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Rhizobium isolates nodulating common bean: A pathway to control *Fusarium* sp, the causative agent of damping-off of seedlings

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ABSTRACT

Obtaining a high-performing inoculant requires combining diversified traits that can strongly contribute to promoting plant growth and protection. This work aims to contribute to developing biological control of *Fusarium* sp., the causative agent of damping-off using rhizobia. Eight rhizobial isolates from the Laboratory of Plant Biology were used. A search for some enzymes (cellulase, proteases, chitinases, and phosphatases) produced by rhizobia and involved in biological control was carried out. The rhizobia isolates were then tested for their ability to biocontrol *Fusarium* sp. in direct and indirect confrontation. Finally, the effect of inoculation with the isolates on the growth of bean plants and the severity of *Fusarium* sp. was assessed. The results show that all the bacterial isolates can synthesize lytic enzymes in varying proportions, while 50% could produce phosphatases. The direct antagonism test against *Fusarium* sp. showed that the isolates exert inhibitions ranging from 20.55% to 65.15% and 30% to 30.8% in indirect confrontation. The results of the effect of inoculation of infected plants on plant growth and pathogen severity indicate that the tested *Rhizobium* isolates promote the growth of infected plants by causing a decrease in disease as well as significant improvement in the height of the stems compared to non-inoculated plants. These results, therefore, indicate that the rhizobia isolates used considerably reduce the growth of *Fusarium* sp. using enzymes and antagonistic substances, leading to improved growth of bean plants.

Contribution/Originality: The study presents a sustainable method to control the damping-off of seedlings in common beans, which is a significant hindrance to bean cultivation.

1. INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is one of the most consumed legumes in the world. Its seeds are rich in proteins, folic acid, dietary fiber, minerals, carbohydrates, and vitamins necessary for human and animal nutrition [1]. In eastern, central, and southern Africa, dry beans are the most important grain legumes, both in terms of cultivated area and consumption [2]. African smallholders cultivate more than 4 million hectares of beans each year, the harvest of which is used as a source of food for more than 100 million Africans [3]. Rural populations of Cameroon in bean production areas cultivate several varieties of common beans for food and commercial purposes. National, sub-regional, and international demand for common beans continues to grow over the years. However, national and African production remains low due to the low availability of nitrogen in the soil, as well as the effects of abiotic

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factors (salinity, acidity, drought, etc.) and parasitic diseases [4]. Thus, more than approximately 84% of production is lost each year in tropical Africa because of these constraints [4]. Damping-off disease, caused by *Fusarium* sp. and *Pythium* sp., is one of the most important pathological constraints [5]. Yield losses due to this disease have been estimated at 80% in Cameroon [6]. The control strategy based on chemical pesticides is one of the causes of environmental pollution when use is poorly controlled. In addition, the high cost of these chemicals is a hindrance for farmers [7]. The use of resistant varieties is another improvement strategy; however, in Cameroon, resistant varieties are insufficient compared to the needs of producers. Furthermore, the development of resistant genotypes remains difficult due to variations in fungal pathogenicity [8]. It is important to limit the damage caused by damping-off in order to maximize bean production yield sustainably. Thus, conventional agriculture should move towards more sustainable and environmentally friendly cultivation systems. The provision of rhizobial inocula capable of promoting plant growth could be exploited for their inhibitory and protective properties in the biological control of phytopathogens responsible for the damping-off of common bean seedlings [9]. The main objective is to evaluate the ability of rhizobia to biocontrol *Fusarium* sp.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material

The plant material consists of common bean (*Phaseolus vulgaris*) grains of the GLP-190 variety for the greenhouse inoculation test and bean plants affected by damping-off collected in the field for *Fusarium* sp. isolation.

2.1.2. Microbial Inoculant

The microbial material consists of eight rhizobial isolates from the Laboratory of Plant Biology at the University of Douala. The isolates used (Figure 1) were obtained from bean nodules collected in Mbouda and Foumbot in the West Region, and Njombe and Nkongsamba in the Littoral Region of Cameroon, along with the reference strain ISRA 352 from the LCM (Laboratoire Commun de Microbiologie) in Dakar, Senegal.

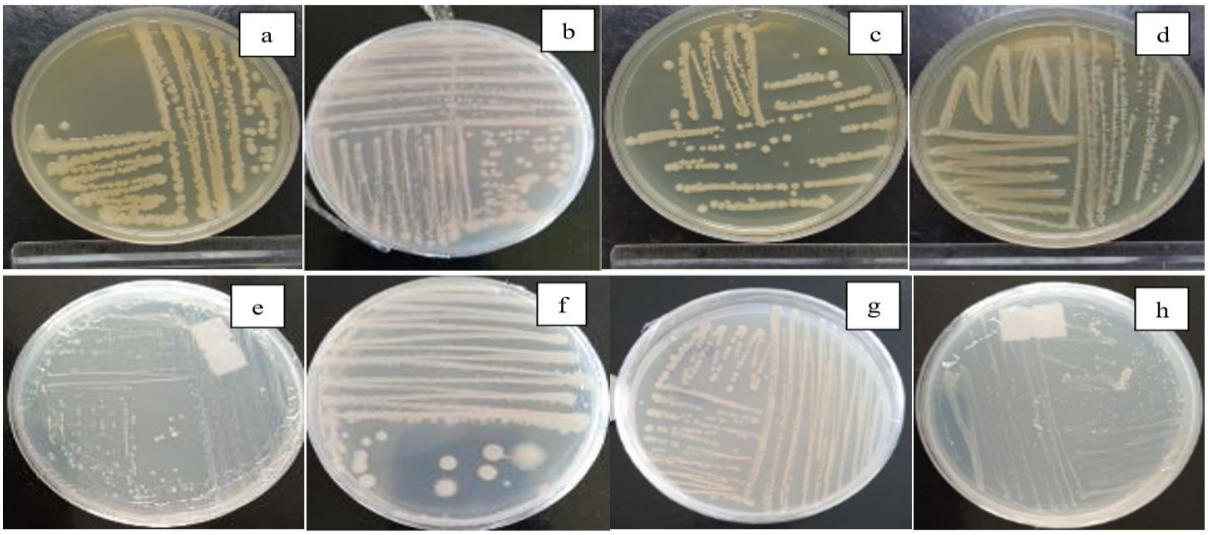

Figure 1. Macroscopic aspect of isolates used. a: PvNj5; b: PvFt3; c: PvMb1; d: PvMb5; e: PvNj8; f: PvNk7; g: PvNk8; h: ISRA 352.

The microbial material also consists of *Fusarium* sp. isolated from diseased common bean plants collected in Foumbot, West Cameroon.

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2.1.3. Soil Sample

The soil sample comes from the fallows of the experimental fields of the Institute of Agricultural Research for Development (IRAD) in Foumbot.

