Antagonistic Effect of Indigenous *Bacillus subtilis* on Root-/Soil-borne Fungal Pathogens of Cowpea

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Abstract: The biopesticide ability of indigenous *Bacillus subtilis* as a biocontrol agent against cowpea fungal pathogens *Fusarium verticilloides, F. equiseti, F. solani, F. oxysporum,* and *Rhizoctonia solani* isolated from diseased cowpea in the northern Guinea savanna of Nigeria was evaluated in the laboratory. Primary *in-vitro* screening for antagonism against these phytopathogenic fungi revealed significant ($P \leq 0.05$) inhibitory effects on mycelial radial growth of the pathogens. Generally, the antibiosis exhibited by *B. subtilis* against *F. verticilloides, F. equiseti,* and *R. solani* was highly significant. However, there were little or no inhibition effects on *F. solani,* and *F. oxysporum*. Differences in times of inoculation between the antagonist and the pathogens were not significantly ($P \leq 0.05$) different in aiding effective and efficient inhibition of the pathogens by *B. subtilis.* The *B. subtilis* strain isolated, identified, and used in this present study is a promising natural biopesticide agent which can be considered as an alternative to chemical pesticides in cowpea disease management strategies and should be further studied and tested for control of other phytopathogenic fungi causing diseases and yield loss in susceptible cowpea germplasm.
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1. Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important grain legume and hay crop in many tropical and subtropical regions, especially in the dry savanna region of West Africa (Fang et al. 2007). Although cowpea is cultivated worldwide, over 75% of the world production is obtained from Africa (Singh et al. 2002). Nigeria is the world's largest producer and consumer of cowpea, as it produces over 2.7 million tonnes of cowpea annually, with an average yield of 417 kg/ha. It produces the white and brown varieties. Nigeria's production in 2005 was estimated to be 110000 tonnes (FAO, 2007). Cowpea is attacked by many diseases caused by viruses, bacteria, fungi, and nematodes (Emechebe and Lagoke, 2002). The two years (2006 and 2007) screening of cowpea field showed that, the most frequently isolated and important root-/soil-borne fungal pathogens that infected cowpea genotypes were *F. verticilloides*, *F. equiseti*, *R. solani F. solani*, and *F. oxysporum* (Killani, 2010). These pathogens attack the underground parts, leading to pre- and post-emergence death, damping-off, seedling and leaf blight, root rot, sunken stem, leaf spot, and leaf rot as reported by Emechebe and Lagoke (2002). Indiscriminate use of fungicides and pesticides in controlling the diseases has polluted both the environment, and the produce (Newsham et al. 1995), thus, the need for proper management of these diseases to sustain the cowpea production at reduced doses of pesticides.

The mechanisms of biological control of plant pathogens by antagonistic bacteria and fungi have been the subjects of many studies in the past two decades (Janisiewicz et al. 2000). Most of these studies were on the control of root- and soil-borne plant pathogens and, to a lesser extent, foliar pathogens. Antagonists are biocontrol agents such as bacteria, fungi, actinomycetes, viruses, and nematodes that reduce the number of disease producing activities of the pathogens (Whipps and Lumsden, 2001). Mechanisms of biocontrol of root and soil-borne pathogens are as a result of the direct action of antagonists on plant pathogens, through antibiosis, predation or parasitism, induced resistance of the host plant, and direct competition for space and limited resources (Janisiewicz et al. 2000). These mechanisms reduce the infection level and bring about the desired results. Linderman (2000) reported that shifts in the microbial community structure and the resulting microbial equilibria can influence the growth and health of plants. Activation of plant defense mechanisms, including the development of systemic resistance, has also been proposed, but the occurrence of this mechanism and its impact in biological control, need further research. Trends in research include the increased

use of biorational screening processes to identify microorganisms with the potential for biocontrol, increased testing under semi-commercial and commercial production conditions, and increased emphasis on combining biocontrol strains with other control methods, thereby integrating biocontrol into an overall production system (Fravel, 2005). Many biocontrol agents have been successfully used in laboratory and greenhouse experiments to control root/soil-borne fungal and bacterial pathogens. Bacteria are important as antagonists of soil pathogens such as *Fusarium* spp. and certain other pathogens that attack roots rapidly through multiple infections. Examples of such bacteria are *Pseudomonas fluorescens* (Georgakopoulos et al. 2002), *P. putida* (Scher and Baker, 1982), *Pseudomonas* spp. (Whipps and Lumsden, 2001), *Agrobacterium radiobacter* (Powell et al. 1990), *Bacillus* spp. (Ikotun and Adekunle, 1990; Thomashow and Weller, 1990). *Streptomyces* spp. and Actinomycetes spp. (Ikotun and Adekunle, 1990). Therefore the aims of this study are to investigate the antibiosis effect of isolated and identify indigenous *B. subtilis* against major root-/soil-borne fungal pathogens isolated from cowpea and its rhizosphere.

