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Pathogenicity And Biocontrol Potentials Of *Bacillus Subtilis* On Postharvest Cassava Root Rot Pathogens

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Abstract
Many species of fungi are known to be associated with cassava root rot deterioration. These pathogens were isolated from the rots and reconfirmed through pathogenicity tests. *Bacillus subtilis*, a non-pathogenic organism with over 10-year history of commercial use as probiotic and as a biocontrol agent was our choice of antagonist. The inhibitory efficiency of this bio-agent was screened on the pathogens and four of the promising isolates were selected based on their suppressive activities and were subsequently identified as strain BA1, BA2, BA3 & BA4. Further investigation revealed that isolates BA2 & BA3 expressed more stable *in vitro* antagonism against *Botryodiplodia theobromae* (1.3±0.34, 1.5±0.13 cm), *Aspergillus niger* (1.7±0.15, 1.0±0.07 cm) and *Fusarium* spp. (1.4±0.33, 1.3±0.12 cm) respectively. Our result collectively, suggests that *Bacillus subtilis* strain BA2 & BA3 suppressed the pathogens through complex mechanisms including secretion of antimicrobial metabolites and aggressive colonization of the microsites of the roots parenchyma.

Keywords: Deterioration, Probiotic, Biocontrol, Antimicrobial Metabolites, Colonization, Microsites

INTRODUCTION

Cassava (*Manihot Esculenta* Crantz) is a dominant staple of primary importance in the south eastern Nigeria. Its resilient nature enables it to grow reasonably well on poor soils and in areas with unpredictable rainfall. One of cassavas major agronomic advantages that endears it to the hearts of farmers in Nigeria is that it has no critical planting date, provided there is enough moisture at planting. The crop is popular among poor farmers because it requires few inputs and less labour to produce reasonable yield. Apart from providing the food energy intake for over 180 million people in Nigeria, it is considered the cheapest source of starch and a multipurpose crop. Besides its important role in food security and good agronomic attributes [1] production is constrained by several factors including biotic and abiotic stresses [2]. Among them is cassava root rot deterioration that renders the roots unappealing and unmarketable within 24-72 h after harvest.

Cassava roots are susceptible to pre- and postharvest rot. Pre-harvest rot occurs mostly due to poorly drained soil while postharvest rots is associated with complex processes that is linked to enzymatic stress response to wounding [3]. Some of the pathogens associated with the rot include *Aspergillus flavus*, *Botryodiplodia theobromae*, *Rhizopus* sp., *Mucor* sp., *Trichoderma harzianum*, *Penicillium* sp. and *Fusarium solani*. The rotten roots are characterized by a blue/black or brown discoloration of the vascular parenchyma, which starts to appear within 24-72h after harvest. The deterioration originates from the wound sites caused during harvesting and handling processes, and spread to storage parenchyma [3]. This phenomenon reduces its market potential and lowers cassava farmer's income and its use as raw material in industries [4].Several methods aimed at delaying postharvest physiological deterioration (PPD) has been proposed and worked on by various researchers. Such approaches include storage under high humidity and limited O_2 which can reduce water loss and oxidative stress [5], storage in polyethylene bags, boxes and coating cassava roots with paraffin wax [6], storage under low temperature (0-4^{0}C) [7] and hot water treatment at 54-56^{0}C for 10 min combined with modified atmosphere packaging [8]. However, these approaches are expensive and are out of reach for poor farmers in most developing countries of the world. In addition, application of chemicals such as ethanol (> 20%), sodium sulphite (10%), sodium dithiocarbamate (10%), saturated sodium chloride, benomyl (550 ppm) and dicloran (1000 ppm) can delay the onset of PPD forseveral days [9], but their potential toxicities and affordability is a major concern especially in some developing nations of the world.

