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Research Article
Biocontrol of Seed Decay, Seedling Damping-off and Plant-Growth Promotion of Cocoa Seedlings with Antagonistic Microbes

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Abstract

Background and Objective: Black pod disease of cocoa affects every part of the pant development from nursery to the pod. Raising healthy cocoa seedlings at the nursery, therefore, ensures better seedling establishment in the field. This study sought to develop a biocontrol option for seed decay and damping-off disease of cocoa and at the same time, promote seedlings growth with antagonistic microbes. **Materials and Methods:** The experiment consisted of two parts; preliminary tube experiment and pot experiment. Oven sterilized topsoil in plastic tubes were infested with *P. palmivora* suspension prepared from a 14 days-old culture and incubated on a planthouse bench. After two weeks, infested soils were separately drenched with broth supensions of the microbial antagonist sowing. Four replicate tubes per treatment were established. In the pot experiment, similar treatment was subjected to a steam topsoil for two weeks. The infested soil distributed in separate tubes or plastic pots and either drenched with NB suspension of the rhizobacterium, PDA suspensions of *Aspergillus* and *Penicillium* spp. or seeds dipped in microbial suspensions before sowing. The setups were maintained in the planthouse for six weeks and disease assessments as well as growth parameters were made or recorded on resulting seedlings respectively. **Results:** Broth suspensions of the microbial antagonists either used as a soil drench on Phytophthora-infested soil or as seed dip, prevented seed decay, damping-off and promoted seedlings growth with significantly higher ($p<0.05$) growth parameters in terms of dry matter, number of leaves, plant height and plant girth, relative to the untreated controls. **Conclusion:** The results from the current study demonstrate that the three microbial antagonists can be formulated and use for the management/prevention of the disease in the nursery.

Key words: Seed decay, damping-off, plant-growth promotion, fungal pathogens

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Competing Interest: The author has declared no competing interest.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Phytophthora pod rot also known as black pod disease is a major constraint to cocoa production worldwide. The disease affects the cocoa plant at all physiological stages of its growth, from the seedlings stage to the cherelle and chupon formation stages[1,2]. The main causal agents of the disease are the *P. palmivora* and the *P. megakarya*. Both *Phytophthora* species caused seed decay, seedling damping-off, seedlings blight, stem canker, flower abortion, black pod and seed discoloration[3-4]. Although the pathogen affects the plant at all physiological stages, since it is a soil-borne and strives well in the soil habitat, the infection starts in the soil, making seed decay, seedling damping-off and seedling blight most important under wet humid conditions in the nursery[5]. Basically, top soils from cocoa-producing farms are used as nursery soils. If such infested soils are therefore used, the possibility of infesting cocoa seeds with *Phytophthora* sp. is greater and will have a negative impact on seedlings' growth and establishment. Awuah and Frimpong[6] reported that seed decay and seedlings blight are also possible especially if infected seeds are planted in uninfected soils. They also suggested that if infected seeds are sown to uninfected soils, would release inocula of the pathogen into the soil from where they can infect the emerging roots and portion of the seedlings in contact with the soil line and would constitute seed to seedling transmission. It is evidently clear that seed decay and seedling damping-off of cocoa (*Theobroma cacao* L.) caused by *Phytophthora palmivora* continue to be a silent killer of cocoa seedlings at the nursery and at the same time continue the life cycle of the pathogens into the field. This aspect of managing the disease has been ignored by nursery managers thus has affected seedling establishment whiles ensuring plant growth and development. Also, this aspect of disease management has not received much attention due to the much emphasis on the economic damage that the pathogen caused to the pods. Seedling growth and development devoid of infections is therefore, a sure way to guarantee good plant establishment, growth and development.

Many reports seem to suggest also that raising seedlings in soils infested with *Phytophthora* sp. results in seeds and roots of seedlings becoming infected causing Pre and post-emergence damping-off[7-8]. In the Philippines, reports indicated that the most common problems of seedlings of cocoa in the nursery is the seed and seedlings damping-off caused by *Phytophthora* spp[9]. In Jamaica, it was reported that seed decay/seedling rot (damping-off) and seedling blight are among the major seedlings problems[10]. This was contained in a nursery manual report where it was indicated that when the seedlings are affected by the damping-off, seedlings collapse and eventually die due to infection by *Phytophthora* sp. It was suggested among management practices options, soil drench with systemic fungicides, media treatment by steam sterilization, solarization or fumigation and preventive measures such as water treatment and/or fungal seed treatment[10]. Richard[11] also reiterated the attempt being made to use chemical fungicides to control black pod, pre-emergence, root rot and post-emergence damping-off caused by *P. palmivora* in Ghana.

Several soil-inhabiting microorganisms play major roles in protecting plants from being attacked by soil-borne pathogens and at the same time benefitting from plant exudates from plant roots[12]. Soil-borne pathogens among them, *Fusarium* spp., *Phytophthora* spp., *Pythium* spp. and some cyst nematodes, *Heterodera* sp., develop well in soils causing severe disease thereby preventing seeds from germinating, causing damping-off, root rots resulting in wilts, etc. Such soils are referred to as conducive soils but these same organisms may develop much less and cause milder disease in other soils in the presence of other soil-inhabiting microorganisms, such as fungi genera Trichoderma, Penicillium, Aspergillus and bacteria; Pseudomonas, Bacillus and Streptomyces. Such soils are referred to as suppressive soils[12]. These antagonistic microorganisms prevent pathogens from causing diseases to the plants through various mechanisms such as antibiosis, lytic enzyme production and competition for nutrients or through direct parasitism of the pathogen, siderophore production, inducing resistance and promoting plant growth that prevents these pathogens from multiplying and causing severe disease[13]. Naturally, suppressive soils added to conducive soils can prevent pathogens to strive in such soils. At the same time, these pathogen-inhibiting microorganisms (antagonists) can be isolated from their natural habitat especially soils and introduced to conducive soils to achieve the same objectives. These processes either naturally or augmented using microbial antagonist, its part or products to suppress other organisms is referred to as biological control and the organism that suppress the pathogen is a biological control agent[12].

Therefore, raising healthy cocoa seedlings at the nursery by augmenting the soils using microbial antagonist(s) would ensure better seedling establishment in the field. The objective of this study was, therefore, to develop a biological control option that will prevent seed decay, controlling damping-off and promotes seedling growth.

MATERIALS AND METHODS

Area of study: The experiment was conducted at the Laboratory and the planthouse, the Department of Crop and Soils Sciences of the Kwame Nkrumah University of Science and Technology (KNUST), Kumasi-Ghana in August-September, 2017. The University is located between Latitude 6.6745°North and 1.5716°West, the second largest city of Ghana, West Africa.