2.2. Methods

2.2.1. Assessing Some Lytic Activities of Rhizobial Isolates

The detection of certain lytic enzymes in bean rhizobia isolates includes the production of cellulases, proteases, chitinases, and phosphatases.

2.2.1.1. Cellulase Production

The search for the production of extracellular cellulases by bacteria is conducted in Petri dishes containing agar media supplemented with CMC (carboxymethyl cellulose) as the sole carbon source, according to the method of Johnsen and Krause [10]. The composition of the medium in g/L distilled water is as follows: Na2HPO4: 6; KH2PO4: 3; NaCl: 0.5; NH4Cl: 1; MgSO4: 0.5; CaCl2: 0.015; glucose: 2; CMC: 10 g; yeast extract: 1.2; pH: 7.2 [11]. The Petri dishes are then incubated at 28°C for 8 days, and the presence of cellulase is checked by flooding the surface of the dish with a 0.5% solution of Congo red for 30 minutes, followed by washing with distilled water. A second wash is then carried out with a 1M NaCl solution. A bright yellow halo around the bacterial colony indicates the cellulolytic activity of the bacterial isolate tested.

2.2.1.2. Protease Production

The search for proteolytic activity in the different isolates of rhizobia is determined by seeding the bacteria on the surface of the Skimmed Milk Agar medium [12], the composition of which per litre of distilled water is as follows: Peptone: 5g; Yeast extract: 2.5g; Glucose: 1g; Skimmed milk: 7%; Agar Agar: 15g; pH 7.2. The presence of a clear zone surrounding the bacterial colonies reflects proteolytic activity (caseinase) in the culture medium, which is determined after 5 days of incubation at 28°C. Three replicates were performed for each isolate. The diameter of the light zone (halo) as well as that of the colonies was measured.

2.2.1.3. Chitinase Production

The composition of the medium in g/L used to screen rhizobia isolates for chitinase activity is as follows: chitin: 5.0; yeast extract: 0.5; K_2HPO_4: 0.5; $MgSO_4 \cdot 7H_2O$: 0.2; NaCl: 0.1; Agar: 20. The pH of the medium was adjusted to 7.0, and the medium was sterilized by autoclaving at 120°C for 20 min. After cooling, the medium is transferred to sterile Petri dishes. Each rhizobia isolate is inoculated into the center of the plate and examined for a clear zone around the colony after incubation at 28°C for up to 7 days. The growth of an isolate on this medium reflects its ability to hydrolyze chitin.

2.2.1.4. Production of Phosphatases

The study of the capacity of bean-nodulating rhizobia isolates to solubilize phosphate was carried out using a qualitative test. The isolates were inoculated using a sterile loop per touch at the rate of three repetitions per Petri dish containing Pikovskaya medium (PVK) composed as follows (g/L): Yeast extract: 0.5; Glucose: 10; Ca3(PO4)2: 5; (NH4) SO4: 0.5; KCl: 0.2; MnSO4 7H2O: 0.1; FeSO4: 0.0001; Agar: 15; Distilled water: 1L; pH: 7.2. The seeded Petri dishes were then incubated at 28°C for 14 days. Naked-eye observations of the Petri dishes were made from the 3rd day. The presence of a yellow halo around the colonies indicates the production of phosphate-dissolving substances [13].

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2.2.2. Evaluation of the Capacity of Rhizobia Isolates to Biocontrol Fusarium sp. in Vitro

The evaluation of the ability of bean-nodulating rhizobia isolates to control *Fusarium* sp. started with the isolation of the pathogen, followed by its identification. A leaf disc pathogenicity test was performed. The ability of rhizobia to control *Fusarium* sp. was assessed in direct and indirect confrontations.

2.2.2.1. Sampling of Common Bean Plants and Soil

Numerous surveys carried out in the region of West Cameroon (Foumbot) made it possible to collect several samples of plants showing characteristic symptoms of damping-off (crown rot, wilting, and yellowing of leaves). The samples were taken to the laboratory for the isolation of the phytopathogen using a steel auger previously sterilized by flaming with 90° alcohol. Soil is taken randomly from the diagonals of the 5m x 5m plot at a depth between 0 and 20 cm. Within the plot, 10 randomly collected samples are carefully mixed to form a composite sample. The composite sample is used for the greenhouse experiment.

2.2.2.2. Sanitization and Isolation of Fusarium

Leaves and stems of bean plants showing symptoms of disease are used for this test. The various organs, cut with sterile pruning shears, are superficially disinfected with ethanol (10%) for 10 seconds and then with 5% sodium hypochlorite for 30 minutes. These samples are rinsed with sterile distilled water to remove all traces of bleach and then dried on sterile filter paper close to the Bunsen burner flame under a laminar flow hood. After disinfection, the organ fragments obtained from diseased bean plants are inoculated (2 to 3 fragments) on potato dextrose agar (PDA) medium autoclaved at 120 °C and poured into Petri dishes with the following composition per litre of solution: apple: 20 g; dextrose: 20 g; agar: 18 g. These dishes are incubated for 8 days at 28°C. Subsequent subcultures on other Petri dishes containing the PDA medium are carried out under aseptic conditions using a sterile loop [14] for purification.

2.2.2.3. Fungi Identification

The identification of fungi is mainly based on macroscopic characteristics such as growth rate, appearance of aerial mycelium, and color of the underside of the colony. Microscopic identification consists of observing under a light microscope the presence of septate mycelium, microconidia, macroconidia, and chlamydospores [15].

2.2.2.4. Pathogenicity Test

The bean leaves are gently rinsed with tap water and then placed in Petri dishes, each containing a cotton pad soaked in sterile water. A 5 mm fragment of the fungal isolate is then applied to each leaf. No fungal isolate fragment is applied to the control. Three replicates are carried out. The presence or absence of symptoms (yellowing or wilting of the leaves) is observed from the 3rd day. The fungi, isolated and purified on new media, are then examined by the naked eye. The identification of the fungi is based on macroscopic (cultural) and microscopic criteria.

2.2.2.5. In Vitro Antagonism of Rhizobia Isolates Against Fusarium sp.

The study of the antagonistic effects of bean-nodulating rhizobial isolates on Fusarium sp. was carried out using direct and indirect confrontation techniques.

2.2.2.5.1. Direct Confrontation Test

The direct confrontation technique makes it possible to demonstrate the production of diffusible substances by the rhizobia. The method of Inam-ul-Haq et al. [16] was used to evaluate the inhibitory effect of rhizobial isolates on the mycelial growth of *Fusarium* sp. A 5 mm diameter explant from a fungal culture is placed in Petri dishes containing 12 mL of nutrient agar medium on one side and bacterial streaks on the other. Controls consist of isolates of *Fusarium* sp. in Petri dishes in the absence of antagonists. Mycelial growth of the isolates is assessed by measuring the radius

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of the colonies after 7 days of incubation. The inhibition of fungal growth was evaluated by calculating the percentage reduction in mycelial growth according to the following Whipps formula [17]:

$$I\% = ((R1 - R2) / R1) \times 100$$

R1 = Radial distance (in mm) of mycelial growth in the absence of the antagonistic strain (control); R2 = Radial distance (in mm) of mycelial growth towards the site of inoculation by bacteria.

