseudomonas* spp., are ideal for use as biocontrol agents of many bacterial and fungal plant diseases. They are known for their high diversity, colonization capacity, plant growth traits involved in stimulation or inhibition plant growth and suppression of different plant pathogens.

Phylogenic identification of *Pseudomonas* strains

Phylogenic identification of the two *Pseudomonas* strains, P4 and P5, was completed by 16S DNAr identification. Agarose gel electrophoresis revealed two clear bands of 1500 bp (Figure 1). This showed that the conserved regions of the 16S rRNA gene were amplified by the SF and SR primer pair. The phylogenetic analysis related to the two *Pseudomonas* strains (100%) to *Pseudomonas stutzeri* NR 116489 and NR 113652.1 was presented (Figure 2).

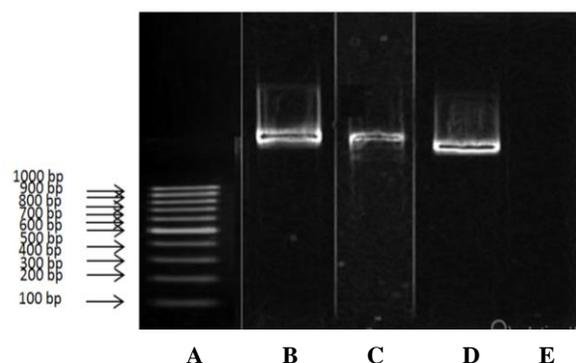


Figure 1. 16SDNAr gene PCR amplicons on 1.5% agarose gel. DNA bands of about 1500 bp were obtained from the two *Pseudomonas* strains after 16S RNAr gene PCR. A. SM: size marker; B. Strains P4; C. Strain P5; D. Positive control established by 16S DNAr of strain P25 *Pseudomonas grimontii* isolated from *Vicia sativa* (Mokrani et al. 2019); E. Negative control

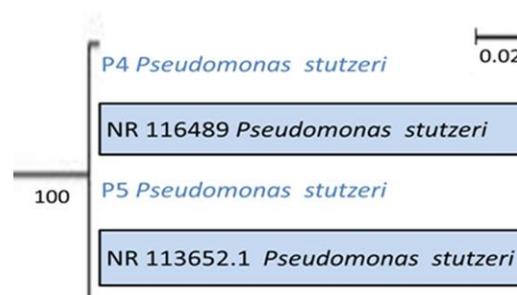


Figure 2 Neighbor-Joining tree obtained using MEGA7, revealing the phylogenetic relationship of the *Pseudomonas* strains. Bar indicates 2% sequence divergence

The molecular identification via 16S DNAr of the two *Pseudomonas* strains attributed them to *Pseudomonas stutzeri* species. The genus *Pseudomonas*, is one of the most varied and ecologically widespread groups of microorganisms on the planet (Spiers et al. 2000). Phylogenetic affiliation of different *Pseudomonas* sp based on 16S rRNA gene suggests that molecular divergence contributes to the genetic range of *Pseudomonas* sp. The strain determination performed on 16S rRNA gene was once related to the nucleotide diversity of *Pseudomonas* sp in soil rhizosphere community amongst distinct agricultural vegetation (Adhikari et al. 2015). *P. stutzeri* is a non-fluorescent bacterium widely distributed in the environment. Over the last few years, many improvements have been made in elucidating the taxonomy of this variety of taxonomical groups, demonstrating the clonality of its populations. The species has obtained a lot of interest due to the fact of its unique metabolic properties (Lalucat et al. 2006). Furthermore, strains of *P. stutzeri* have structured in at least seven DNA-DNA similarity groups known as genomovars (Ursing et al. 1995). Confirmation of this system of internal subdivisions was once said following different methods to bacterial phylogeny, which included 16S rRNA gene sequencing (Bennasar et al. 1996). Two individuals of the same genomovar share increased than 70% DNA-DNA similarity, the common species threshold,

and these values are reduced and typically much less than 50% when individuals of distinct genomovars are compared (Rosselló et al. 1991).

Production of HCN

Pseudomonas strains are known to synthesize a large variety of antibiotics that act effectively in controlling plant pathogens. From those, cyanide hydrogen is the most volatile antibiotic largely described as *Pseudomonas* biocontrol agents. Evaluation of the capacity of the two *P. stutzeri* strains to produce hydrogen cyanide, revealed a qualitative change of Whatman paper N°1. Color changes from yellow to reddish or reddish-brown confirmed HCN production (Figure 3).

