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In Vitro evaluation of some fungicidal bioactive chemicals against fusarium spore germination and survival

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Abstract

The genus Fusarium are the most important pathogens associated with different plants including crop plants, fruits, vegetables, etc. causing of constrained production and productivity crops. Bioactive compounds are environmentally friendly and amenable. This study aimed to evaluate the effectiveness of some bio-active compounds derived from Bacillus strain for the in vitro control of Fusarium mold. Seven (7) isolates of the Fusarium complex were isolated from various stored fruits, vegetables and food grains using Potato Dextrose Agar (PDA) media supplemented with antibacterial agent. The growth and survival of the respective spores were challenged with supernatants of overnight-grown culture of Bacillus subtilis TC17 for 20 minutes. The supernatant prevented the germination of spores of some fungi strains in the order; *F. solani, F. oxysporum, F. equiseti, F. acuminatum, F. proliferatum, F. moniliforme* and *F. tricinctum* against control. Therefore, this research showed that the bio-active agent can be used to control the pervasive plant pathogenic fungal species with outstanding result and therefore should be developed further for registration for commercial applications.

Keywords: Bio-control; Fungi; Bacillus; Fusarium; Spore; Survival and Supernatant

1. Introduction

Fruits, vegetables among other agricultural products are important in human diets because they provide essential growth factors such as vitamins and minerals necessary for proper body metabolism and healthy and normal growth (Ozel *et al*., 2017). Despite the dependence of human on these food materials, fruits are easily spoilt by many ways including microbial deterioration. The active metabolism during the storage stage though may be responsible for spoilage, microorganisms especially molds are associated where they also cause risks when their toxic materials are injected (Sun *et al*., 2016). The presence of high concentration of the sugars in the materials couples with vitamins and minerals enhances the successful growth and survival of various parasitic and saprophytic forms of fungi (Rahmani *et al*., 2013). Several reports have indicated that a lot of fruits and vegetables are lost to spoilage, especially during post-harvest stages (Emana *et al*., 2015; Figure 1).

Spoilage fungi which can be toxigenic or pathogenic are often controlled using different types of chemical compounds and some toxin-producing fungi have been identified and isolated from spoilt fruits by previous researchers. It is now documented through some research findings that some soil-borne bacteria that are antagonistic to plant pathogens could make a substantial contribution to prevention of plant diseases, and therefore represent an alternative to the use of chemical pesticides in agriculture (Bantayehu *et al*., 2018). Various methods have therefore emerged to understand the mechanisms of their operations and how their roles in plant health and soil fertility could be further enhanced through research into antagonistic activities that have previously been observed especially in organic-rich soils. This type of soil and the rhizosphere have frequently been used as a model environment for screening of putative agents for use in biological control of soil-borne plant pathogens. The genera of bacteria such as *Bacillus* spp., *Pseudomonas* spp.,

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and *Streptomyces* spp. are widely used as bio-control agents and *Bacillus* spp. has been reported to produce several antibiotics some of which are at the verge of commercialization (Devi *et al*., 2018). Many non-pathogenic rhizobacteria like *Bacillus* sp., form endospores and can tolerate extreme pH, temperature, and osmotic conditions. This thus, offer several advantages over other organisms. It was previously reported that *Bacillus* sp. was found to colonize the root surface, increase plant growth, and cause lysis of fungal mycelia which signals to the fact that they could be used as potential protection against fungi attack. Most of the strains are regarded as safe biological agents and their potential is considered high in the biological control measures (Fessard and Remize, 2019). The potential antagonistic activity is due mainly to production of antifungal antibiotics which appear to play a major role in biological control of plant pathogens and could be explored on larger scale. Generally, the spoilage of farm produces due to fungal attack should no longer be tolerated owing to the high cost of both production and storage. Also, the high cost of importation of exotic chemical control agents is discouraging. The need to look inward for the production of indigenous, biological chemicals that would be environmentally friendly and affordable to common farmers in order to be able to produce more food for the ever-growing populations on sustainable bases cannot be over stated.