Evolution of biological control dates back to the end of nineteenth century, but was stagnated in the first half of the twentieth century due to insufficient theoretical basis. Moreover,in the 1950's the successfulintroductionof chemical control methods further limited the development of biological control. Recently, greater " health and environmental awareness" and improved theoretical knowledge have all boosted biological control techniques. Interestingly, all plants have natural enemies that

suppress the proliferation of their pathogens. Biological control uses precisely these natural enemies (predators, parasites, or competitors) to maintain phytophagous pest and microbial populations within acceptable limits and to consequently increase the number of species in the ecosystem.

Bacillus subtilis is ubiquitous in nature with wide applications in biotechnology, industries and agriculture [10]. First discovered by [11], *B. subtilis* has been proven over the years as a phytopathogenic antagonist and as a plant growth promoter. Apart from growth stimulation, *B. subtilis* has demonstrated active suppressive activities against disease pathogens through multiple mechanisms including production of extracellular lytic enzymes and secondary metabolites [12], cell lysis, competitive exclusion and induced systemic resistance in plants [13], and cell wall degradation. Commercial strains of *B. subtilis* (e.g. Serenade) have been marketed as biocontrol agents for fungal diseases of crops [14]. This biofungicide has demonstrated impressive suppressive effect against a variety of pathogenic bacteria, including *Erwinia, Pseudomonas* and *Xanthomonas* strains [15]. Other *B. subtilis* commercial products include Kodiak (with demonstrable PGR activity), Subtilex (biofungicide), Vault (for soy beans and pea nuts growth enhancement), RhizoPlus (plant growth promoting rhizobacterium and biocontrol), Taegro (biofungicide) and Pomex (microbial fungicide) [16]. Though the mechanism of this biofungicides are in doubt; however, it has been established that *B. subtilis* can secrete diverse antibacterial agents as well as a broad spectrum of lipopeptides, such as surfactin, that are powerful biosurfactants [17]. On safety, *B. subtilis* has been used in functional foods or as probiotics such as in Italian probiotic Lactipan Plus and Japanese fermented soybean product Natto. Probiotic therapy is very attractive as it recreates natural flora rather than disruption [18].

To further strengthen its safety on humans, *B. subtilis* does not carry genes that encode one or more of the four known enterotoxins (Hb1, Nhe, Cytk, and BceT) [19]. Other potential virulence factors found absent in *B. subtilis* are haemolysis and the production of the phospholipase lecithinase, although the later did give a weak reaction with *B. subtilis*, Natto [20]. However, the only issue associated with the use of *B. subtilis* is its intrinsic resistance to clindamycin, which may require a more comprehensive study [20].

Pathogens or antagonists may act as biocontrol agents through competitive exclusion or secretion of antibiotics. Their relative pathogenicity is dependent on their rapid growth rate.Defined as "the use of living organisms to suppress the population density or impact on a specific pest organism [21], the end goal is to use biology to serve man in an environmentally friendly way to ensure healthy crops or other products.However, the continuous use of pesticides, fungicides and other agricultural chemicals in agricultural production over the years has triggered resistance in pests and organisms with its increasing ecological and environmental consequences [22]. Above all, the attraction is that biocontrol is sustainable, target specific and safer. In this study, we investigated the colonization and the biocontrol efficacy of *B. subtilis* on cassava root rot fungal pathogens and our findings are hereby discussed.

MATERIALS AND METHODS

Isolation of Fungal Pathogens

Rotten cassava root samples from five markets in Anambra, Imo, Bendel, Cross-River, Rivers and Benue States of Nigeria were first washed under running tap water and surface sterilized with 70% ethanol solution. They were cut open with sterile surgical blade to expose the boundary margin between the rot and the healthy parts. About 3 mm in diameter portions were excised from the margin of the rotten boundary and inoculated on previously prepared potato dextrose agar (PDA) plates. Incubation was at room temperature (28 ± 2^0C) for 5 days and the culture plates were examined daily for fungal growth. The isolated fungal pathogens were sub-cultured three times in order to obtain pure cultures and were subsequently stored in PDA slants at 4^0C.