2. Materials and Methods

2.1 Isolation and Identification of Bacterial Antagonist (*B. subtilis*)

Bacillus subtilis was isolated from the soil collected from the cowpea rhizosphere in the experimental field of the International Institutes of Tropical Agriculture (IITA) in the northern Guinea savanna, in Shika, Kaduna State, Nigeria using pour plate methods (Janisiewicz, 1988 and Roberts, 1990). Colony counting was done by means of a Gallenkamp colony counter (Model Leica Quebec Darkfield Colony Counter). The numbers of bacteria per gram of soil were calculated using the following formula:

$$\text{CFU per gram soil} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Plate}}$$

After 96 hours incubation, attention was paid to bacterial and fungal colonies showing a clear zone of inhibition against other microorganisms. These were sub-cultured, and purified to obtain pure cultures and identified. A pure culture of *B. subtilis* was maintained on sucrose peptone agar slants in 28 ml McCartney screw capped bottles; containing sucrose, 2 g; peptone, 0.5 g; KH_2PO_4, 0.05 g; $MgSO_4.7H_2O$, 0.025 g; and agar, 1.5 g (Lelliott, 1965).

2.2 Isolation and Identification of Root-/soil-borne Fungal Pathogens

Root-/soil-borne fungi were isolated from naturally infected roots/stems of cowpea plants collected from the experimental plot site. Infected plant tissues were surface sterilized in 3% sodium hypochlorite (NaClO) for 3 min, rinsed in three changes of sterile distilled water, and then blotted dry with a sterile paper towel pad. Approximately 2 mm × 7 mm tissue sections were cut from the advancing portion of the lesion of surface sterilized tissue using a sterile scalpel blade. The sections were plated on specific *Fusarium* spp. medium [Peptone Penta- chloronitro-benzene Agar (PPA)] modified by Nash and Snyder (1962). The PPA contained the following: Diffco agar powder (15 g l^{-1}), peptone (15 g l^{-1}), KH_2PO_4 (1 g l^{-1}), $MgSO_4.7H_2O$ (0·5 g l^{-1}). The medium was autoclaved at 120°C for 20 min. The medium was then amended with Chloramphenicol (0·05 g l^{-1}), Pentachloronitrobenzene (0·75 g l^{-1}), Chlorotetra-cycline (0·5%), water (10 ml l^{-1}) as suggested by Ros et al. (2005). For *R. solani*, full strength Difco Potato Dextrose Agar (PDA), prepared according to manufacturer's specifications, was employed. The plates were incubated at 28°C in an incubator (Model Gallenkamp Cooled Incubator) for 7 days. Young active growth of different fungal mycelia from each isolate on the plated tissue was sub-cultured on to PDA and *Fusarium* spp. isolates were purified using the single spore technique on PPA. The pour plate method was used for the isolation of fungi from the soil. Fungal isolates were characterized and identified based on their colonial morphology and microscopic characteristics using different identification keys and methods developed by Nelson et al. (1983). Bacterial isolates were maintained on nutrient agar (NA) slants while fungal pathogens were maintained on PDA slants. Slant cultures were stored at 4°C in the refrigerator until used.

2.3 *In-vitro* Antagonistic Experiment

Three different types of experiments were carried out to evaluate the antagonistic effects of *B. subtilis* using the pairing and spreader methods. Nutrient broth yeast agar (NBYA) was prepared and poured into sterilized Petri dishes and allowed to solidify. *B. subtilis* was separately cut with 5 mm sterile cork borer. The agar discs were inoculated at four equidistant positions adjacent to one another. The fungal pathogens were equally inoculated against *B. subtilis* at the opposite ends on the same diameter of 9 cm Petri dishes of sterile NBYA for *B. subtilis* and vice versa for the fungal pathogens. Inoculation of the antagonist was done 24 hours before the pathogens, simultaneous with the pathogens and 24 hours after the pathogens. All the plates were incubated at a temperature of 28°C for 7 days to allow adequate antagonist - pathogen interaction to take place. All the cultured plates were periodically observed for mycelial growth and zone of inhibition. The growth diameter and zone of inhibition (cm) of both the antagonists and the pathogens were measured. Using NBYA prepared agar, a loopful of pure culture of *B.*