2.2.2.5.2. Indirect Confrontation Test

This technique consists of placing in the center of each Petri dish containing the nutrient agar medium an explant of approximately 5 mm in diameter taken from young colonies of the pathogen and in another dish bacterial streaks. The lids of the two dishes are removed, and the bottoms are sealed with a strip of parafilm to prevent the loss of volatile substances. The whole setup is incubated at 28 °C for 7 days. As a control, a Petri dish containing only the nutrient agar medium is inoculated with the pathogen [18]. The rate of inhibition of fungal growth is determined according to Whipps' method [17].

2.2.3. Evaluation of the Effect of Inoculation with Rhizobia Isolates on the Growth of Plants Infected with Fusarium and the Severity of the Disease

The isolates with the best results in enzymatic tests and the ISRA 352 strain are used for greenhouse inoculation.

2.2.3.1. Sanitizing and Pre-Germination of Seeds

Phaseolus vulgaris seeds are treated with 3% sodium hypochlorite for 3 minutes and then thoroughly rinsed with sterile distilled water. They are then soaked in 70% alcohol for 2 minutes and rinsed several times with sterile distilled water. The disinfected seeds are germinated in sterile plastic trays containing sterile filter paper soaked in sterile distilled water for 24 hours in the dark.

2.2.3.2. Sowing of Seeds

Pre-germinated seeds with short radicles are aseptically transferred into plastic bags using sterile tweezers at a rate of one seed per bag. After sowing, the bags are first soaked with sterile distilled water.

2.2.3.3. Preparation of Bacterial and Fungal Inocula

The bacterial isolates to be tested were cultured on Yeast Extract Mannitol (YEM) medium with the following composition per litre of distilled water: Mannitol: 10 g; K2HPO4: 0.5 g; MgSO4·7H2O: 0.22 g; NaCl: 0.1 g; yeast extract: 0.5 g. The pH of the medium is adjusted to 6.5. The cultures are placed in a thermostatically controlled shaker set at 125 rpm at 28 °C for 2 days. The spore suspension is prepared by flooding the agar surface with 5 ml of sterile distilled water. The fungal culture is then scraped off with a sterile spatula. The conidial suspension obtained is stirred for 30 s and then filtered through Whatman No. 1 paper to remove mycelial debris. The spore concentration is adjusted to 1,106 spores/ml using a Malassez cell [19].

2.2.3.4. Inoculation of Plants

Seedlings were obtained from seeds sown in polystyrene bags containing sterile soil. 30 mL of a fungal suspension and 30 mL of a bacterial suspension of each isolate tested, previously prepared and adjusted to 108 and 109 CFU/mL, respectively, are placed at the base of the stem of each seedling [20]. The control batches consist of a set of seedlings treated with sterile distilled water and a set of seedlings grown in soil infected with *Fusarium* sp. To allow better contamination, regular watering with sterile distilled water is carried out to maintain a humidity level sufficient for the growth of the phytopathogen. The bean plants are assessed for growth and susceptibility to disease. The plant

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growth parameters measured are stem length and leaf area [21]. Disease severity was scored on a 1-5 scale developed by CIAT(International Center for Tropical Agriculture) [22].

2.2.4. Statistical Analysis

The data collected were subjected to an analysis of variance (ANOVA) using SPSS 12.0, and means were separated by the Duncan test at the 5% probability level.

3. RESULTS

3.1. Production of Lytic Enzymes by Bacterial Isolates

The production of lytic enzymes by bacterial isolates is shown in Figure 2.

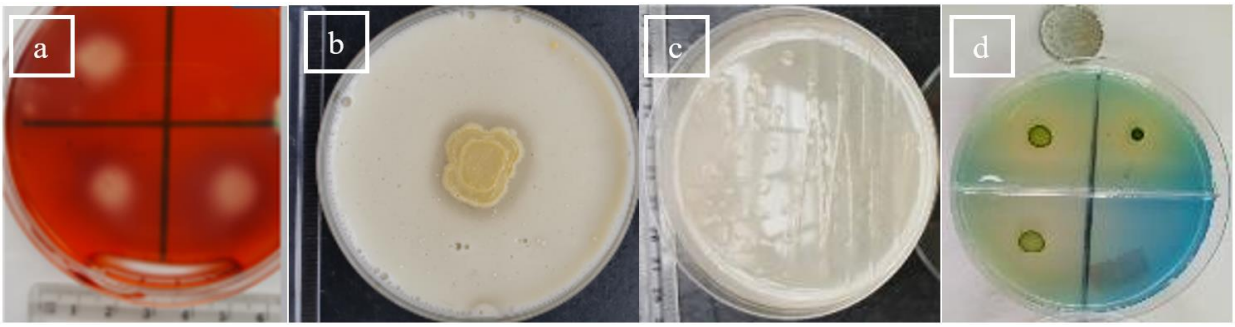

Figure 2. Production of lytic enzymes by bean nodulating rhizobia isolates (a. cellulase; b. protease; c. chitinase; d. phosphatase).

3.1.1. Production of Cellulases

Figure 3 shows the diameters of the halos produced by cellulase-producing rhizobial isolates on agar supplemented with CMC. All rhizobial isolates (100%) had the ability to produce halos on specific agar media, although the diameters varied between isolates. The diameter of the halos varied from 0.5 to 3.2 cm. Cellulase production is significantly higher for isolate PvFt3 (3.2 cm) and significantly lower for PvNj8 (0.55 cm).

Isolates PvMb1 (2.55 cm), PvNk7 (2.65 cm), and PvNj5 (2.55 cm) do not show any significant difference. These isolates had significantly larger halo diameters than isolates PvMb5 (1.1 cm) and PvNk8 (1.25 cm), which in turn had significantly smaller halo diameters than the reference strain ISRA 352 (1.2 cm).