Soil microorganisms make contributions to soil fertility, soil structure, biodiversity and have a few PGP traits (Barea et al. 1996). One secondary metabolite produced commonly through rhizosphere *Pseudomonads* is Hydrogen Cyanide (HCN), a gas identified to negatively have an impact on root metabolism and root extension (Alström and Burns 1989; Schippers et al. 1990). This suggests that cyanogenesis, by way of rhizobacteria in the rhizosphere, can adversely influence plant growth (Kremer and Souissi 2001). The degree of HCN produced by means of the use of the rhizobacteria *in vitro* does not correlate with the determined biocontrol effects, as a result disproving the biocontrol hypothesis. But alternatively, is implicated in geochemical procedures in the substrate, indirectly developing the availability of phosphate (Rijavec and Lapanje 2016). Also, the function of HCN manufacturing by using *Pseudomonas fluorescens* strain CHA0 as an antagonistic aspect that contributes to biocontrol of *Meloidogyne javanica*, the root-knot nematode, *in situ* (Siddiqui et al. 2006).

Siderophores synthesis

It has been suggested that the siderophore-mediated competition for iron with soil-borne pathogens is an important mechanism for biological control. Qualitative production of siderophores on CAS agar media by the two *P. stutzeri* strains, showed appearance of characteristic orange halos surrounding growth. This indicated Fe chelation and revealed siderophores production (Figure 4).

To facilitate iron (III) acquisition, plants and microorganisms, such as fungi and bacteria, produce and excrete strong iron (III) chelators called siderophores (Neilands 1984). Important groups of siderophores consist of hydroxamate siderophores, catecholate (phenolates) siderophores and carboxylate siderophores (Pattan et al. 2017). Various species amongst non-fluorescent *Pseudomonas* group are capable to produce siderophores (Meyer et al. 2002; Bultreys et al. 2001). The *P. stutzeri* strain CCUG 36651, was once shown to produce ferrioxamine E (nocardamine) as the major siderophore. This collectively with ferrioxamine G and two cyclic ferrioxamines having molecular masses 14 and 28 atomic mass units decrease than that of ferrioxamine E, suggested to be ferrioxamine D2 and ferrioxamine X1, respectively (Essén et al. 2007). Furthermore, siderophores act as a possible biocontrol agent in opposition to negative

phytopathogens and hold the potential to alternative hazardous pesticides (Saha et al. 2016).

IAA production

Indole acetic acid is one of the most physiologically active and important auxins regulating a range of plant cellular and physiological processes. Qualitative determination of the indole acetic acid revealed that the two strains produced IAA on Luria-Bertani agar supplemented with 5 mm L-tryptophan. The color obtained was pink-red for the two strains, signifying a moderate IAA production (Figure 5).

Production of IAA was confirmed by paper chromatography compared with the authentic control (10 mg/mL). After revelation with Salkowski's reagent, chromatogram of IAA extracts showed two pinkish spots ($R_f = 0.53$) corresponding to the authentic IAA (Figure 6).

Indole acetic acid production is another essential PGPR trait implicated directly in stimulation of plant increase. IAA is a major property of rhizosphere bacteria that plays a main function in plant growth and development; specifically in modifying cell division, cell differentiation, cell expansion, and lateral root formation (Pant and Agrawal 2014). Equally, IAA incites in the production of longer roots with multiplied wide variety of root hairs and root laterals which are involved in nutrient uptake (Datta et al. 2000).

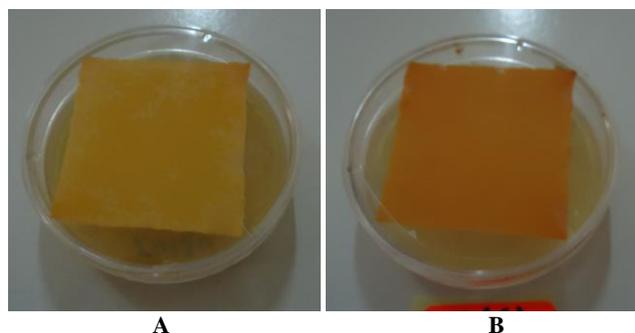


Figure 3. Qualitative production of hydrogen cyanide by *P. stutzeri* strains on agar medium (a: weak reddish color for strain P4 indicating low production; b: reddish-brown color for strain P5 meaning moderate production)