subtilis was taken and seeded fully on sterile NBYA plates. Mycelia discs of young actively growing cultures of the fungal pathogens were cut separately with sterilized 5-mm cork borers and inoculated right at the centre of the cultured plates. There were three replicates of each treatment. The plates were incubated at 28°C±2 and were periodically observed for 7 days to allow adequate antagonist - pathogen interaction to take place. The growth diameters (cm) of the pathogens were measured. The percentage inhibition was calculated according to Odebode et al. (2004). Laboratory data were collected for 3-9 days.

2.4 Statistical Data Analysis: Statistical analyses were performed using General Linear Modeling (GLM) procedure, with Duncan Multiple Range Test (DMRT), of SAS® (2009) System for Windows Version 9.1 software (SAS Institute, Cary, North California, USA), to compare different treatments with respect to degree of inhibition. Laboratory bioassay experiments' dependent variables were subjected to analysis of variance (ANOVA). The least square means (LSM) test at 5% level of significance was used to compare treatment means for each measured parameter. Standard error (SE) and coefficient of variation (CV in %) were also computed.

3. Results

In-vitro effect of *B. subtilis* on fungal pathogens (*F. verticilloides*, *F. equiseti*, *F. solani*, *F. oxysporum*, and *Rhizoctonia solani*) after day 3 showed that *B. subtilis* had established no contact with the mycelia of all the fungal pathogens except for *R. solani* which recorded a zone of inhibition of average diameter of 0.30 cm; this trend was maintained throughout the period of observation. However, *F. verticilloides* finally established a zone of inhibition of average diameter 0.10 cm, after day 5. ANOVA revealed that *B. subtilis* significantly (P<0.0001) inhibited growth of *F. equiseti*, *R. solani* , *F. solani*, and *F. oxysporum* to an average zone of inhibition of 4.10 cm, 3.00 cm, 3.20 cm 3.00 cm, and 3.20 cm in diameter, respectively after day 5 and this continued till day 7 (Plate 1A). As regards the simultaneous inoculation of *B. subtilis* and the fungal pathogens, the results indicated a zone of inhibition of average diameter of 0.1 cm between *B. subtilis* and the fungal pathogens after day 3, which was maintained throughout the period of observation although *F. equiseti* zone of inhibition was increased to 0.20 cm at day 3. ANOVA showed that all the pathogens were significantly inhibited after day 3 but the effects were very significant ($P \leq 0.0001$) on *F. verticilloides*, *F. equiseti*, *R. solani*, and *F. oxysporum*. However, *B. subtilis* had greater antagonism ($P \leq 0.0001$) on *F. verticilloides* and *F. oxysporum* (Plate1B). Inoculation of *B. subtilis* after the fungal pathogens revealed that both the *B. subtilis* and the pathogens grew close to each other and an average zone of inhibition of diameter 0.10 cm was established between each pathogen and the *B. subtilis*. Incubating the cultures beyond the three days showed that *F. verticilloides* and *R. solani* maintained their zone of inhibition while*F. equiseti* and *F. oxysporum*, increased their zones of inhibition to 0.20 cm in diameter. Throughout the periods of observation, *B. subtilis* significantly (P<0.0001) inhibited only *F. solani*. It was generally observed that, the inoculation of *B. subtilis* before the pathogens and simultaneously with pathogens was the best inoculation option for the *in-vitro* experiment when antagonist was inoculated at four equidistant position and vice–versa for each of the fungal pathogens (Table 1).

In the second *in-vitro* experiment, each fungal pathogen was directly inoculated opposite of *B. subtilis* and vice-versa. Within the first 3 days of pairing, both *B. subtilis* and each of the fungal pathogens had not made any mycelial contact. No contact was established until after day 3. At day 5, the mycelia radial growth was terminated at 3.70 cm (*F. verticilloides*), 4.05 cm (*F. equiseti*), 4.90 cm (*R. solani*), 4.65 cm (*F. solani*), and 3.75 cm (*F. oxysporum*). When *B. subtilis* was inoculated simultaneously with pathogens, only *R. solani* grew faster and established contact with *B. subtilis* after day 3 while other fungal pathogens established mycelia contact after day 5 but this was later halted on day 7 with the average growth diameter measuring 2.60 cm, 2.85 cm, 5.50 cm, 4.90 cm and 3.95 cm for *F. verticilloides*, *F. equiseti*, *R. solani*, *F. solani* and *F. oxysporum*, respectively.