Sources and maintenance of microbial antagonists: In this study, microbial antagonists viz: *Bacillus amyloliquefaciens, Aspergillus* sp. and *Penicillium* sp. were used. The *B. amyloliquefaciens* was previously isolated from yam (*Dioscorea* sp.) rhizosphere soils, kept on nutrient agar and refrigerated at 4°C till needed whilst the fungal antagonists (*Aspergillus* sp. and *Penicillium* sp.) were obtained as anti-Phytophthora laboratory contaminants of *P. palmivora* and maintained on Green Cocoa Mucilage Agar (GCMA) and kept at 26±2°C with frequent sub-culturing till needed.

Protection of cocoa seeds from damping-off disease with the *Bacillus amyloliquefaciens* (plastic tube experiment): Plastic centrifuge tubes (50 cc) measuring about 30 mm in diameter and 115 mm deep were filled with five grams of oven-sterilized topsoil and infested with a mixture of mycelia and sporangia of *P. palmivora* (10 mL sterile distilled water) suspension prepared from a 14 days-old culture. The tubes were incubated on a planthouse bench. After two weeks, the infested soils were separately drenched with (i) 10 mL NB suspension of the *B. amyloliquefaciens* prepared from a 24 hrs culture of the bacterium and diluted to a concentration of 10^8 CFU mL^{-1} and (ii) Fungicide (seed star) containing 20% Metalaxyl, 20% Imidacloprid and 4% Anthraquinone (Nova Agro Ltd, Hong Kong), prepared according to the manufacturer's instruction. Two cocoa seeds were then sown into each tube and tinned to one seedling after germination. Four replicate tubes per treatment were established. Sterilized infested soils with *P. palmivora* drenched with NB and infested soils with only *P. palmivora* served as controls. The setups were kept in a planthouse for six weeks and disease assessments were made on seed decay and the resulting seedlings. For the recovery of the bacterium from rhizosphere soil, representative tubes of each treatment were assayed for *B. amyloliquefaciens* by sprinkling particles of soil on NA in Petri plates and were observed after 24 hrs. Roots of plants were also biopsied by plating pieces on NA plates and the resulting cultures observed for the diagnostic characteristics of *B. amyloliquefaciens*.

Evaluation of microbial antagonists for the prevention of damping-off/seedling blight and plant-growth promotion of cocoa seedlings (Pot experiments): Steamed sterilized topsoil (20 kg) was placed on a black polyethylene sheet on a laboratory floor and infested manually by mixing with two liters of Green Cocoa Mucilage Broth (GCMB) suspension of *P. palmivora.* The suspension was prepared by homogenizing a two-week-old culture of the fungus with a laboratory blender at low speed for 10 min. The infested soil was covered with another polyethylene sheet and left to incubate on a laboratory floor at room temperature of 28±2°C. An uninfested control soil was also established. Particles of the infested soil were bioassayed after two weeks, by sprinkling on GCMA to ensure that the *P. palmivora* had been established in the soil. Uninfested control soil was similarly assayed to ensure that it did not contain any *Phytophthora* inoculum. Ten kilograms of the *P. palmivora*-infested soil was distributed into 450 cc (90 mm diameter and 70 mm deep) plastic pots (250 g of soil/pot) and each of five pots drenched with a NB suspensions of the bacterium *B. amyloliquefaciens* (100 mL/pot). Sets of five pots were similarly, but separately drenched with Potato Dextrose Broth (PDB) suspensions of an *Aspergillus* sp. and a *Penicillium* sp. (10^8 spores mL^{-1}). Treated soils were incubated for 14 days in a planthouse. Infested soil drenched with the fungicide Seed star and undrenched control soils were also established as positive and negative controls respectively. Soils with only NB, GCMB, PDB and sterilized water were similarly setup as other controls.

Cocoa seeds were extracted from pods (Hybrid: Amelonado×Amazon) obtained from the Seed Production Unit of Cocobod, Jamasi in the Ashanti region. Pods, collected from the same tree were used. The seeds were bulked and sown in the pots (one seed per pot) and kept in a planthouse. They were equally watered with 10 mL water when needed. On germination, the seedlings were grown for an additional six weeks and the following data were taken on each plant (i) the girth of stem-taken with Vernier calipers two centimeters from the soil line, (ii) the plant heights were taken with a 30 cm meter rule from the soil line to the tip of growing stem, (iii) the number of leaves per plant and (iv) the dry matter. Dry matter analysis was done according to the method of Awuah[14] by carefully removing plants from soil, washing soil off the roots, drying the entire plants in an oven at 80°C to constant weight and dry matter obtained by weighing the plants.

Concurrently, cocoa seeds were dipped in suspensions of the microbial antagonists before sowing in *P. palmivora*-infested soils contained in 450 cc plastic pots (five replicate pots per treatment). Seeds dipped in a fungicide (Seed star)

suspension, NB, GCMB, PDB and sterilized distilled water were also setup as control. Plants were grown as in the drenching experiment and similar plant growth data taken after 6 weeks.

Recovery of microbial antagonists: For the recovery of microbial antagonists (*B. amyloliquefaciens*, *Aspergillus* sp. and *Penicillium* sp.) and the pathogen (*P. palmivora*), soils were assayed before sowing and 6 weeks later at the end of the experiment to confirm the presence or the absence of the antagonists in the soil. Assays were done by introducing a sterile wire loop into the soil and streaking it on Nutrient Agar (NA) plates (for the bacterium), Chloramphenicol Potato Dextrose Agar (CPDA) plates (for the *Aspergillus* sp. and the *Penicillium* sp.) and green cocoa mucilage agar (GCMA) plates for *P. palmivora*. The experiments were repeated in the same soils and pots after six weeks (which constituted Pot experiment II).