Estimation of the Percentage Occurrence of the Fungal Pathogens

Determination of the frequency of occurrence of the fungal pathogens isolated was carried out with 10 cassava roots from each state. The obtained pathogens were cultured separately on PDA plates and the occurrences of each of the isolates from the 10 samples were recorded. The percentage was calculated and expressed as follows:

Percentage occurrence = X/N x 100/1

X = Total number of each organism in all the samples

N = Total number of all the organisms in all the samples screened**.**

Isolation and Selection of Antagonistic Bacteria

Fresh 23 soil samples were collected from the rhizospheres of cassava roots and the bulk soil at cassava field in sterile polythene bags from different points at a depth of 2 cm. Soil samples were taken to the microbiology laboratory for analysis. Ten grams of each of the samples were homogenized in sterile 10 ml distilled water and were serially diluted. Ten soil samples were boiled in a water bath for 10 minutes at 80^0C in order to aid isolation of spore-forming bacteria, while the remaining samples were

not boiled in order to isolate both spore-forming and non-spore forming bacteria [23].A 0.1 ml portion of the soil suspension samples were plated directly onto previously prepared nutrient agar (NA) medium and incubated at 37^0C in triplicate. After 24 h incubation, individual colonies were picked and purified by streaking three times on NA plates. Purified isolates were screened for starch hydrolysis as described by [24]. This was done with NA containing 1% soluble starch. Amylolytic activity was demonstrated by streaking the isolates on the NA. Incubation was at 37^0C for 24 h before flooding with iodine. In situ enzyme production was observed with the clearing zones of whitish appearance in the areas surrounding the isolate. Based on the satisfactory performance of some of the isolates in the amylolytic activity tests, they were selected for further experimental studies.

Identification of the Putative *Bacillus Subtilis*

Morphological characterization of the isolates was carried out by Gram stain, motility test, spore staining as well as biochemical test (catalase, nitrate reduction, Voges–Proskauer, indole production, and sugar fermentation) as described by [25].

Pathogenicity Tests

The previously isolated fungal pathogens from the rotten cassava roots were tested to ascertain their degrees of disease inducement in healthy cassava roots. The selected cassava roots were thoroughly washed with tap water, sterile distilled water and surface sterilized with 70% ethanol solution. A 30mm diameter sterile cork borer was used to remove a cylindrical core from the cassava roots. Sterile paper disk impregnated with each of the fungal isolates were individually introduced aseptically into five cassava roots and sealed with sterile petroleum jelly under a lamina airflow hood. Observation was for ten days at room temperature. Inoculums of the developed rots on the cassava roots were further cultured. The resulting cultures were sub-cultured 3 times on PDA in order to obtain pure cultures. These pure cultures were characterized, identified and matched with the previously isolated cassava root rot pathogens as described in [26]. The confirmation was based on the match between the previously isolated pathogens from the rotten cassava roots and the pathogens from the pathogenicity tests.

***In vitro* Antagonism Study**

The dual agar plate method was used to test for antagonism. The mycelia of each of the isolates (*B. theobromae, A. flavus, A. niger, Fusarium* spp., *Rhizopus* spp. and *Penicillium* spp.) were dual–culture plated with the previously isolated and identified potent strains of *Bacillus subtilis* (BA1, BA2, BA3 and BA4) as described by [27]. Agar disks (3 mm ø) of each of the isolates and the antagonists were cut-out with a sterile cork borer and placed equidistantly at 80 mm from each other in a petri dish containing 15 ml of PDA medium. Petri dishes containing only a disk of the isolates served as the controls. The experimental set-up were replicated three times and incubated for six days. Results were determined as the means of percentage inhibition of the growth of the fungal isolates in the presence of one of the *B. subtilis* isolates (BA1, BA2, BA3 and BA4). Percentage inhibition was determined using the formula of **[**27**]:**

% inhibition = 1-[Diameter of the lawn/Control growth] x 100.