When *B. subtilis* was inoculated after the pathogens, mycelia contact was established between each of the pathogens and the *B. subtilis* after day 3, with an average zone of inhibition of 0.10 cm. By day 5, the mycelia radial growth had become restricted to 4.60, cm for *F. verticilloides*, 5.00 cm for *F. equiseti*, 4.45 cm for *F. solani* and 2.65 cm for *F. oxysporum* in However, mycelial radial growth was halted on day 7 for *R. solani* (Plate 1C). It was also observed that the zones of inhibition established between *R. solani* and *B. subtilis* faded away and became invisible on day 7 (Table 2). When the spread plate method was used to evaluate the antagonistic potentials of *B. subtilis* it was observed that *B. subtilis* had a greater significant ($P \leq 0.0001$) effect on the mycelial radial growth of all the fungal pathogens (Plate 1D). There was no significant ($P<0.05$) variation in their percentage mean of inhibition.

In summary, *B. subtilis* significantly ($P \leq 0.0001$) controlled *F. oxysporum* and *R. solani*, irrespective of the antagonistic method used. On the basis of overall performance, the effect of timing, pairing, and treatment interactions, the study concluded the simultaneous inoculation of the antagonist (*B. subtilis*) and the fungal pathogens was the most effective.

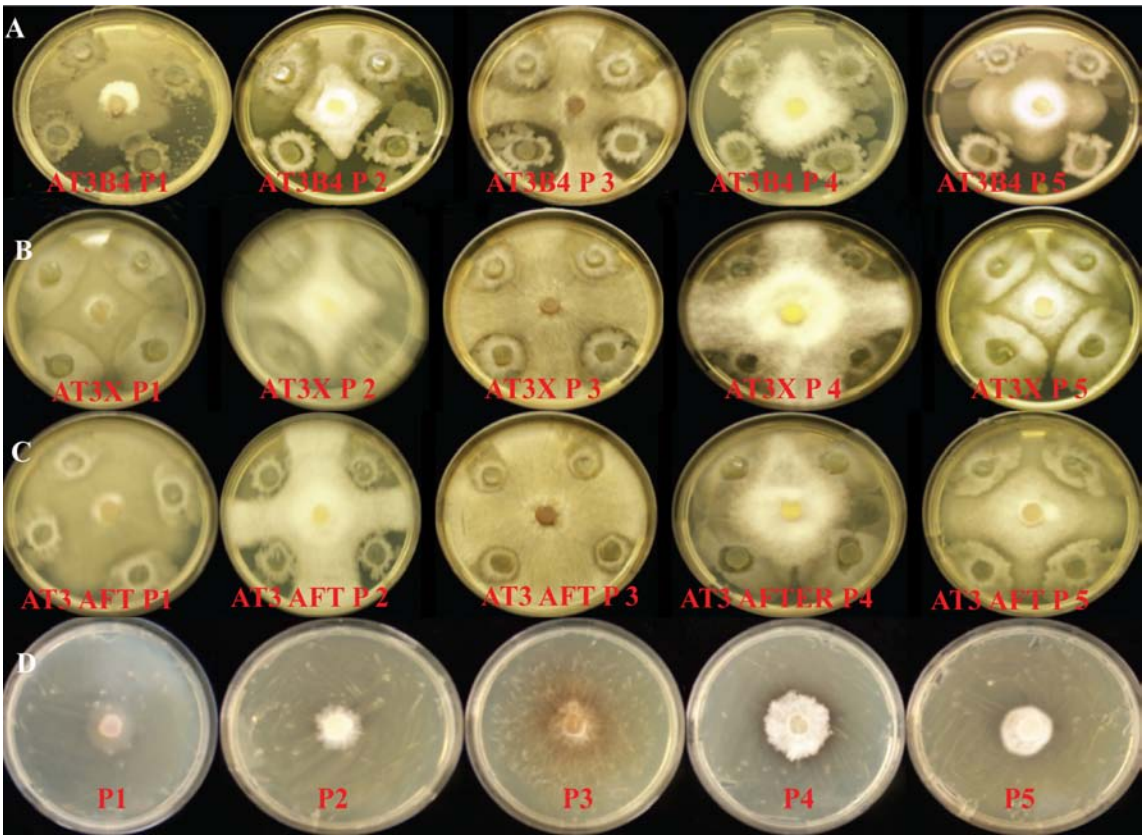

Plate 1 A-D: Effect of pairing of *B. subtilis* with pathogens P1-P5 (A) before pathogens (B4), (B) simultaneously with pathogens (X) and (C) after pathogens (AFT) using agar disc and (D) pairing using spreader method (P1-P5), photograph taken 7 days after inoculation and incubation at 28±2°C. P1 = *F. verticilloides*, P2 = *F. equiseti*, P3 = *R. solani*, P4 = *F. solani*, P5 = *F. oxysporum.*