Determination of antimicrobial metabolite production in soil by the rhizobacterium, *B. amyloliquefaciens,* ESI: In an initial experiment, plastic pots (150 cc; 65 mm diameter and 60 mm deep) each containing 125 g sieved, oven sterilized topsoil were either drenched with 50 mL, 24 hrs old ESI Nutrient Broth (NB) culture or ESI in sterilized distilled water suspension. Potted soils were maintained by watering with an equal amount (10 mL) of water when needed to keep them moist. There were four sets of treatments viz. (i) Potted soil amended with 0.7 g of urea (40% N) and drenched with ESI NB culture, (ii) Soil amended with 0.7 g of urea (40% N) and drenched with ESI in Sterilized Distilled Water (SDW) suspension, (iii) Soil drenched with ESI NB culture and (iv) Soil drenched with ESI SDW suspension. Controls consisted of the above treatments but without ESI were maintained. Also maintained as controls were ESI NB culture and NB alone without passing through the soil. Four replicates pots were maintained per treatment. The experiment was repeated once but in the repeat experiment, controls consisted of only ESI NB culture and NB alone without passing through soil were maintained. After three weeks, soil in each pot was mixed thoroughly, 10 g added to eight milliliters of distilled water into 25 cc vials and left to stay for 1 hr. The decanted supernatant was autoclaved and allowed to stand for 24 hrs for the fine soil particle to settle. Five milliliters of the resulting clarified supernatant was placed in 25 cc vials and autoclaved again to obtain the final ESI filtrates from the soils. Each vial was seeded with a 20 µL of seven-day-old *A. niger* conidial suspension (1×10^6 conidia mL^{-1}) and incubated on a laboratory bench at room temperature ($28\pm2^\circ C$) for 28 days. Growths of mycelial mats from the resulting cultures, if any were carefully harvested by using loop onto pieces of A4 paper, oven-dried at 20°C for 3 hrs and weighed. The experiment was repeated simultaneously with non-sterilized soil.

Statistical analysis: The study was conducted using a Complete Randomized Design (CRD) with three replicates where necessary. The analysis of variance (ANOVA) was performed on the data using GenStat statistical package[15]. When there was significance, mean separation was accomplished using the Least Significant Differences (LSD) test at 5% probability. For the basic statistical analysis of data, MS Excel 2010 was used.

RESULTS

Protection of cocoa seeds with the *B. amyloliquefaciens* (Plastic tube experiment): Result from this study shows that cocoa seeds were totally protected when *P. palmivora*-infested soil in plastic tubes was drenched with the *B. amyloliquefaciens* (ESI) broth suspension giving a percentage of infection of zero (0%) indicating a 100% germination of seeds comparable to the *P. palmivora*-uninfested control soil and the fungicide-treated soil which recorded 100% disease infection (a total seedling decay/pre-emergence damping-off) in Table 1 and shown in Fig. 1a-e.

Prevention of damping-off/seedling blight in cocoa and promotion of plant growth by microbial antagonists: Similar results were obtained when the experiment was conducted in plastic pots that included two other microbial antagonists (*Aspergillus* and *Penicillium* spp.) apart from the rhizobacterium *B. amyloliquefaciens* (ESI), revealed that the percentage of damping-off was zero (0%) indicating 100% germination of seeds for all the three antagonists in both the drenched *P. palmivora*-infested soils as well as seeds dipped in antagonists before planted into *P. palmivora*-infested soils (five seed germinated out of the five replicate pots)and these were comparable to the uninfested soil and the fungicide treated soil (+ controls) which also recorded 0% damping-off (100% seed germination) that did not receive the antagonists. The *P. palmivora*-infested negative control soils, however, resulted in damping-off of 80% indicating 20% seeds germination and 60% damping-off (40% germination) in soils when drenched or seeds dipped in microbial suspension before sowing respectively in Fig. 2a-f and Table 2.

In the repeat experiment which was conducted in the same soils and pots after six weeks, total damping-off (100% damping-off) indicating 0% seed germination was

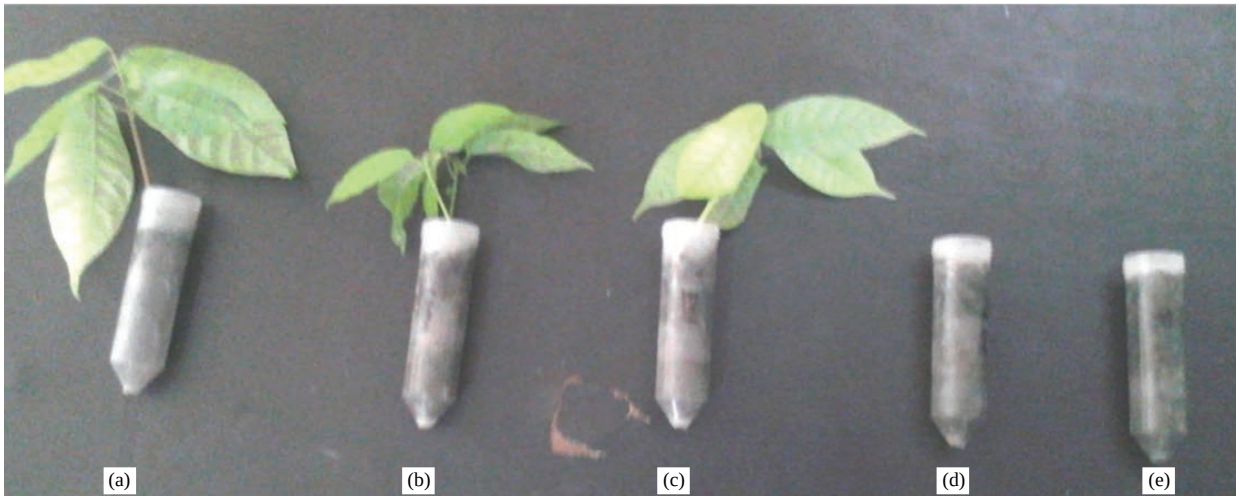

Fig. 1(a-e): Cocoa seedlings growing in plastic tubes containing;
(a) Untreated soil, (b) *P. palmivora*-infested soil drenched with *B. amyloliquefaciens*, ESI, (c) *P. palmivora*-infested soil drenched with fungicide (seed star), (d) *P. palmivora*-infested soil drenched with nutrient broth (NB) and (e) *P. palmivora* infested soil not receiving any protective drench, demonstrating the protective ability of the bacterial antagonist, ESI. The last two tubes had ungerminated, damped-off seeds

Table 1: Percentage damping–off infection of cocoa seeds in *P. palmivora* infested soils treated with ESI broth culture suspension (plastic tube experiment)

Soil treatment	Number of germinated seeds/number of seeds sown	Percentage damping-off (%)
Infested soil+ESI	4/4	0
Infested soil+seed star (fungicide) (+control)	4/4	0
Uninfested soil (+control)	4/4	0
Infested+NB (-control)	0/4	100
Infested soil (-control)	0/4	100

ESI: Rhizobacterium, *Bacillus amyloliquefaciens*

Table 2: Percentage damping-off of cocoa seedlings in *P. palmivora* infested soils treated with microbial antagonists (pot experiment I)