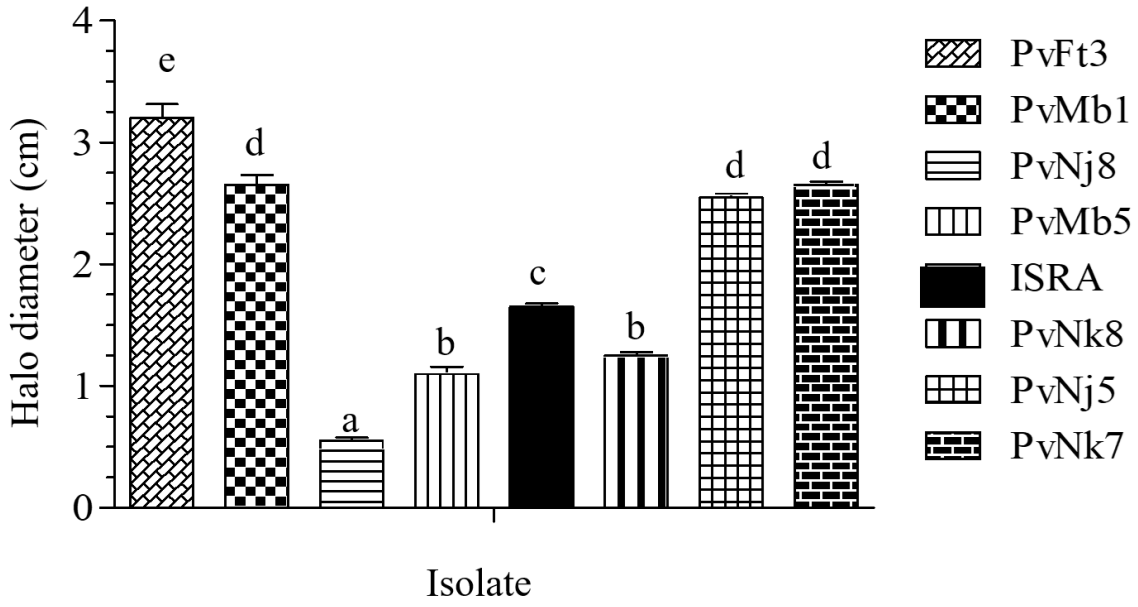

Figure 3. Diameters of halos obtained by cellulase-producing rhizobial isolates.

Note: Bars with the same letter (a, b, c, d, e) are not significantly different by Duncan's test with a mean probability of 5%.

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3.1.2. Production of Proteases

The production diameter (Figure 4) ranges from 1.08 cm to 2.5 cm. Protease production is significantly higher for isolates PvNj5 (1.8 cm) and PvMb5 (1.7 cm) and significantly lower for isolates PvNj8, ISRA 352, and PvNK8 with values of 1.21 cm and 1.08 cm, respectively. The ANOVA test shows that there is no significant difference between isolates PvNj5 and PvMb5 (1.19 cm). No significant difference was found between the reference strain ISRA 352 (1.2 cm) and isolates PvNj8 (1.21 cm) and PvNk8 (1.3 cm).

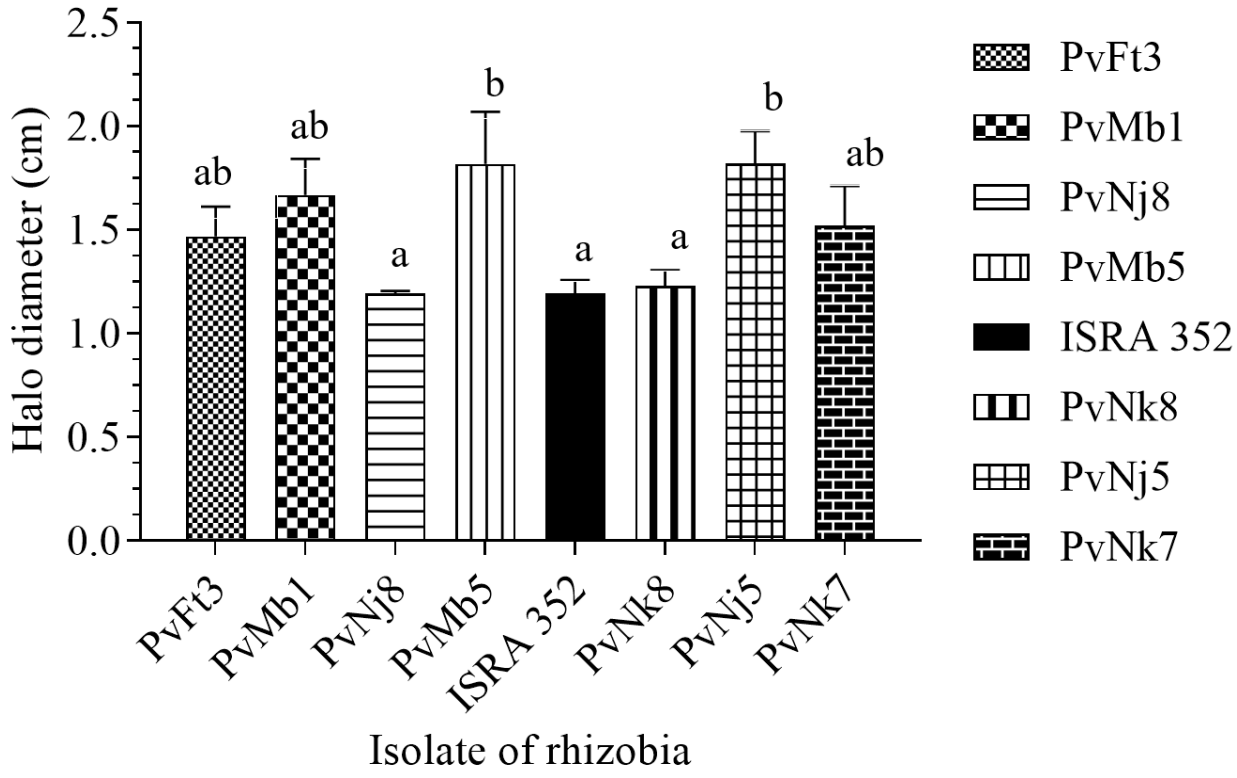

Figure 4. Production of proteases by rhizobia isolates.

Note: Bars with the same letters (a, b) are not significantly different at 5% average probability by the Duncan test.

3.1.3. Production of Chitinases

The production of chitinases by the different bacterial isolates is presented in Table 1. Isolates PvFt3 and PvNj5 presented distinct colonies and good growth compared to ISRA 352, PvMb1, PvMb5 and PvNk8, which represent 50%. PvNj8 and PvNk7 had weak growth.

Table 1. Production of chitinases by rhizobia.

Isolates of rhizobia	Chitinases
PvFt3	+++
PvMb1	++
PvNj8	+
PvMb5	++
ISRA 352	++
PvNk8	++
PvNj5	+++
PvNk7	+

Note: -: No growth; +: Low growth; ++: average growth; +++: Strong growth.

3.1.4. Production of Phosphatases

Table 2 shows the results of the phosphatase production test. Good production of phosphatases, reflected by the formation of the yellow halo around the colony, was recorded by isolates PvMb5 and PvNk7 (25%) compared to all

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isolates. ISRA 352, the reference strain from Senegal, and PvMb1 (25%) showed low phosphatase production, while isolates PvFt3, PvNj8, PvNk8, and PvNj5 (50%) did not produce a solubilization halo.

Table 2. Production of phosphatases by rhizobia.