Figure 1 Tomato Fruit Rot Caused by Fusarium sp. (A) and Fusarium Dry Rot of Potatoes (B)

Looking at antagonistic activities of microorganisms from the biotechnological point of view, one important feature of Bacillus species is their diverse secondary metabolism and the ability to produce a wide variety of antimicrobial agents with diverse antagonistic potentials (Leyva *et al*., 2018). Strains of Bacillus subtilis have been especially reported to have such high tendencies to be active producers. Therefore, in recent years, the interest in biological control of plant pathogens has significantly increased, due to the need for introduction of more environmentally friendly alternatives to the massive use of chemical pesticides so as to reduce their menaces (Alvarez-Sieiro *et al*., 2016). Therefore, the present study was aimed at isolation of potential antagonistic *Bacillus subtilis* with potential antifungal components for the in vitro control of Fusarium pathogenic fungus

2. Material and methods

2.1. Sample Collection and Material Sterilization

Organic-rich soil was collected from corn farm and transported in previously sterilized container to the laboratory for analysis. Meanwhile, all apparatus and materials used for the research were sterilized at 121°C for 15 minutes(autoclave) and 180^0C (Hot-air oven). This was done to avoid contamination during the sample processing (Saha *et al*., 2012).

2.2. Isolation and Identification of Antagonistic Bacillus Strains

Using sterilized media and materials, 1g of the collected soil sample was added to 9ml of sterile peptone water and serially diluted to 10^{-6}. Then, aliquot 0.1 ml of 10^{-3} and 10^{-5} dilutions were spread plated on sterilized nutrient agar medium and incubated for 24 hours at 35^0C. Pure colonies with morphology characteristics of Bacillus subtilis were carefully selected, and purified by sub-culturing three consecutive times (Hossain and Rahman, 2014). The bacteria isolates were further characterized using standard morphological technique (Gram staining method and observed by optical microscope under the oil-immersion lens) and biochemical techniques including carbohydrate fermentation test, indole formation, methyl red (MR) and VP tests, respectively. Thereafter, the antagonistic isolates were selected according to their inhibitory efficacy against Fusarium mold (Shrestha *et al*., 2016). The identified isolates were stored at -70°C in 15% glycerol with Tryptic soy broth at 4°C for use in further studies.

2.3. Isolation and Identification Indicator Fungi

Isolation of the fungi (Fusarium spp.) was isolated from decaying tomatoes collected from the stored. Sterilized Potato dextrose agar (PDA) supplemented with Streptomycin antibiotic was used for the isolation of fungi. One gram of the spoilt tomato was added to 9 mL of sterile peptone water and serially diluted to 10^{-6}. Aliquot 0.1 ml of 10-4 was pour-plated in the molten agar. The set-up was kept in an incubator at 28°C under dark conditions (Ali *et al.,* 2014. All the procedure were carried out in laminar hood under sterilize condition. After five days of incubation, distinct colonies of fungus that appeared were picked with a sterilized loop and transferred to fresh PDA plates. Morphological characterization of the isolated fungi was done and a total of sixteen isolates of Fusarium were identified on the basis of colony morphology, morphological characteristic of macro- and micro-conidia and conidial measurement. Among these Fusarium isolates, *Fusarium oxysporum* species was identified using manual of Booth, (1971).

Figure 2 Spoilt Tomato; Source of Fungi

2.4. Pathogenicity Tests

All the isolates of F. oxysporum were grown on nutrient agar and the respective sporeswere harvested, carefully introduced into fresh, disinfectant-washed tomatoes that was previously incited with serile knife. The wounds were covered with sterile vasline, kept in humid environment for 5 days and observed for the development of fusarium with the same morphological characteristics (Bensch *et al.,* 2012).

2.5. Preparation of Bacteria Supernatants

Figure 3 Biochemical Test and Centrifugation of Bacillus growth Broth

A single pure colony of the Bacillus strain was sub-cultured into 10 ml sterile nutrient broth and grown for 24 hours. Afterwards, the pure culture was introduced into 250 ml flask containing sterile nutrient broth and incubated with

shaking at 37°C for 48 hr. Then the culture was centrifuged at 8,000 rpm at 4°C for 10 min and the supernatant was collected as the crude extract of bacteria for further tests (Lee *et al*., 2017).