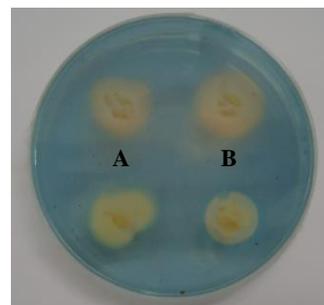


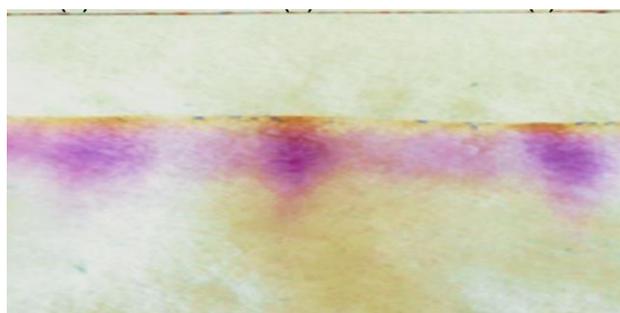
Figure 4. Qualitative productions of siderophores by *Pseudomonas stutzeri* strains on CAS-medium (a: small orange halo surround growth for strain P4 meaning low production; b: large orange halo surround colonies for strain P5 revealing moderate production)



Figure 5. Production of indole acetic acid by strain P5 *Pseudomonas stutzeri* (red color reflecting moderate IAA production)



Figure 7. Leaf blight caused by fungus isolate St-bt on *Phaseolus vulgaris* leaves



A B C

Figure 6. IAA chromatogram. A. Control; B. IAA extract of strain P4; C. IAA extract of strain P5

Also, it stimulates cell elongation via enhancing positive conditions like: extend in osmotic contents of the cell, rising in permeability of water into cell, limiting in wall pressure, amplifying in cell wall synthesis, inducing RXA, and protein synthesis. Furthermore, IAA enhances embial activity, inhibits or prolongs abscission of leaves, induces flowering and fruiting (Zhao 2010).

Isolation and identification of fungus

Isolation of fungus

The fungus isolates St-bt was isolated from *P. vulgaris* showing leaf blight disease symptoms. Characteristic small yellow-brown tasks were observed on the leaves area (Figure 7).

In this study, isolation of fungal diseases occurring in *P. vulgaris* cultures revealed fungus St-bt showing characteristic symptoms of leaf blight disease. Riley et al (2002) had reported that control measures of plan pathogens depend on proper identification of diseases and the causal agents. Therefore, diagnosis is one of the most important aspects of a plant pathologist's training. It includes identification of affected plants, recognizing healthy plant appearance, identifying attribute symptoms, and checking distribution of symptoms.



Figure 8. Macroscopic aspect of isolate St-bt on PDA medium

Macroscopic and microscopic identification

After isolation on PDA medium, fungus isolate St-bt revealed characteristic black colonies surrounded with white color in the edge (Figure 8). Furthermore, colonies were velvety with irregular shape, black in the revers, marked by less speed growth forming colony of 26 mm at the end of 7 days of incubation.

Microscopic examination of isolate St-bt showed mycelium characterized by ramified septa, typical mature and immature conidia. Evenly, conidia were always isolated and never in chains, an essential distinguishing feature between genera *Alternaria* and *Stemhyllium* (Figure 9). Immature conidia were ovate to cylindrical, brown with one to two transverse septa. Mature conidia were dark brown cylindrical, with a pointed apex; they assume cottony longitudinal and transverse septations with an irregular outline.

In this present study, macroscopic and microscopic identification of the isolate St-bt had attributed it to *Stemphylium botryosum*. Hosen et al (2009) mentioned that the colonies of *S. botryosum* on PDA medium are velvety to cottony in texture with a grey, brown, or brownish-black color colony. Also, conidia are colored and septated were determined amongst *S. botryosum* strains (Caudillo-Ruiz et al. 2017). Furthermore, Boutkhal (2012) had reported that *S. botryosum* is a frequent fungus isolated from bean cultures. The fungus *Stemphylium botryosum* has been suggested to be very widespread in temperate and subtropical regions, where it is established in a wide range of economically essential vegetation (Mwakutuya 2006).