Isolates	Phosphatases
PvFt3	-
PvMb1	+
PvNj8	-
PvMb5	++
ISRA 352	+
PvNk8	-
PvNj5	-
PvNk7	++

Note: -: No production; +: Low production; ++: Good production.

3.2. Ability of Rhizobia Isolates to Biocontrol Fusarium sp. in Vitro

To evaluate the biopesticidal capacity of rhizobial isolates, the isolation of the phytopathogen was carried out using plants affected by damping-off of common bean and then identified. A pathogenicity test was conducted, and finally, direct and indirect confrontation tests were performed against *Fusarium* sp.

3.2.1. Appearance of the Phytopathogen Isolate Obtained from Diseased Bean Plants

Samples of diseased stems and leaves used to isolate the plant pathogen resulted in a pure *Fusarium* isolate. Macroscopic observation of the colonies shows that they grow rapidly. On PDA (Figure 5A), the colony is pale with well-developed mycelium. Microscopic observation of *Fusarium* sp. revealed the presence of septate filaments accompanied by microconidia and macroconidia (Figure 5B), straight or slightly curved, small, rifle-shaped.

Figure 5. Macroscopic (A) and microscopic (X40) (B) appearance of the *Fusarium* isolate.

3.2.2. Ability of Bean Nodulating Rhizobia Isolates to Control Fusarium sp. in Direct Confrontation

The results show that 62.5% of the isolates have antagonistic activity. The most significant inhibitions were obtained with isolates PvMb1 (Figure 6a) and PvFt3 (Figure 6b), which caused a significant reduction in the diameter

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of *Fusarium* sp. compared to all isolates. Isolate PvNk7 (Figure 6c) caused weak inhibition of the phytopathogen compared to the diameter of the *Fusarium* sp. control (Figure 6d), which completely invaded the dishes 8 days after sowing. Isolates PvNJ8 and ISRA 352 showed no inhibition of *Fusarium* sp.

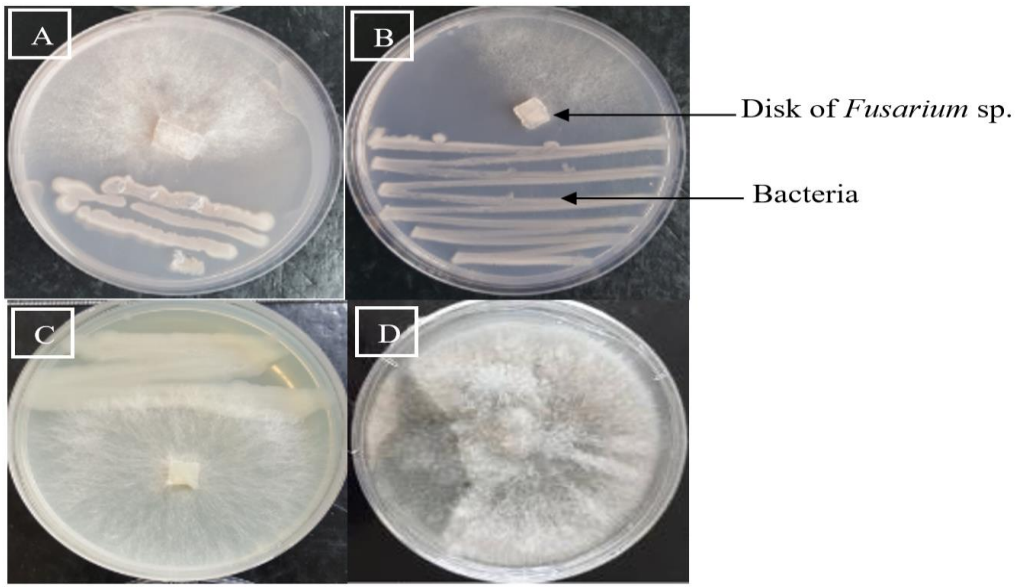

Figure 6. Direct confrontation test of bean nodulating rhizobia - *Fusarium* sp: a) inhibition of *Fusarium* by PvMb1; b: inhibition of *Fusarium* by PvFt3; c: **N**o inhibition of *Fusarium* by PvNk7; d: **Co**ntrol dish containing only *Fusarium*.

Figure 7 shows the inhibition percentages of *Fusarium* sp. by isolates of rhizobia nodulating bean and the reference strain ISRA 352 in direct confrontation. The inhibition percentages of *Fusarium* sp. varied from 20.55% for the reference strain IRSA 352 to 65.15% for the PvFt3 isolate. Statistical analysis of the results using the ANOVA test shows a highly significant effect ($p<0.05$) of the PvFt3 and PvMb1 isolates with inhibition rates of 65.15% and 64.44%, respectively on the growth of *Fusarium* sp. The PvNj8 (41.66%) and PvNj5 (38.66%) isolates show non-significant inhibition percentages between them but differ from the PvMb5 isolate, which shows an inhibition percentage of 31.1%. There is no apparent inhibition for isolates PvNk7 (14.99%) and PvNk8 (16.66%), which have inhibition percentages of less than 20%. These percentages are not significantly different from those observed for ISRA 352, which shows a slight inhibitory activity. Among the eight rhizobial isolates tested, five (62.5%) inhibited the development of *Fusarium* sp. by more than 20%.

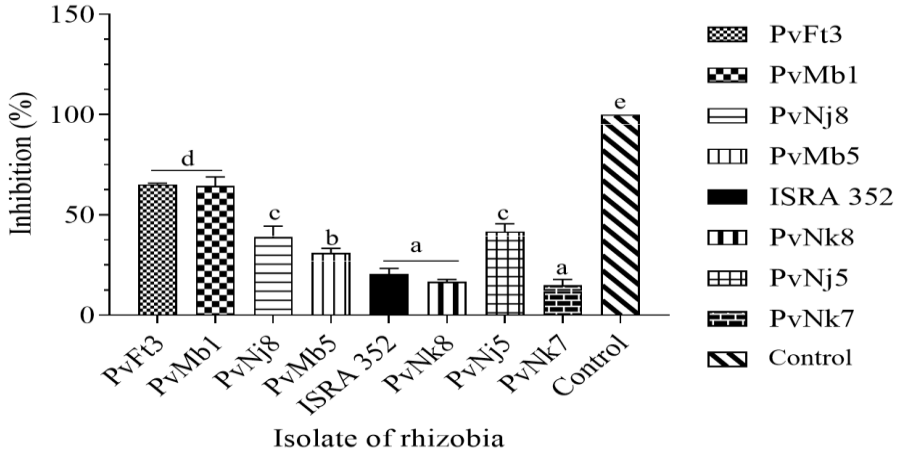

Figure 7. Percentage inhibition of *Fusarium* sp. by rhizobia isolates in direct comparison.
Note: Bars with the same letters (a, b, c, d, e) are not significantly different at 5% average probability by the Duncan test.