2.6. Preparation of Fusarium Spore Suspensions

Pure culture of the test indicator fungi was sub-cultured on sterile PDA and incubated for 5 days at 28^0C for mass multiplication of inoculum of *F. oxysporum* (Degraeve *et al*., 2016). After the incubation, the spores of the cultures were carefully collected into 10 ml normal saline with tween80 added to keep the conidia spores apart.

2.7. *In vitro* Antagonistic Challenge

Five milligrams of each spore suspension of the respective isolate of *F. oxysporum* at the concentrations of 10^3, 10^4, 10^5, 10^6 and 10^7 Cfu/ml) were added to five milligram of Bacillus supernatants respectively. The spore suspension/supernatant mixture in triplicates was allowed to stand for 20 minutes. According to the method of Gossen *et al*. (2016), the spore germination test was performed by spreading 0.1 ml of each mixture on sterile PDA (Supplemented with streptomycin) and incubated for 5 days at 28^0C. fungi colony formation as a sign of germination of spore were recorded as supernatant not being effective while lack of germination was recorded as positive. PDA media inoculated with spore suspension without supernatant was used as control. The percent inhibition in the growth (germination) of the pathogenic *F. oxysporum* was recorded comparing it with control. The percent inhibition in growth was calculated according to the following formula;

Percent inhibition= (C-T)/C X 100; Where C= indicated as the growth (germination) of the test pathogen in control plate; T= Germination (growth) of the test pathogen in treatment.

2.8. Antifungal Spectrum Detection

2.8.1. Preparation of Fungal Cultures

Using the method of Madrid *et al*. (2014) with little modifications, the pure cultures of fungi; *A. niger, Penicillium sp., Mucor sp., A. fumigatus* and *A. flavus* were mass-produced and the spores carefully collected, respectively, prepared into suspension and then were challenged with the bacillus supernatant as described above.

3. Results and Discussion

3.1. Isolation and Identification of Antagonistic Bacillus Strains

In this study, Bacillus bacteria species were isolated from the soil and they showed different biochemical and morphological characteristics from day 1 to 7. The combination of cultural, morphological and biochemical characteristics of the bacteria was in conformity with those in the Bergey's manual of Determinate Bacteriology (Foysal *et al*., 2011). The Bacillus subtilis isolated included, *Bacillus subtilis* TC28, *Bacillus subtilis* TC17, *Bacillus subtilis* TC06, *Bacillus subtilis* TC11 and *Bacillus subtilis* TC54. However, *Bacillus subtilis* TC17 was selected for further applications. A combination of morphological and biochemical reactions was used to identify the isolates based on standard bacteriological manuals (Sastry and Bhat, 2016).