Table 1: *In-vitro* inhibition of mycelial radial growth of pathogens by *B. subtilis* using equidistant and opposite inoculation methods after 3, 5 and 7 days of incubation at 28 °C.

	Equidistant inoculation method			Opposite inoculation method		
Treatment	3DAI	5DAI	7DAI	3DAI	5DAI	7DAI
BS AFT PATH	2.78a	3.65a	3.86a	2.20b	3.04ab	3.42b
BS B4 PATH	2.60b	3.53b	3.77a	2.01c	3.03ab	3.46b
BS ×PATH	2.84a	3.65a	3.86a	2.40a	3.12a	3.51ab
CV (%)	8.52	7.86	6.61	9.61	8.14	8.33

*Means within columns, followed by the same letters are not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test (DMRT), CV (%) = Coefficient of variation in percentage, DAI = Days after inoculation. BS AFT PATH = *B. subtilis* inoculated after 24 hours with pathogens. BS B4 PATH = *B. subtilis* inoculated 24 hours before pathogens. BS X PATH = *B. subtilis* inoculated simultaneously with pathogens.

Table 2: Zone of inhibition exhibited by the *B. subtilis* over the pathogens after 7 days of incubation at 28°C ± 2°C using four equidistant inoculation methods and direct opposite inoculation method.

	Equidistant inoculation method			**Opposite inoculation method**		
Treatment	**3DAI**	**5DAI**	**7DAI**	**3DAI**	**5DAI**	**7DAI**
Z1btwATpwP1	0.07a	0.07a	0.10a	0.00a	0.07a	0.07a
Z1btwATpwP2	0.07a	0.20a	0.20a	0.02a	0.03a	0.05a
Z1btwATpwP3	0.15a	0.18a	0.18a	0.07a	0.17a	0.08a
Z1btwATpwP4	0.08a	0.13a	0.13a	0.00a	0.00a	0.03a
Z1btwATpwP5	0.03a	0.10a	0.15a	1.15b	1.60b	2.02b

* Within columns, means followed by the same letters are not significantly different ($P \leq 0.05$) according to DMRT. ns = not significant ($P \leq 0.05$). Z1btwATpwP = zone of inhibition between the antagonists and the pathogens after 7 days of incubation at 28 ± 2°C, BS = *B. subtilis*, P1 = *F. verticilloides*, P2 = *F. equiseti*, P3 = *R. solani*, P4 = *F. solani*, P5 = *F. oxysporum.*

Table 3: Cumulative effect of comparison of timing, pairing, treatment and interaction between timing * treatment, pairing*treatment, timing*pairing*treatment on the mycelial radial growth of both the pathogens and the antagonists using four equidistant inoculation methods and the direct opposite inoculation method.

Source	3DAI	5DAI	7DAI
Timing	0.25*	23.48***	101.73***
Pairing	33.46***	40.03***	368.86***
Timing*pairing	4.40***	9.71***	108.06***
Treatment	13.38***	25.75***	1528.90***
Timing* treatment	1.32***	5.41***	341.57***
Pairing*treatment	1.90***	1.41***	142.27***
Timing*pairing*treatment	0.30***	0.39***	50.07***
CV (%)	7.95	7.04	6.75
Equidistant inoculation	2.68a+	3.54a	3.79a
Opposite inoculation	2.65a	3.77b	4.49b

CV (%) = Coefficient of variation percentage. DAI = Days after inoculation. + Means within columns, followed by the same letters are not significantly different. ($P \leq 0.05$) according to Duncan's Multiple Range Test (DMRT).* = F value level of significant ($P \leq 0.05$). ** = F value level of significant ($P \leq 0.01$). *** = F value level of significant ($P \leq 0.0001$).