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3.2.3. Ability of Bean Nodulating Rhizobia Isolates to Biocontrol Fusarium sp. in Indirect Confrontation

The indirect confrontation test makes it possible to screen rhizobia for volatile substances capable of inhibiting the growth of *Fusarium* sp. The results show a distant inhibitory effect of rhizobial isolates on the growth of *Fusarium* sp. Figure 8a illustrates the inhibitory effect of the PvFt3 isolate on the growth of the phytopathogen in comparison with the control dish (Figure 8b), in which the fungi invaded the entire Petri dish.

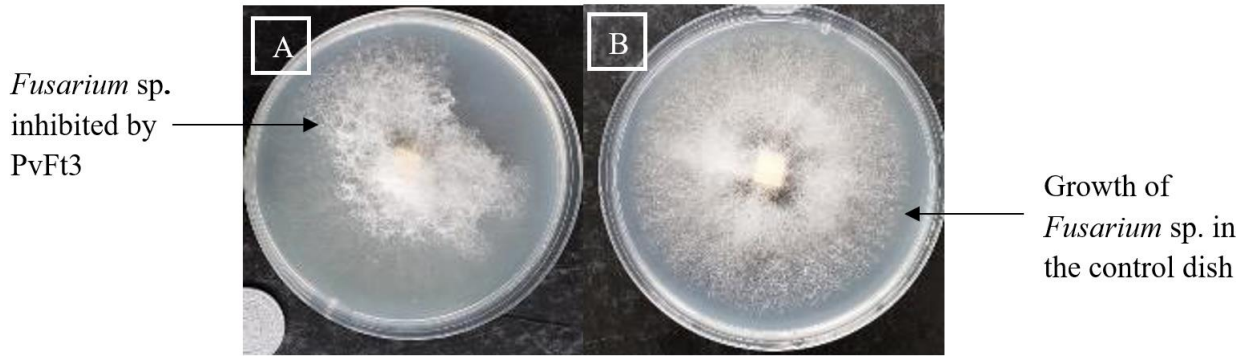

Figure 8. Indirect rhizobia - *Fusarium* sp. confrontation test: A) Inhibitory effect of PvFt3 against *Fusarium* sp.; B: Control.

Figure 9 shows the percentages of inhibition of *Fusarium* sp. by the isolates tested in indirect confrontation. The percentage of inhibition varied between 30% and 30.8% depending on the isolate. Not all isolates are able to produce volatile substances that inhibit the growth of the phytopathogen. Isolates PvNj8, IRSRA 352, PvNk8, and PvNj5 did not induce inhibition (50%). The highest inhibition rates were recorded by the isolates PvFt3 and PvMb1, with inhibition percentages of 30% and 30.8%, respectively (25%). The inhibition percentage of PvFt3 is significantly higher, followed by the PvMb1 isolate. PvMb5 (17.18%) and PvNk7 (13%) showed negligible antagonistic effects, with inhibition rates below 20%.

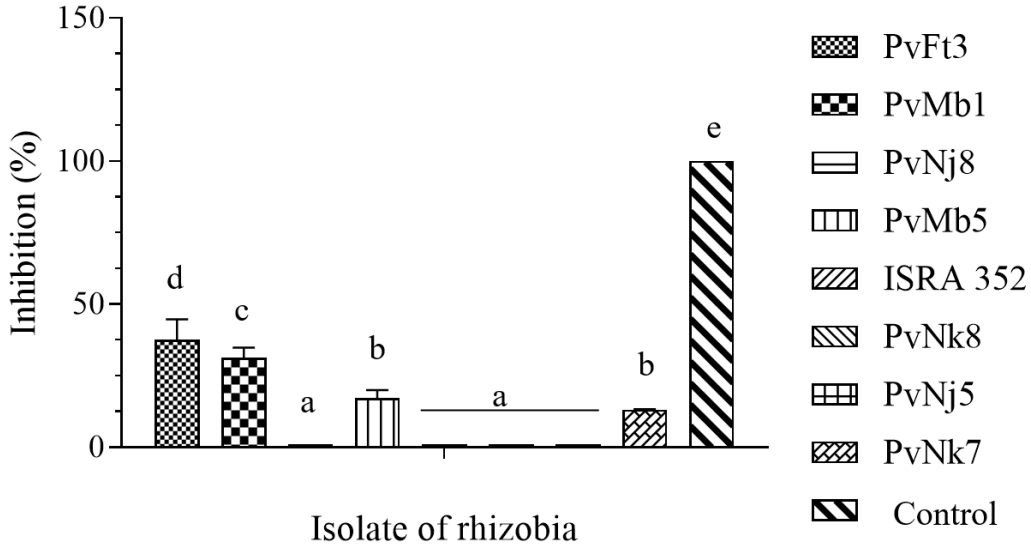

Figure 1. Percentage inhibition of *Fusarium* sp. by bean nodulating rhizobia isolates in indirect confrontation.
Note: Bars with the same letters (a, b, c, d, e) are not significantly different at 5% average probability by the Duncan test.

3.3. Results of the Effect of Inoculation with Rhizobia Isolates of Plants Infected with Fusarium sp. on their Growth and the Severity of the Phytopathogen

3.3.1. Results of the Effect of Inoculation of Rhizobia Isolates on the Growth of Infected Bean Plants

Inoculation resulted in a significant improvement in stem height compared to non-inoculated and non-infected controls. Infection of common bean plants with *Fusarium* sp. significantly reduced the height of the plant stems in

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some cases. The stem length of plants infected with *Fusarium* sp. is significantly higher for plants inoculated with isolates PvFt3, PvMb1, PvNj5, and PvMb5, with values of 24.3 cm, 23 cm, 22.15 cm, and 21.5 cm, respectively and significantly lower for strain ISRA 352 (19.75 cm). The stem length of uninfected plants (F-) is significantly higher for plants inoculated with isolate PvFt3 (26.1 cm), followed by isolates PvMb1 (25 cm) and PvNj5 (25.75 cm), with no significant difference between them, and significantly lower for strain ISRA 352 (24 cm). Inoculation with PvFt3 improves the stem growth of infected plants (F+). A significant difference in stem length was observed between plants infected with *Fusarium* sp. and inoculated with PvFt3 (24 cm) and uninfected plants (26.1 cm) inoculated with the PvFt3 isolate. An improvement of 1.9 cm was observed in the stems of plants inoculated and infected with *Fusarium* sp. compared to infected control plants. The improvements in stem length of isolates PvMb1, PvNj5, and PvMb5 are 2.8 cm, 3.2 cm, and 3.5 cm, respectively. Strain ISRA 352 shows no significant difference (Figure 10). Inoculation with strain ISRA 352 showed no significant difference between infected and uninfected plants. An improvement in stem height of 2.4 cm was observed in inoculated and infected plants.