***In Vivo* Study of the Suppressive Activities of *B. Subtilis* Isolates (BA2 and BA3) Against Thefungal Pathogens**

Ten healthy cassava roots were selected and washed for 15 min. in running tap water. They were surface sterilized with 1 % NaOCl solution for 10 min. Further washing was with sterile distilled water followed by air-drying at room temperature (30 ± 2^0C). Sterile cork borer was used to create a core in the tissues of the roots under aseptic condition. Inoculations of the fungal pathogens were done aseptically under a laminar air flow hood by inserting a 0.6 cm diameter agar discs from the margin of a 5-day-old culture on PDA into the cores that have been previously created. This was followed by inoculating with 0.6 cm diameter agar discs of one of the *B. subtilis*strains (BA2 and BA3) grown on NA for 3 days. Roots inoculated only with *B. subtilis* served as control. The treated and the control roots were kept at room temperature (30 ± 2^0C) in a loosely covered polyethylene bags. The roots were examined for rotting after 10 days of incubation by cutting diametrically across the point of inoculation. Rot symptoms were assessed and recorded.

Statistical Analysis

Experiments were designed as a completely randomized design (CRD). All experiments were replicated three times and the means were compared by Duncan Multiple Range Test (DMRT) at $P\leq 0.05$.

RESULTS AND DISCUSSION

Postharvest root rot is an economically significant disease of cassava that limits its market potential and reduces incomes accruable to cassava farmers. By nature, the rhizospheres of healthy plants are thickly populated with numerous groups of microbes with multiple beneficial biocontrol abilities. Research has severally proven that such rhizospheres cohabited by soil-borne pathogenic fungi could be a possible and useful source for isolation of microbes with promising antibiotic activities. The presence of bacterial isolates and identification of copious numbers of antagonistic *B. subtilis* among the teeming numbers of organisms in the rhizospheres of healthy cassava roots is an indication that *B. subtilis*

holds promise in our effort to effectively and efficiently suppress the activities of the cassava root rot pathogens. To date, available traditional and chemical storage techniques are either expensive or toxic and have not provided the much needed solution. In this study, we explored the inhibitory activities of these bioagent as an alternative for the management of the root rot pathogens.

Diverse groups of fungal isolates were recovered from the rotten cassava roots. The isolates grew rapidly on PDA, and were subsequently identified and characterized (Tables 1 & 2). *A. niger, Fusarium* spp., *B. theobromae, Penicillium* spp., *Rhizopus* spp., and *A. flavus* were frequently isolated (Table 3). When we subjected the fungal isolates to pathogenicity tests, *A. niger* which occurred more frequently in culture (Table 3) was the most virulent based on its aggressive growth pattern, spread and abundance in culture suggesting that the organism is the primary cause of cassava root rot postharvest physiological deterioration (PPD) after harvest in the rain forest zone of the southern Nigeria particularly in Anambra, Imo, Delta, Cross-River, Rivers and Benue States since the samples were sourced from these parts of the country.

To investigate the antagonistic activities of *B. subtilis* on the isolated postharvest cassava root rot pathogens, we co-cultured the isolated *Bacillus* strains (BA1, BA2, BA3 and BA4) with each of the pathogens. Distinct zones of inhibition were observed on the culture plates, but beyond 48h of incubation, the zones remained stable while the culture plates of the control were overgrown by the fungal pathogens (Table 4). We observed further that strains BA2 and BA3 secreted more extracellular metabolites than BA1 and BA4 based on the area of their inhibition zones (>0.8). We therefore concluded that **t**he pathogens may have exhibited significant difference in their susceptibility to the antagonists. Though their inhibitory response to the antagonists were not significantly different (P>0.5), their sensitivity to antagonists BA2 and BA3 were significantly different (P<0.5) as strains BA2 & BA3 demonstrated strong inhibition patterns when tested on the cassava root rot pathogens (*B. theobromae, A. niger, A. flavus, Fusarium* spp., *Rhizopus* spp. and *Penicillium* spp.) (Table 5).Disease severity reduction pattern (*in vivo*) as captured in table 5 decreased significantly by the activities of the two *Bacillus* strains (BA2 & BA3) suggesting rapid induced systemic resistance or competitive exclusion of the pathogens