4. Discussion

The biological control of plant diseases is one of the viable alternatives to chemical control in sustainable agriculture. *B. subtilis*, used in this study successfully inhibited the growth of all the root-/soil-borne fungal pathogens isolated from cowpea *in-vitro*. This result corroborated that of Chandanie et al. (2006) which reported the use of Plant Growth Promoting Fungi (PGPF) isolate (*Phoma* sp. GS8-2, GS8-3 or *P. simplicissimum* GP17-2) to successfully control *Collectotrichum orbiculare* causing diseases in cucumber plants. Both pairing inoculation methods used for the *in-vitro* experiment were significantly effective in the suppression of the pathogens. Simultaneous inoculation of *B. subtilis* with each of the fungal pathogens was the best of the three methods of inoculation. This result was contrary to that of Shobowale (2002), who reported that the inoculation of the antagonist before the pathogens (AG×B4P) aided the growth inhibition of *F. moniliforme* significantly better than the simultaneous inoculation of the antagonist with the pathogens, and inoculation of the

antagonist after the pathogens. Also, physical examination of zones of inhibitions revealed that the simultaneous inoculation of the antagonist and fungal pathogens aided growth inhibition significantly more than the other method.

The production of antibiotics by the *Bacillus* spp. and their uses in the biological control of plant pathogens have been reported in many reviews. *B. cereus* produces lytic enzymes and antibiotics; and *B. subtilis* possesses a lytic factor in its cell wall (Pukall et al. 2005). In an earlier report, Young et al. (1974) stated that *B. subtilis* produces at least five different antibiotics, namely: subtillin, bacitracin, bacillin, subtenolin, and bacilonycin. The indigenous *B. subtilis* used in this present study may possess some of these chemical compounds which might have accounted for the zones of inhibition recorded. The production of toxins by *Bacillus* spp. has been reported by several researchers. Pukall et al. (2005) identified four toxin producing strains of *Bacillus* spp., namely *B. pumils, B. fusiformis, B. subtilis,* and *B. mojavensis* apart from normal toxin producer, *B. cereus*.

Mohammed and Amusa (2003) reported that *B. cereus* and *B. subtilis* inhibited the mycelial growth of a range of fungi causing seedling blight with the zone of inhibition ranging from 35.5% to 57.8%. Likewise, Bankole and Adebanjo (1998) stated that soil inoculated with *B. cereus, B. subtilis* and *Trichoderma* spp. reduced seedling infection by *Fusarium* spp. and the efficacy of their antagonists increased with dosage. The mycelial growth inhibition of root-/soil-borne fungi pathogens observed in this present study might therefore be due to antibiotics and specific cellular enzymes produced by the strain of *B. subtilis* used. Mechanisms of biocontrol suggested by other researchers included antibiosis (Fravel, 1988), and rhizosphere competence (Howell, 2003). These factors might be responsible for the observed antibiosis in this present study. The varying biocontrol mechanisms that led to the growth inhibition of the root-/soil-borne fungal cowpea pathogens by the antagonist might be indicative, among other reasons, for the different mechanisms of antibiosis exhibited by different antagonistic agents against different pathogens. The results of the interaction between the antagonistic *B. subtilis* and the fungal pathogens agreed with the conclusion of Sharma and Sankara (1988) that a good biocontrol agent should have a good degree of persistence and aggressiveness but be non-pathogenic to the host. The beneficial effects of bioprotectants on plants have also been previously reviewed by Luz (1996).

The clear zone of inhibition produced in the *in-vitro* experiment is an indicative of antibiosis by the biocontrol agent against the fungal pathogens. This mechanism could have included any of the compounds reported by Fravel (1988), and/or the extracellular hydrolytic enzymes produced by *B. subtilis* that are important determinants of antibiosis. Moreover, the production of a colourless zone of inhibition by the antagonist *B. subtilis* suggested the probable production of colourless metabolites by the *B. subtilis* which diffused into the agar and inhibited the radial mycelial growth of the root-/soil-borne cowpea fungal pathogens. Therefore, the antibiosis activities of *B. subtilis* evaluated in this study suggests that it is a promising biocontrol agent against these fungal pathogens.

4. Conclusion

Application of indigenous *B. subtilis* strain isolated, identified and used in this present study as a biopesticide in the control of fungal pathogens of cowpea shows that it is a promising natural biopesticide agent. It exhibited sufficient antibiosis capability due to its good inhibitory performance against *F. verticilloides*, *F. equiseti, F. solani, F. oxysporum,* and *R. solani in- vitro* in the laboratory. It can be considered as an alternative to chemical pesticides in disease management strategy and should be further studied under field condition and possibly scaled-up for the control of numerous phytopathogenic fungi causing diseases and great yield loss in cowpea production.

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