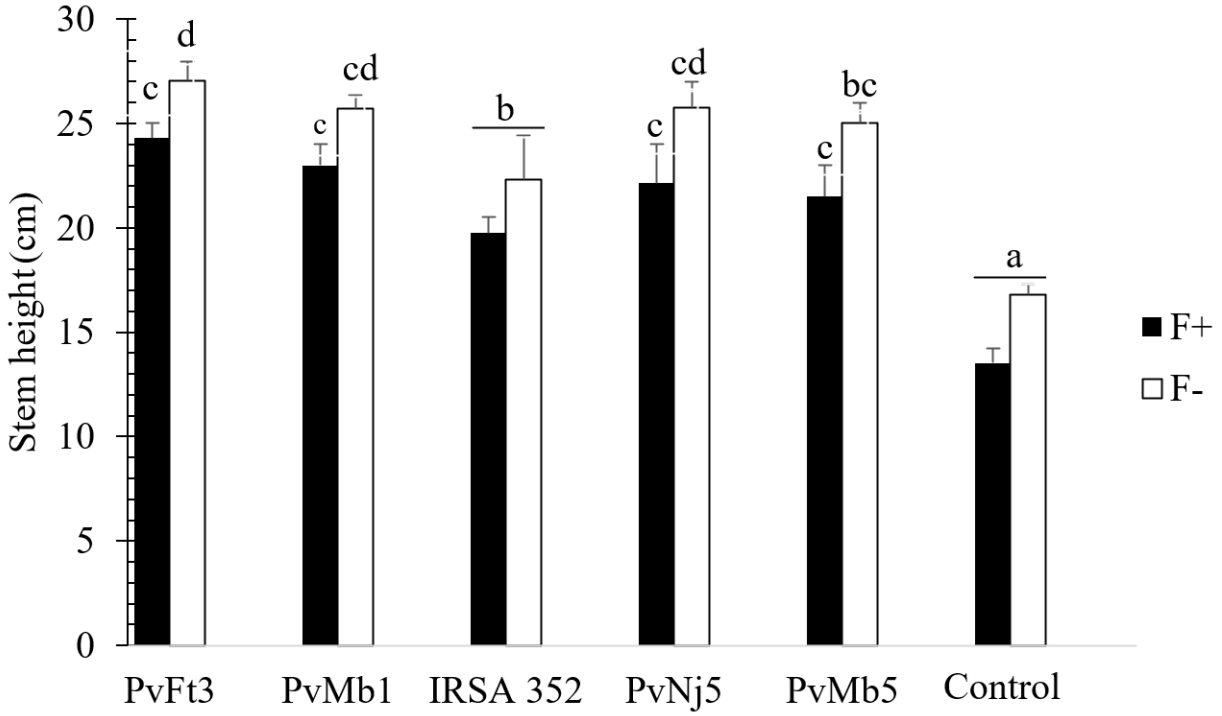

Figure 10. Stem height of common bean plants with rhizobia isolates and infected with *Fusarium* sp.

Note: Bars with the same letters (a, b, c, d, e) are not significantly different at 5% average probability by the Duncan test. F-: No infection by *Fusarium* sp.; F+: Infection by *Fusarium* sp.

The growth of infected and inoculated plants was also investigated by assessing the leaf area of plants infected with *Fusarium* sp. and inoculated with rhizobia. Infection of common bean plants with *Fusarium* sp. reduced the leaf area of infected plants (29.33 cm2) compared to uninfected control plants (33 cm2), but this difference is not significant. All rhizobia isolates caused an improvement in leaf area of infected and rhizobia-inoculated plants compared to infected and non-inoculated plants, with variation between rhizobia isolates (Figure 11). The leaf area of plants infected with *Fusarium* sp. is highest in plants inoculated with isolate PvFt3 (50.6 cm2), followed by isolates PvMb1 (49 cm2), ISRA 352 (42 cm2), PvNj5 (40.66 cm2), and PvMb5 (38 cm2). The leaf area of plants not infected with *Fusarium* sp. is larger for plants inoculated with isolate PvMb1 (55.6 cm2), followed by isolates PvFt3 (50.33 cm2), IRSA 352 (51 cm2), PvNj5 (43 cm2), and PvMb5 (39 cm2). Slight improvements in leaf area were recorded for all isolates, but these improvements were not significant.

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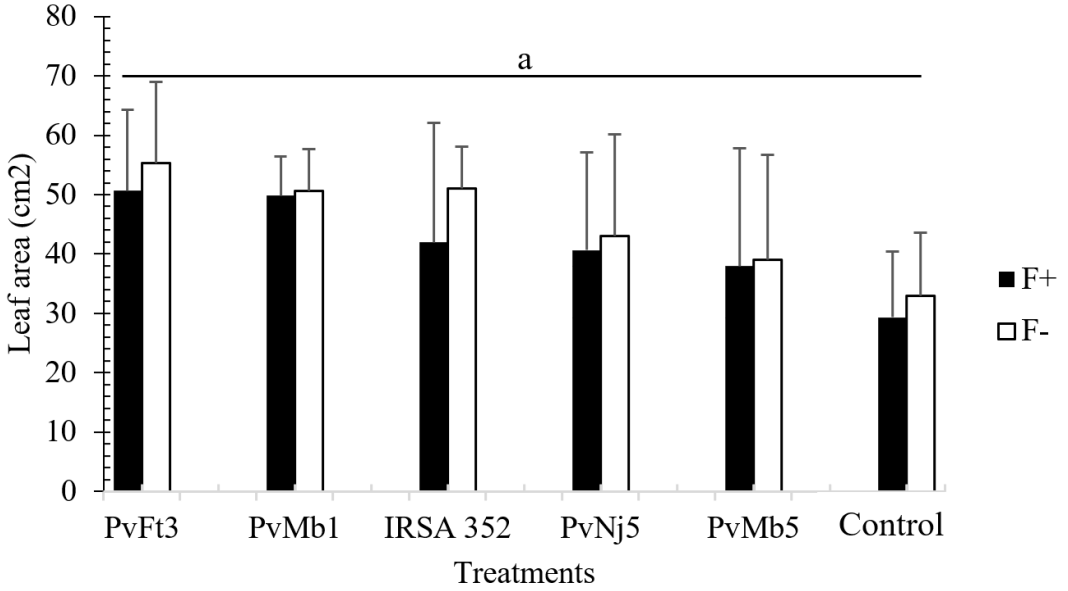

Figure 11. Leaf surface of bean plants infected with *Fusarium* sp. and inoculated with rhizobia.

Note: Bars with the same letter (a, b, c, d, e) are not significantly different according to Duncan's test with an average probability of 5%. F-: No infection by *Fusarium* sp.; F+: Infection by *Fusarium* sp.