	Drenched[2]			Dipped[2]		
Soil treatment[1]	No. of seeds germinated/ No. sown	Post emergence damping-off[3]	Damping -off (%)	No. of seeds germinated/ No. sown	Post emergence damping-off[3]	Damping -off (%)
Infested soil+ESI	5/5	0/5	0	5/5	0/5	0
Infested soil+*Aspergillus* sp.	5/5	0/5	0	5/5	0/5	0
Infested soil+*Penicillium* sp.	5/5	0/5	0	5/5	0/5	0
Infested soil+seed star (fungicide) (+ control)	4/5	0/5	20	5/5	0/5	0
Uninfested soil+NB suspension (+control)	4/5	0/5	20	5/5	0/5	0
Uninfested soil+PDB suspension (+control)	5/5	0/5	0	5/5	0/5	0
Uninfested soil+GCMB suspension (+control)	5/5	0/5	0	4/5	0/5	20
Uninfested soil (+control)	4/5	0/5	20	5/5	0/5	0
Infested soil (-control)	2/5	1/5	80	3/5	1/5	60

[1]Infested soil-sterilized soil infested with *P. palmivora* and allowed to stand for two weeks before microbial/chemical treatment with ESI, *Penicillium* and *Aspergillus* sp. Uninfested soil-sterilized soil uninfested with *P. palmivora.* [2]Drenched soil-soil watered with 50 mL microbial suspensions, fungicide, nutrient broth (NB), potato dextrose broth (PDB) orgreen cocoa mucilage broth (GCMB) before seeds were sown into them, Dipped soil-seeds placed for 10 min in microbial suspensions, fungicide, NB, PDB or GCMB before sowing. [3]Seed that died after emergence

recorded only when seeds were planted in soil that previously was infested with *P. palmivora* whiles 0% damping-off (100% germination) recorded in soils treated with microbial antagonists respectively in Fig. 3a-f and Table 3.

Plant-growth promotion with microbial antagonists (Pot experiments I and II)

Dry matter analysis: Dry matter weights of cocoa seedlings in *P. palmivora*-infested soils receiving ESI, *Aspergillus* and *Penicillium* spp. drenched soils ranged from 0.88-4.62 g and

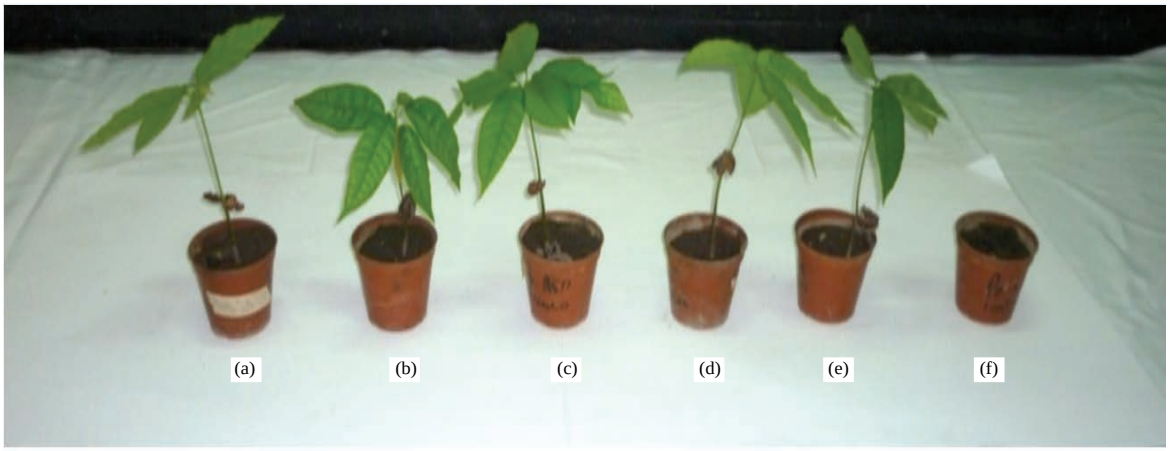

Fig. 2(a-f): Stages of development of cocoa seedlings in drenched soils infested with *P. palmivora;*
(a) Uninfested soil (positive control), (b) Infested soil treated with *B. amyloliquefaciens* ESI (bacterial antagonist), (c) Infested soil treated with *Aspergillus* sp. (fungal antagonist), (d) Infested soil treated with *Penicillium* sp. (fungal antagonist), (e) Infested soil treated with fungicide (seed star) (positive control) and (f) Untreated infested soil (negative control)

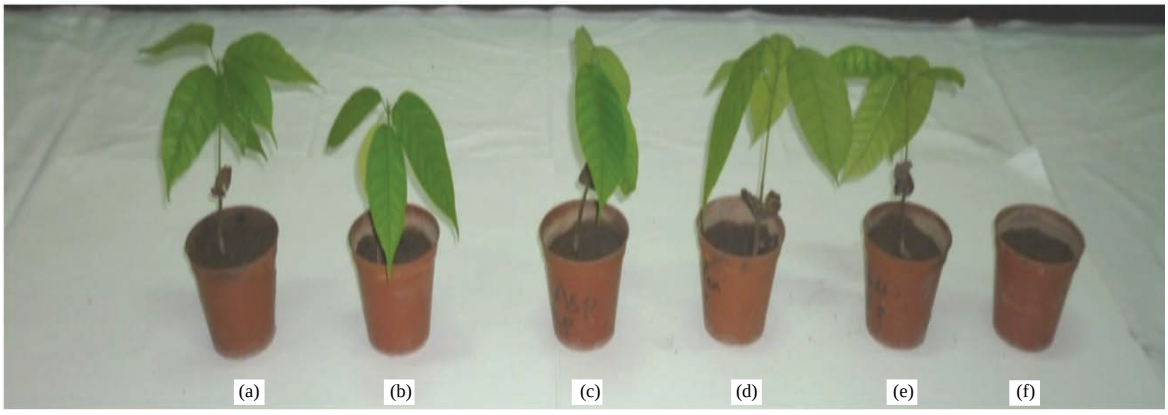

Fig. 3(a-f): Stages of development of cocoa seedlings when seeds dipped in soils infested with *P. palmivora*;
(a) Uninfested soil (positive control), (b) Infested soil with seeds dipped in the *B. amyloliquefaciens*, ESI suspension (bacterial antagonist, (c) Infested soil with seeds dipped in *Aspergillus* sp. suspension (fungal antagonist), (d) Infested soil with seeds dipped in *Penicillium* sp. suspension (fungal antagonist), (e) Infested soil with seeds dipped in fungicide suspension (seed star) (positive control) and (f) Infested soil (negative control)