Table 1: Microscopic and biochemical characteristics of Bacillus subtilis

Parameters	**Isolates**			
	BA1	**BA2**		**BA3**
	BA4			
Colony features	Dull and creamy brown	Dull and creamy brown	Dull and creamy brown	Dull and creamy brown
Microscopy				
Gram reaction	+	+	+	+
Motility	+	+	+	+
Cell arrangement	Blunt ended, singly and in pairs	Blunt ended, singly and in pairs	Blunt ended, singly and in pairs	Blunt ended, singly and in pairs
Spore stain	+	+	+	+
Biochemical reaction				
Catalase	+	+	+	+
Oxidase	+	+	+	+
Indole	-	-	-	-
Voges-Proskauer	+	+	+	+
NO_3	+	+	+	+
Carbohydrate utilization				
Glucose	+a	+a	+a	+a
Lactose	ND	ND	ND	ND
Maltose	ND	ND	ND	ND
Mannitol	ND	ND	ND	ND
Urease	-	-	-	-
Hydrogen sulphide	ND	ND	ND	ND
Hydrolysis of:				
Starch	+	+	+	+
Gelatin	+	+	+	+
Identity	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***	***Bacillus subtilis***

Key: + = Positive, - = Negative, ND = Not done, +a =Positive with acid production.

Table 2: Characterization and identification of the fungal isolates

Isolate	Colony features	Microscopy	Identity
A	Fast growing colonies that covered the agar surface with a dense whitish growth. On sporulation, the cotton or whitish growth turned grey or yellowish brown.	Non-septate.. Rhizoids and sporangia brownish and branched. Sporangiophores were visualized.	***Rhizopus* spp.**
B	Rapid growth on PDA. Culture surface was slightly brownish with smooth surface on PDAand with a contrasting yellow colour on the reverse side of the culture plate	Conidiophores were upright, simple and terminating in a swelling bearing phialides at the apex with many colours.	***A. Niger***
C	Rapid growth on PDA. Colonies were initially whitish but later dull green with grey centre and whitish margin after 7days at 28^0C.	Conidiophores were observed. The phialides at the apex of the conidiophores carried brush-like clusters referred to as "penicilli"	***Penicillium* spp.**
D	Culture surface was pale brown on sabouraud glucose agar and czapek dox agar.	Powdery mass of yellow green spores observed on the surface of the culture and light yellow on the reverse side. Hyphal growth was thread-like branching, and producing mycelium.	***A. flavus***
E	Pinkish and moderate growth on PDA compared to vigorous growth on malt extract agar.	After 4 days of culture, mycelium covered the entire culture plates. Sporulation was observed after 7 days. Colony colour varied from pink to white, purple and violet on the two media (PDA & malt extract agar)	***Fusarium* spp.**
F	Rapid growth with fluffy colonies. Colony colour was dull white which latter changed to black.	Sporulation was observed on culture medium. Pycnidial production was between 20 to 34 days.	***A. Theobromae***

within 2-4 days. According to [28] genes coding for lipopeptides are present in most biocontrol strains that have already been commercialized and such strains have enhanced capacity to produce antibiotics that can suppress the growth of fungal root rot pathogens. Moreover, the protective action of *B. subtilis* lipopeptides to plants have been described and reported under pre-and postharvest conditions, either by inhibiting the pathogenic fungi directly or inducing systemic resistance of the host plants [29, 30]. In addition further colonization of the pathogens exhibited by both antagonistic *B. subtilis* strain BA2 and BA3 during *in vivo* experiments suggests that they may be good antagonists against cassava root rot pathogens (Table 5).