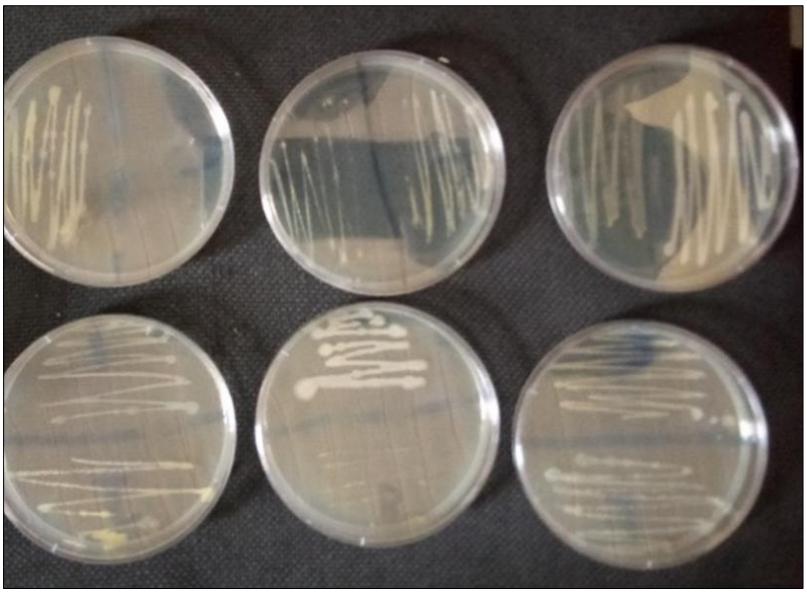

Figure 4 Pure Culture of Bacillus Strains

3.2. Isolation and Characterization of Fusarium mold

In the present study, Fusarium fungi strains were isolated from spoilt tomatoes. They were identified morphologically and on further study, all the isolates were found to be pathogenic to tomato fruits. Several reports have indicated that identification of Fusarium species from the spoilt fruits and vegetables has always been recorded. The fungal macroscopic characteristics and variations observed in the size and shape of the microconidia, macroconidia, and chlamydospores (Leslie and Summerel, 2006) suggested that the isolates collected may have corresponded to *Fusarium* spp. Their presence is reported to be responsible for several losses and wilt diseases in many crops. A diverse population of non-pathogenic and pathogenic F. oxysporum isolates have been reported to be associated with the rhizosphere and endorhizosphere of tomato plants (Manikandan *et al*., 2018). In the pathogenic test, the persistence of the fungus under tomato deterioration for seven days showed that they were the fungal associated with and responsible for the spoilage of the produce. ().

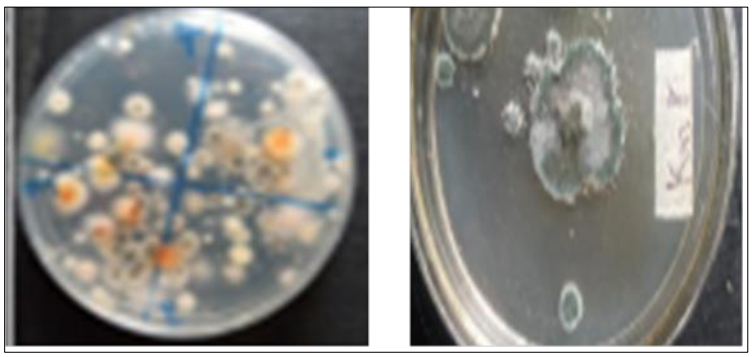

Figure 5 Cultural and morphological features of Fusarium sp. on PDA agar

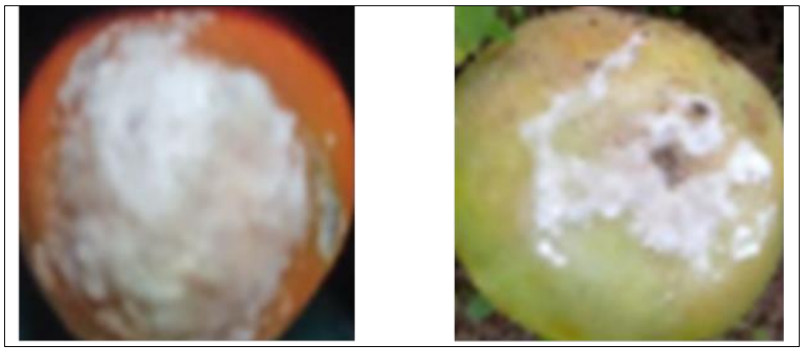

Figure 6 Pathogenicity Test Results

3.3. *In vitro* Antagonistic Challenge and spectra of Activity

The spore plate count of the respective treated fungi culture was in the following order; *F. solani* (102), *F. oxysporum* (10^1), *F. equiseti* (-), *F. acuminatum* (-), *F. proliferatum* (9), *F. moniliforme* (-), *F. tricinctum* (10^3) and control (10^4). The supernatant (metabolites) was able to prevent the germination of some fungi strains (-), it was not effective against some others giving that the spores germinated after incubation.