Table 3: Percentage damping-off disease of cocoa seedlings in *P. pamivora*-infested soils treated with microbial antagonists (pot experiment II)

	Drenched[2]			Dipped[2]		
Soil treatment[1]	No. of seeds germinated/ No. sown	Post emergence damping-off[3]	Damping -off (%)	No. of seeds germinated/ No. sown	Post emergence damping-off[3]	Damping -off (%)
Infested soil+ESI	5/5	0/5	0	5/5	0/5	0
Infested soil+PDB suspension of *Aspergillus*	5/5	0/5	0	5/5	0/5	0
Infested soil+PDB suspension of *Penicillium*	5/5	0/5	0	5/5	0/5	0
Infested soil+seed star (fungicide)	5/5	0/5	0	5/5	0/5	0
Uninfested soil+NB broth suspension	5/5	0/5	0	5/5	0/5	0
Uninfested soil+PDB broth suspension	5/5	0/5	0	5/5	0/5	0
Uninfested soil+GCMB broth suspension	5/5	0/5	0	5/5	0/5	0
Uninfested soil (+control)	5/5	0/5	0	5/5	0/5	0
Infested soil (-control)	0/5	0/5	100	0/5	0/5	100

[1]Infested soil: Sterilized soil infested with *P. palmivora* and allowed to stand for two weeks before microbial/chemical treatment with ESI, *Penicillium* and *Aspergillus* sp. Uninfested soil-sterilized soil uninfested with *P. palmivora*. [2] Drenched soil: Soil watered with 50 mL microbial suspensions, fungicide, nutrient broth(NB), potato dextrose broth (PDB) or green cocoa mucilage broth (GCMB) before seeds were sown into them, Dipped: Seeds placed 10 min in microbial suspensions, fungicide, NB, PDB or GCMB before sowing. [3]Seed that died after emergence

Table 4: Dry matter of cocoa seedlings in *P. palmivora*-infested soils treated with microbial antagonists after eleven weeks of seedlings growth

	Dry matter (g)			
	Pot experiment I[2]		Pot experiment II[2]	
Soil treatments[1]	Soil drenched with microbial antagonists	Seeds dipped in microbial antagonists	Soil drenched with microbial antagonists	Seeds dipped in microbial antagonists
Infested soil+ESI	3.83	3.33	3.72	3.77
Infested soil+Asp.	3.81	4.44	3.78	3.30
Infested soil+Pen.	2.93	4.36	2.89	3.33
Infested soil+fungicide	2.21	3.45	3.70	2.57
Infested soil+NB	4.50	5.21	4.79	3.82
Infested soil+PDB	4.62	3.98	4.86	3.62
Infested soil+GCMB	2.34	4.69	4.44	2.86
Uninfested soil	3.26	3.21	4.92	4.41
Infested soil	0.88	0.88	0.00	0.00
LSD (0.05)	1.78	1.61	1.52	1.44
CV (%)	43.8	33.4	32.0	36.1

[1]Infested soil: Sterilized soil infested with *P. palmivora* and allowed to stand for two weeks before microbial/chemical treatment with ESI, *Penicillium* (PEN) and *Aspergillus* sp. (ASP). Uninfested soil-sterilized soil uninfested with *P. palmivora*, [2] Experiment 1: Main experiment conducted to evaluate plant growth promoting ability of the microbial antagonists, Experiment 2 was conducted in the same soil six weeks after the initial pot experiment. Seedlings were five weeks old when data was taken. NB: Nutrient broth, PDB: Potato dextrose broth, GCMB: Green cocoa mucilage broth

0.88-4.69 g in seed dipped in microbial suspensions. The least dry matter of 0.88 g was associated with the infested soil that did not receive the microbial antagonists and this was significantly lower ($p<0.05$) than all the dry matter in Table 4. There were also no significant differences ($p<0.05$) between the three microbial antagonists. Similarly, the lowest result associated with infested soil was recorded when seeds were dipped in suspensions of the microbial antagonists in experiment I (Table 4).

In the repeated experiment (experiment II) conducted in the same soils six weeks after the initial pot experiment (experiment I), the dry matter ranged from 0.00-4.92 g (drenched) and 0.00-4.41 g (dipped). The least of 0.00 g indicating no dry matter was obtained from *P. palmivora* infested soil. Dry matter of 4.92 and 4.41 g were associated with the sterilized uninfested soil of drenched and dipped respectively and this was significantly similar generally to those obtained with the microbial antagonists (Table 4).

Number of leaves (Pot experiment I): Infested soils drenched with suspensions of the microbial antagonists produce seedling with a significantly higher mean number of leaves of 4-10 ($p<0.05$) compared to those obtained from the soil infested with *P. palmivora* of 1-3 leaves in Fig. 4a. Similar results were obtained when the seed dipping method was used in Fig. 4b.

Plant height: Seedlings heights 14-17 cm were highest in *P. palmivora*-infested soils drenched with suspensions of microbial antagonists. There were significantly different ($p<0.05$) from those associated with the infested soil without biocontrol treatment of 4-5 cm in Fig. 4c. In the experiment where seeds were dipped in suspensions of the microbial antagonists before being sown, the results obtained with the microbial antagonists were generally similar (14-18 cm) to the drenched experiment with regard to the plant height and differed in plant height significantly ($p<0.05$) with *P. palmivora*-infested soil (control) of 3-5 cm in Fig. 4d.

Plant girth: The widest plant girths of 0.25-0.55 cm were obtained in infested soils drenched with suspensions of the three microbial antagonists and these were significantly higher ($p<0.05$) than those obtained with the *P. palmivora*-infested soil (-control) of 0.00-0.22 cm. Generally, there were also no significant differences in seedling girth among the microbial treatments in Fig. 4e. These trends were similar to those obtained using seeds dipping method with 0.22-0.48 and 0.00-0.18 cm for microbial antagonists and *P. palmivora*-infested soils respectively in Fig. 4f.

With respect to the growth parameters studied (number of leaves, plant height and plant girth) in the pot experiment II shown in Fig. 5a-f, conducted in the same soils, 6 weeks after the experiment I, the following results were obtained.

Number of leaves (Pot experiment II): Infested soils drenched with suspensions of the microbial antagonists produce seedling with a significantly higher mean number of leaves of 2-5 ($p<0.05$) compared to those obtained from the soil infested with *P. palmivora* of zero (0) leaves indicating that none of the seeds germinated in Fig. 5a. Similar results of 2-7 leaves were obtained for the microbial antagonists and zero (0) seeds for soil infested with *P. palmivora* when the seed dipping method was used respectively in Fig. 5b.