3.3.2. Severity of Fusarium sp. on Plants

The protective effect of the different isolates selected against damping-off disease in common bean plants was evaluated by measuring the severity of the disease (Table 3) in seedlings previously inoculated and infected with bacterial suspensions. Inoculation with rhizobia (antagonists) has a positive effect on disease reduction compared with infected but non-inoculated control plants. No obvious damage was observed in plants inoculated with PvFt3 and PvMb1. In other words, these isolates confer resistance to the pathogen. These isolates resulted in a 90% reduction in disease. For PvNj5 and PvMb5, 16-25% damage was observed, showing that the isolates reduced disease severity by 75%. These isolates are intermediate between resistant and susceptible isolates. For the reference strain ISRA 352, 26-50% damage was observed. The ISRA 352 strain reduced the disease by 50%. None of the plants showed symptoms severe enough to cause yield loss or plant death.

Table 3. Severity of bean plants affected by the damping off of seedlings.

Isolates	Severity range	Reduction of the disease (%)
PvFt3	1 - 5 %	90
PvMb1	1 - 5 %	90
PvMb5	16 - 25 %	75
PvNj5	16 - 25 %	75
ISRA 352	26 - 50 %	50
Control	26-50%	50

Note: 1 - 25%: No visible symptoms; 26 - 50%: Visible symptoms.

4. DISCUSSION

4.1. In Vitro Biocontrol of Fusarium sp. in Direct and Indirect Confrontation

Fusarium sp. is a ubiquitous plant pathogen that causes root rot, vascular wilt, and damping-off in a wide range of host plants. The isolation steps in the laboratory allowed a fungus to be obtained from the leaves and stems of diseased bean plants. Microscopic observations revealed the presence of a septate mycelium with two categories of conidia: more or less numerous microconidia and macroconidia. The presence of these two types of spores is considered one of the main characteristics for the identification of *Fusarium* sp. [23]. *Fusarium* sp. was recovered from the stems and leaves of plants showing symptoms of crown rot specific to wilt. Analysis of the morphological appearance of the colonies revealed whitish filamentous hyphae. This result is in agreement with the work carried out

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by Nelson et al. [24] and Hibar et al. [25], who claim that this pigmentation produced by the phytopathogen is generally specific to *Fusarium oxysporum* species. The demonstration of antagonistic activity against bean seedling wilt (*Fusarium* sp.) was carried out *in vitro* using the direct confrontation technique in Petri dishes containing a PDA agar medium. All isolates induced a reduction in pathogenic mycelial growth. The basic mechanisms of such inhibition may be due to antibiosis and parasitism. The fact that the fungal colony does not completely colonize the box in the presence of the bacteria reflects the ability of the antagonists to produce diffusible substances that limit the mycelial growth of the pathogen. According to Nourozian et al. [26], the formation of zones of inhibition between the pathogen and the antagonist bacteria in the confrontation test is probably due to the production and diffusion in the culture medium of substances with antimicrobial activity produced by the antagonists. In addition, the use of nutrient-rich media eliminates the possibility of this zone occurring due to a phenomenon of competition for nutrients [20]. The results obtained show that the inhibition rate varies from 20.55% to 65.15%. These results are in agreement with those of Youagang Gougueu [9], who showed that rhizobia can significantly reduce the growth of *Fusarium* sp. and Rhizoctonia, phytopathogens responsible for the damping-off of bean seedlings. The variability in the rate of inhibition is probably due to both the concentration of the diffusible substance produced by the antagonist isolate [27] and the nature of this substance [28]. Rhizobia inhibit *Fusarium* sp. in indirect confrontation with inhibition rates varying from 30 (PvMb1) to 30.8% (PvFt3). This activity was detected in 25% of the isolates tested. This inhibition may be due to antibiosis or to the production of volatile compounds. According to Kai et al. [29], more than 200 volatile organic compounds produced by bacteria and molds have been identified. The majority of these molecules are involved in multiple interactions and exert antimicrobial or modulatory effects on the growth of microorganisms and plants.

4.2. Inoculation Effect of Inhibitory Rhizobial Isolates in Vitro on Bean Growth and Disease Severity

The results obtained show that the majority of isolates improve bean growth and are able to reduce disease in the presence of the pathogen. Disease severity was reduced at rates ranging from 50% to 90%. The isolates PvFt3 and PvMb1 were the most effective in reducing disease severity, with a reduction of 90%. These results are in agreement with those of Asseng et al. [30] and Youagang Gougueu [9] who reported the beneficial effect of endophytes such as Rhizobium on plant growth and the reduction in the incidence of fungal diseases.

The protective activity of isolates is not linked to a single mechanism, but rather to the synergy of several parameters [31]. Although the effects of PGPR are divided into two distinct categories: growth-promoting effects and growth-promoting biological control effects, there is always a close relationship between the two [32]. PGPRs promote overall plant growth, leading to increased disease tolerance in the plant, and conversely, biological control of plant diseases by PGPRs can indirectly lead to promotion of plant growth [33]. Numerous studies have shown that the protective effect of PGPR is most often the result of the presence of multiple traits that can act directly or indirectly on disease development in the plant.

The reduction in disease severity after treatment with antagonists (rhizobia) is most often accompanied by an improvement in plant growth compared to infected controls [34, 35]. The results of the present study confirm these observations. A significant improvement ($p < 0.05$) was observed in the different growth parameters. The stem length and leaf area of infected bean seedlings were significantly improved after inoculation with the five selected isolates. PvMb1 and PvFt3 isolates showed the best performance compared to the other isolates. In addition to the action of antibiotics, volatile or diffusible substances, as the main agents in reducing the growth of pathogens, promoting plant growth through the synthesis of growth regulators and making mineral elements available to the plant, can also contribute indirectly to preventing the installation of the pathogen in the rhizosphere of the plant [10, 32].

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5. CONCLUSION

The use of PGPR for the sustainable improvement of agricultural production is becoming increasingly common in agriculture. The aim of this study was to contribute to the development of biological control of *Fusarium* sp., the causal agent of damping-off in common bean, through the use of rhizobia. Eight isolates from the collection of the Plant Biology Laboratory were tested for their ability to synthesize lytic enzymes. The results obtained showed that all isolates were positive in the cellulase, chitinase, and protease production tests, but 50% of the isolates were positive in the phosphatase production test. The *in vitro* antagonistic activity of the eight bean-nodulating isolates against *Fusarium* sp. showed an inhibitory effect on the growth of the mycelium of *Fusarium* sp. in direct and indirect confrontation on Petri dishes. The inhibition rate varied from 20.55% to 65.15% in direct confrontation and from 30% to 30.8% in indirect confrontation. It seems that the most effective mechanism of inhibition of *Fusarium* sp. by rhizobia is the production of diffusible substances. The effect of rhizobial inoculation of infected plants on the growth and severity of *Fusarium* sp. showed a clear improvement in stem length and leaf area of bean plants and a reduction in damage caused by the pathogen. The best isolates in the whole study are PvFt3 and PvMb1.

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