Table 1 The Effect of the Bacillus Supernatant TC17

Fusarium	Reduction %				
	10^{-7}	**10^{-6}**	**10^{-5}**	**10^{-4}**	**10^{-3}**
F. solani	4	23	26	31	34
F. oxysporum	7	17	19	35	38
F. equiseti	-	12	24	26	28
F. acuminatum	-	23	25	32	28
F. proliferatum	-	19	27	38	43
F. moniliforme	-	20	25	29	37
F. tricinctum	-	15	22	46	42

The *in vitro* effect of antagonistic activity of the cell-free supernatant of the Bacillus strain understudied against the germination of the spores of the Fusarium complex is as presented on Table 1 presents the result showed that the supernatant at 10^{-3} dilution factor had the highest effect on the fungi isolates. The range being 28% to 43%. Generally, all the concentrations except at 10^{-7} suppressed the colony growth of the pathogen at varying percentages. The supernatant at the lowest concentration did not have any effect against F. solani and F. oxysporum. It has been reported that antagonistic activity of Bacillus metabolites against fungi are specific in their target and against specific fungi. This observation agrees with several reports that bacteria strains such as Bacillus could produce “iturin and surfactin”, “surfactin and fengycin” (BLB277), and “fusaricidin and polymyxin” (BLB267) which exhibit broad spectrum activity against several phytopathogenic fungi with different sensitivities. It was also alluded that the secondary metabolites were cyclic peptides which could coexist in the cell as a mixture of several peptide variants with different aliphatic chain length according to Mora et al., (2015). They also observed that the metabolites are resistant to heating and proteinase K treatment and they could interact and then disintegrate fungal cell wall materials of the fungus leading to their death. Also, the proportion of the produced antifungal metabolites could be specific for the bacterial strain. In fact, Cawoy et al. (2015) in their research pointed out that different *B. subtilis* and *B. amyloliquefaciens* strains cultivated in an optimum medium had different proportions of the surfactin, fengycin, and iturin. Hence, the differences in activities. Bacteria with potential antifungal activity could also produce proteases which could bind to the outer mannoprotein, open the protein structure, and expose inner glucan layers and chitin microfibrils of the fungus thereby killing them (Choudhary *et al*., 2014). Bacteria metabolic product have also been found to breakdown the micelles of fungal pathogens. Therefore, this can be explained in the confrontation assay that while the supernatant recorded reduction of 43% against F. proliferatum, 28% reduction was recorded against both F. equiseti and F. acuminatum. This might be

due to different genetic abilities of the fungi that account for varying pathogenicity and resistance (Cowger, and Arellano, 2013). In terms of spectrum of activity, strain TC17 showed growth inhibitory activity against all the fungal pathogens tested in this study, especially Fusarium complex. The colony reductions ranged from 43% to none at all. (Table 1) when the fungal spores were sub-cultured on the agar surface with B. subtilis FC17 was retarded. Similarly in the previous study, it has been reported that the Bacillus strains had high disease suppression (Choudhary *et al*., 2014). Based on the current study, it could be established that the metabolites obtained from Bacillus subtilis strain studied was able to inhibit the germination of the fungi spores that cause different tomato spoilage at least to a different extent. Their various capabilities to inhibit spore germination of fungi with pathogenic behavior could be harnessed for the effective management of the diseases caused by the fungi in single formulation or in combination with other antifungal products. It has been reported by several authors that one effective means of controlling Fusarium wilt appears to be practical and economically efficient control measure is the possibility of biological method which are environmentally safe. Therefore, for the management of Fusarium wilt and other spoilage caused by the same, it was concluded that among various isolates of Bacillus, Bacillus subtilis has the potential to suppress growth of pathogenic Fusarium complex and could be used as a biocontrol agent against the management of several fungal disease in tomato.

4. Conclusion

A wide range of pathogenic microorganisms including molds have been controlled using *Bacillus*-based biocontrol agents. This result agrees with other observations that the supernatant as a biological control agents could strategically weaken the target spore wall thereby preventing their germination. In other words, the bacteria supernatant quite significantly suppressed the hyphal growth of the fungal species, Fusarium spp., as indicated by the varying percentage inhibitions respectively. Therefore, the *in vitro* analysis from this research showed that the bio-active agent can be used to control the pervasive plant pathogenic fungal species with outstanding result and therefore should be developed, register the product for commercial applications.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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