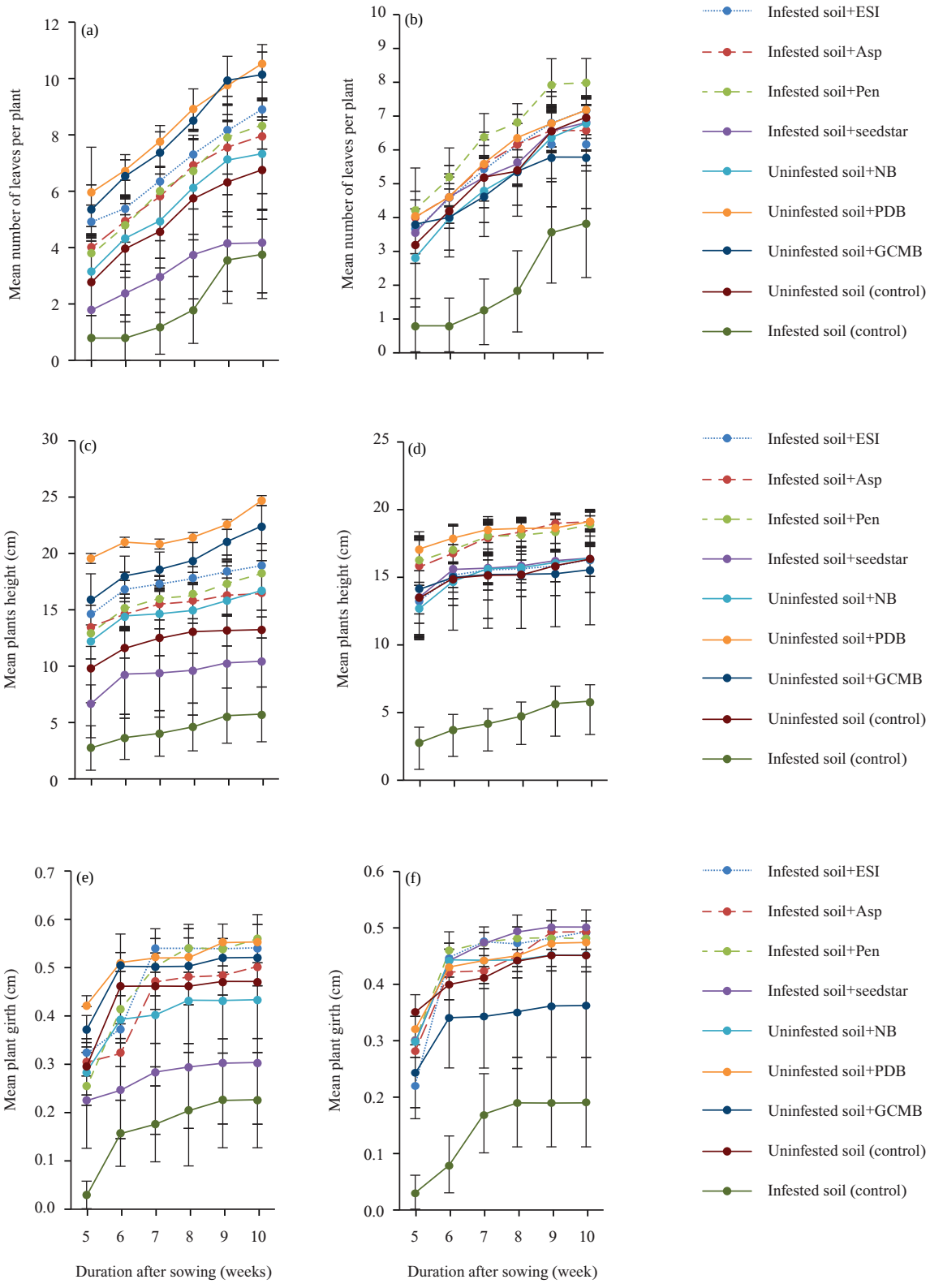

Fig. 4(a-f): Graph showing plant growth promoting ability of the microbial antagonists (Pot experiment I);
(a-b) Mean number of leaves for the drenching method and dipping method, (c-d) Drenching method and dipping method: plant height and (e-f) Plant girth for the drenching method and dipping method. Vertical bars represent standard errors of means