Table 3: Percentage frequency of occurrence of the isolated fungal pathogens from the rotten cassava roots

Isolate	Frequency of occurrence (%)
Rhizopus spp**.**	8.5
Botryodiplodiatheobromae	19.5
Aspergillus niger	28.6
Penicillium spp**.**	13.4
Aspergillus flavus	6.6
Fusarium spp**.**	23.4

Data are means of three replications

B. subtilis is a non-pathogenic organism that secretes copiousamount of antimicrobial metabolites that acts against fungal pathogens. The bioagent is our choice as it has a well-documented history of commercial use for more than 10 years as a probiotic in the treatment of gastrointestinal diseases and as a biocontrol agent [20]. Production of antimicrobial substances is a major bacterial adaptive strategy for effective control of pathogens. Earlier studies suggest that biocontrol mechanisms could be as a result of antimicrobial metabolitessecretion andcompetitive exclusion which could protect against pathogenic infections [28-32]. We therefore concluded that *B. Subtilis* may have inhibited the pathogens either through secretion of antifugal substances or through colonization of microsites faster than the fungal pathogens [35].

This assumption may explain the suppressive ability of the antagonists against the cassava root rot fungal pathogens. Thus, the apparent ability of the *B. subtilis* strains BA2 and BA3 to control these cassava root rot pathogens may be directly proportional to their colonization through antibiotic secretion and perhaps through competitive exclusion. However, the exact mechanisms by which these antifungal metabolites act is still unknown, but may be traced to its ability to interrupt the growth of the fungal mycelium [17] or through modification of the physical and chemical properties of the cells of the pathogens [36].

Table 4**:** Inhibition zone produced by *Bacillus subtilis* isolates against the cassava root rot Pathogens

Pathogen	**Antagonist Inhibition zone (cm)**			
	BA1	BA2	BA3	BA4
B. theobromae	0.8±0.03	1.3±0.34	1.5±0.13	0.3±0.10
Aspergillus niger	1.0±0.11	1.7±0.15	1.0±0.07	0.6±0.12
Rhizopus spp**.**	0.4±0.12	0.9±0.03	1.6±0.13	0.7±0.14
Penicillium spp**.**	0.6±0.01	0.8±0.02	0.9±0.04	0.6±0.03
Aspergillus flavus	0.6±0.02	1.1±0.12	0.9±0.03	0.8±0.04
Fusarium spp**.**	0.7±0.13	1.4±0.33	1.3±0.12	0.5±0.02

Data are means of three replications while values are expressed as mean±standard deviation

Table 5: In vivo activity of the antagonists (BA2 & BA3) against the pathogens

Antagonist	**Day**	**Pathogens**					
BA2		***Rhizopus spp***	***B. theobromae***	***A. niger***	***Penicillium spp***	***A. flavus***	***Fusarium spp.***
	2	42±0.012	28±0.13	38±0.06	46±0.12	35±0.04	39±0.03
	4	68±0.09	59±0.06	62±0.01	63±0.02	54±0.03	68±0.12
	6	39±0.01	42±0.03	46±0.02	44±0.04	38±0.16	42±0.14
	8	22±0.16	26±0.014	33±0.04	31±0.05	27±0.02	28±0.03
	Control	0	0	0	0	0	0
BA3	**2**	48±0.012	38±0.03	28±0.02	34±0.17	33±0.4	29±0.03
	4	60±0.02	64±0.14	56±0.13	52±0.01	62±0.12	57±0.10
	6	39±0.1	48±0.02	37±0.01	38±0.014	44±0.12	38±0.02
	8	20±0.04	35±0.04	28±0.03	29±0.16	26±0.01	24±0.014
	Control	0	0	0	0	0	0

Data are means of three replications while values are expressed as mean±standard deviation.

CONCLUSIONS

The application and exploitation of biological agents in agriculture offers both healthy and environmentally friendly alternatives over chemically synthetic agents in sustainable agricultural production. Thus, the use of beneficial microbes such as *B. subtilis* to control pathogenic organisms associated with postharvest diseases as described in this paper offers an attractive alternative compared to the use of synthetic chemicals. The approach is sustainable and environmentally friendly.

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