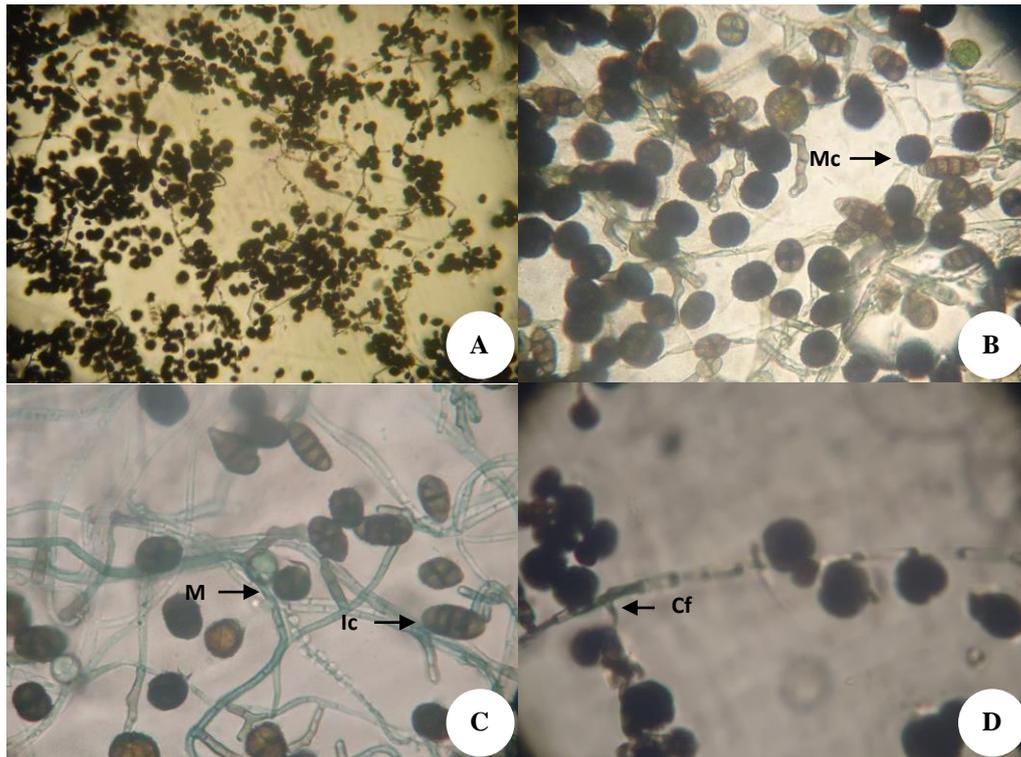


Figure 9. Microscopic observation of isolate St-bt (A and B: showed abundant spores after activation of fungus culture by UV light for 16 h at room temperature (10x) and (40x), respectively; C: plentiful mycelium without culture activation (40x); D: indicated conidiophore (Cf) (40x); Ic: immature conidia; Mc: mature conidia; M: Mycelium

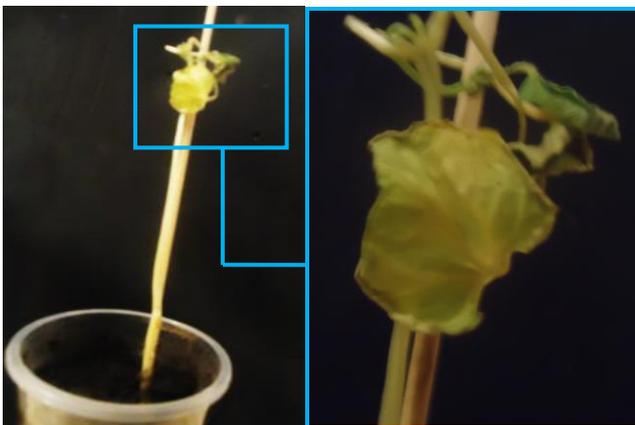


Figure 10. Pathogenicity test of isolate St-bt *Stemphylium botryosum* on *Phaseolus vulgaris* (A and B: characteristic symptom indicating yellow-brown tasks of the infected leaves)

Pathogenicity test

The purpose of the pathogenicity test was to reproduce disease symptoms observed on *P. vulgaris* leaves a moment of isolation to confirm the pathogenicity of the isolate St-bt. This test revealed symptomatic stemphyliose leaf blight disease. It was characterized by yellow-brown tasks observed throughout the leaves area (Figure 10).

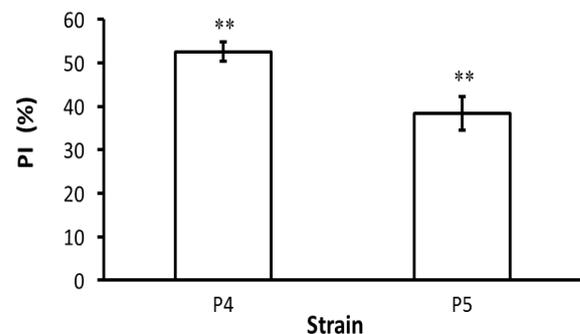


Figure 11. Antifungal activities of *Pseudomonas stutzeri* strains against isolate St-bt (PI: percent inhibition; **, very significant difference at $p < 0.05$).

In conclusion, current findings led to conclude that *P. stutzeri* strains are potent antifungal agents against isolate St-bt *in vitro* trials. Thus, by producing effective PGP traits like HCN and siderophores involved in the pathogen suppression. However, further evaluation of the effectiveness of strains P4 and P5 for biological control of bean leaf blight caused by isolated St-bt on-field culture and the design formulations and applications should be done in the field.

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