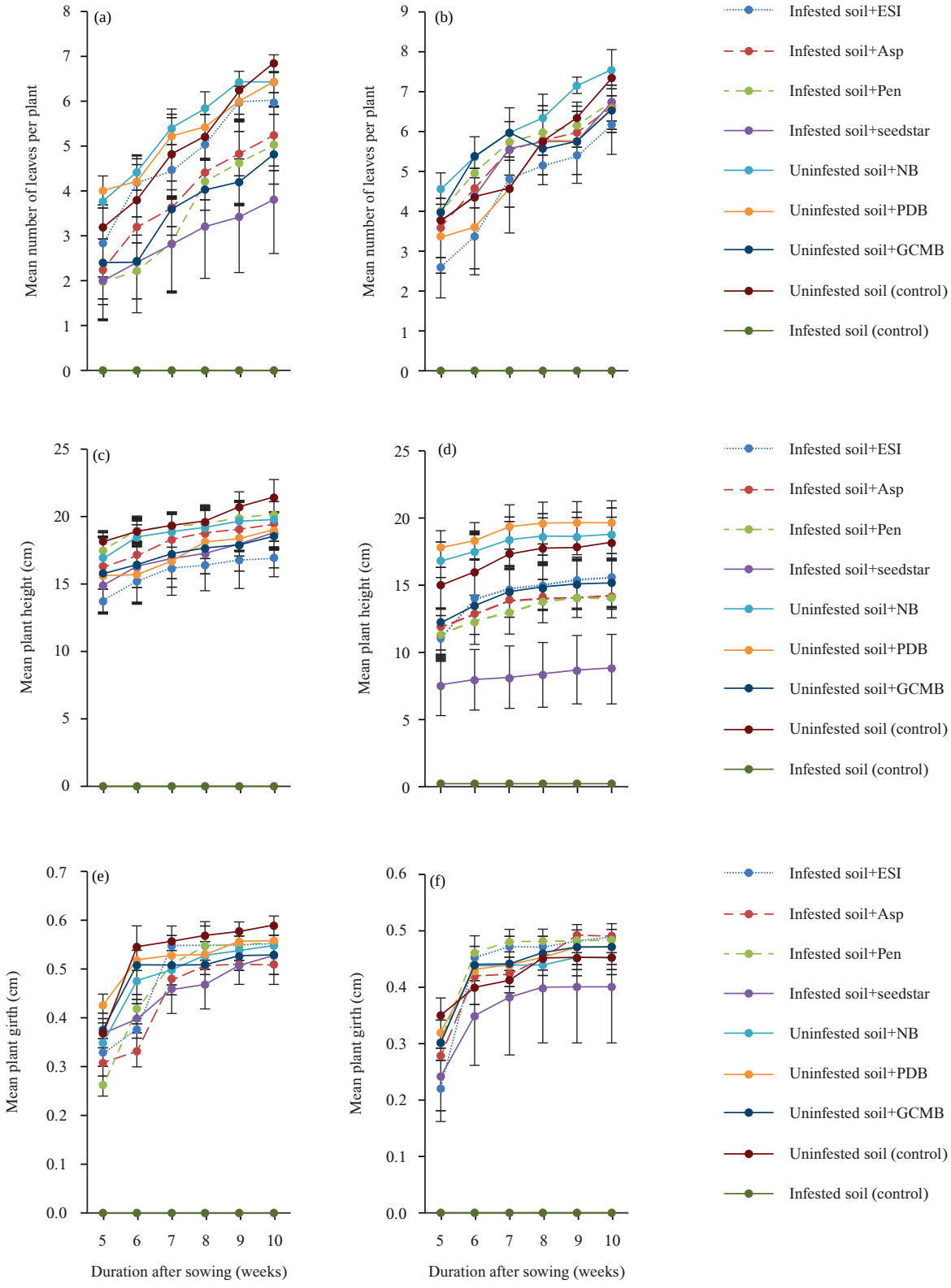

Fig. 5(a-f): Graph showing plant growth promoting ability of the microbial antagonists (Pot experiment II);
(a-b) Mean number of leaves for the drenching method and dipping method, (c-d) Plant height for the drenching method and dipping method and (e-f) Plant girth for the drenching method and dipping method. Vertical bars represent standard errors of mean

Table 5: Presence or absence of microbial antagonists and *P. palmivora* from drenched soils and those of seeds dipped in suspensions of microbial antagonists before and after seeds sown

	Pot experiment I				Pot experiment II			
	Drenched		Dipped		Drenched		Dipped	
Soil treatments	Before sowing	After 6 week	Before sowing	After 6 week	Before sowing	After 6 week	Before sowing	After 6 week
ESI	+	+	-	+	+	+	+	+
Aspergillus sp.	+	+	-	+	+	+	+	+
Penicillium sp.	+	+	-	+	+	+	+	+
P. palmivora (control soil)	+	+	+	+	+	+	+	+

+: Present, -: Absent

Table 6: Growth inhibition of *Aspergillus niger* in soil extract of antimicrobial metabolite produced by the bacterial antagonist, ESI

Soil treatment	Sterilized soil	Unsterilized soil
ESI+NB+urea	-	-
ESI+H_2O+urea	-	-
ESI+urea	-	-
ESI+H_2O	-	-
ESI+NB	-	-
NB control	+	+

Control: Without the rhizobacterium, +: Growth of *A. niger*, -: No growth of *A. niger*, NB: Nutrient broth, ESI: Rhizobacterium, *B. amyloliquefaciens*

Plant height: Seedlings heights 14-18 cm were highest in *P. palmivora*-infested soils drenched with suspensions of microbial antagonists. There were significantly different ($p<0.05$) from those associated with the infested soil without biocontrol treatment of 0.00 cm in Fig. 5c. In the experiment where seeds were dipped in suspensions of the microbial antagonists before being sown, the results obtained with the microbial antagonists were generally similar (10-18 cm) to the drenched experiment with regard to the plant height and differed in plant height significantly($p<0.05$) with *P. palmivora* infested soil (control) of 0.00 cm in Fig. 5d.

Plant girth: The widest plant girths of 0.25-0.55 cm were obtained in infested soils drenched with suspensions of the three microbial antagonists and these were significantly higher ($p<0.05$) than those obtained with the *P. palmivora*-infested soil (-control) of 0.00 cm. Generally, there were also no significant differences in seedling girth among the microbial treatments Fig. 5e. These trends were similar to those obtained using seeds dipping method with 0.22-0.48 and 0.00 cm for microbial antagonists and *P. palmivora*-infested soils respectively in Fig. 5f. The value of zero (0)recorded in the *P. palmivora*-infested soils that did not receive any microbial antagonists, the seedlings completely died indicating total or complete damping-off.

Bioassay of soil for the microbial antagonists and *P. palmivora*: The microbial antagonists ESI, *Aspergillus* sp. and *Penicillium* sp. were recovered from soils into which they were incorporated in Table 5 whilst *P. palmivora* was recovered only from soils infested with the pathogen and did not receive anti-microbial treatments in both drenched soils and seeds dipped in microbial suspensions. The pathogen could not be isolated from soils that had been treated with the microbial antagonists. The presence of either the antagonists or the pathogen is indicated as positive (+) and absence as negative (-) (Table 5).

Anti-microbial metabolite production in soil by the *B. amyloliquefaciens*, ESI: In an initial study, when 24 hrs nutrient broth culture of ESI amended with urea fertilizer (43% N) or without urea fertilizer was used to drench the soil for three weeks, production of the anti-microbial metabolite was enhanced in the soil, resulting in complete growth inhibition (indicated as negative) of the test fungus, *Aspergillus niger*, grown in the soil extract in Table 6. Similarly, when sterilized distilled water was used instead of the nutrient broth and amended with either Urea or without Urea, the resulting soil extract also completely inhibited the growth of the test fungus (indicated as negative), compared to the control which was without the bacterial antagonist, *B. amyloliquefaciens* ESI (indicated as positive) (Table 6). In other control, traditionally the nutrient broth was used to culture the *B. amyloliquefaciens* ESI and to produce the antibiotics which was used as a positive control and also resulted in complete inhibition of the test fungus (also indicated as positive control). Similar results were obtained when the experiment was repeated with each of the treatments with its own control which did not have the metabolite produced by the bacterial antagonist, *B. amyloliquefaciens* ESI (also indicated as negative) (Table 7).

Table 7: Growth inhibition of *Aspergillus niger* in soil extract of the antimicrobial metabolite produced by the bacterial antagonist, *B. amyloliquefaciens* ESI with each of the treatments with its control

Treatment	Sterilized soil	Unsterilized soil
ESI+NB+urea	-	-
NB+urea (control)	+	+
ESI+H_2O+urea	-	-
H_2O+urea (control)	+	+
ESI+NB	-	-
NB control (soil extract)	+	+
ESI+H_2O	-	-
H_2O control (soil extract)	+	+
ESI+NB (non soil extract)	-	-
NB control (non soil extract)	+	+

Control: without the rhizobacterium, +: Growth of *A. niger*, -: No growth of *A. niger*, NB: nutrient broth, ESI: Rhizobacterium, *B. amyloliquefaciens*

DISCUSSION

Seed decay and seedlings damping-off of cocoa (*Theobroma cacao* L.) caused by *Phytophthora* species continue to be a silent killer of cocoa seedlings at the nursery. Prevention of this menace is crucial using microbial antagonists for sustainable control. The results obtained in this study show that broth suspensions of the three microbial antagonists used as drenched or as seed dip in *P. palmivora*-infested soil, prevented damping-off and protected seedlings growth. The soil is a primary source of inoculum for *Phytophthora palmivora*[5], therefore, soil with a history of Phytophthora infestation will require treatment before being used to raise seedlings. This, however, is rarely done but the soil is not the only source of inocula. According to Erwin and Ribiero[7] and Opoku and Wheeler[8], if soil infested with *Phytophthora* sp. is used in raising seedling, the roots of the seedlings become infected and if such seedlings are planted in the field, they would spread the pathogen to pathogen-free fields.

The three microbial antagonists also offered protection against cocoa seed decay and seedling damping-off, comparable to the fungicide Seed star. The ability of the microbial antagonists to protect cocoa seeds from decay and seedling damping-off is a positive development since healthy cocoa seedlings could be established not only in the nursery but even in the field during the early stages of transplanting for seedlings establishment, using the antagonists. When these microbial antagonists are introduced during the early stage of cocoa establishment, seedlings protection from Phytophthora root rot will be ensured and plant growth promoted.

Dipping the cocoa seeds in the suspensions of microbial antagonists or drenching the soil with microbial suspensions gave similar results with respect to protection against damping-off and plant growth, even when the soils were replanted after six weeks. This shows how early these microbes are established in the soil and how persistent these microbes are in the soil and this might due to how the microbial antagonists might have used the soil nutrients and root exudates for their maintenance in the soil. This study, therefore, suggests that the use of the seed dipping method of applying or introducing the microbial antagonists to the soil would be the most appropriate since it is easier to implement than the soil drenching method. Ahmad and Baker[16] have indicated that biocontrol agents can colonize the rhizosphere when added as seed treatment and may protect the underground parts of plants from pathogen attack.

Biocontrol of damping-off of seedlings with bacterial and fungal antagonists, especially *Bacillus* spp. is known for *B. amyloliquefaciens*[16], *B. subtilis*[17-21], *Pseudomonas flourescens*[21], *P. putida*[21], *Burkholderia cepacia*[21-22], *Trichoderma viride*[21-22], *Gliocladium virens*[21-22]. *Bacillus* spp. are capable of colonizing the root environmment[23-27]. This study found this to be true as the rhizobacterium, ESI was detected on root surfaces of cocoa seedlings and seed coats perhaps providing physical protection as reported by[16]. This result also suggests that the rhizobacterium ESI would not only offer physical protection to the seeds but also may use other mechanisms such as antibiosis to achieve this objective since it is established that it possesses the genes for the production of secondary metabolites confirmed as antibiotics iturin A, bacillomycin D and surfactin in another studies[28]. Therefore, the protection of cocoa seeds from decay and seedlings from damping-off by the bacterium could be attributed to the three antibiotics as well as the physical protection offered by the biofilm of the bacterium.

In this study, an indirect approach was used to determine the potency of antifungal metabolites production in soil by the rhizobacterium, ESI. The method involved drenching and leaching out treated soil with water and using a known amount of the leachate to culture the test fungus *Aspergillus niger*. Inhibition of the mycelial mat weight of the *A. niger* then would indicate that antifungal metabolites have been produced in the soil environments. This approach and/or its modifications were also used to determine whether metabolites were produced on root surfaces. Even though the soils were found to contain inhibitory substances (possibly antibiotics), such substances could not be detected on root surfaces possibly because small quantities were produced on such surfaces which rendered the method used insensitive for detection. Production of antibiotics *in situ* by biocontrol agents indicates that the effective quantities are difficult to estimate since they are produced in small amount relative to

other, less toxic organic compounds such as salicylic acids, indole acetic acids, jasmonic acids, lipopolysaccharide, etc. present on root surfaces[29].

Plant-growth promotion is an attribute of biocontrol agents and is due to the production of growth hormones or regulators such as indole acetic acid, gibberellins, cytokinins and auxins that directly enhance growth[30-31] and indirectly through the production of antibiotics[32-33]. In this study, cocoa seedlings growth, in terms of dry matter, the number of leaves, seedlings height and seedling girth were promoted when the antagonistic micro-organisms were added to the soil. Meena and Marimothu[34] observed the boosting effect of *Pseudomonas fluorescens* formulation on plant height, leaf area index, root length, nodules per plant and dry matter on beans. However, this study attributed the boosting effect to the suppression of *Phytophthora palmivora* in the soil to antibiotics production. This study has therefore demonstrated that the three microbial antagonists can be formulated to prevent damping-off and promote seedling growth of cocoa in the nursery environment.

CONCLUSION

This study has appreciated that seed decay and seedlings damping-off of cocoa (*Theobroma* cacao L.) caused by *Phytophthora* species which continues to be a silent killer of cocoa seedlings at the nursery, can no longer be considered unimportant. Prevention of this menace is therefore crucial using microbial antagonists for sustainable control. The findings of this study have demonstrated that broth suspensions of the three microbial antagonists used as a drench or as seed dip in *P. palmivora*-infested soil, prevented seed decay, seedling damping-off and promoted seedlings growth. However, the seed dip method is recommended over the drenched method due to ease of application. It is also noteworthy that the microbial antagonists as soil inhabitants, persisted in the soil, thereby offering lasting protection to cocoa seedlings. The microbial antagonist's viz. *Bacillus amyloliquefaciens* (ESI), *Aspergillus* and *Penicillium* spp. have demonstrated inhibitory capacity against *P. palmivora* in the soil and therefore potential biocontrol agents against seed decay and seedling dumping-off of cocoa seedlings.

SIGNIFICANCE STATEMENT

This study has highlighted the role of microbial antagonists that could be beneficial for the prevention of seed decay, seedling damping-off and their growth promotion ability. The outcome has therefore helped to uncover the critical areas of the need to pay particular attention to protecting the cocoa seedlings at the nursery stage from eminent collapse and possible transfer of black pod disease to the field that many researchers in the field have not paid particular attention to.

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