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Effect of bio-fungicide in the control of fungi isolated from selected farm soils across Nnamdi Azikiwe University, Awka

Achugbu Adaeze Nnedinma [1,*], Ibedu Oprah Chidinma [1], Orakwelu Ncheta Obianuju [2], Unachukwu Chibuzor [1] and Nwigbo Regina Chinelo [3]

[1] *Department of Botany, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Nigeria.*
[2] *Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Nigeria.*
[3] *Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Nnamdi Azikiwe University, Awka, Nigeria.*

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Abstract

Evaluating the effect of bio-fungicide in the control of fungi isolated from selected farm soils across Nnamdi Azikiwe University, Awka was carried out in this study. Soil samples were collected from 4 different farm sites across Nnamdi Azikiwe University main campus viz: farm site behind Bioscience auditorium (site A), farm site beside Unizik security block (site B), farm site beside GTB building (site C) and farm site opposite Elmeda hostel (site D). Identification of fungal species isolated were based on physical observation of the growth of the fungi and the structures that were observed under the microscope to reveal the type of hyphal growth, fruiting bodies and resting spores. Biofungicdes used in the control of fungi isolated from soil in this study were *Trichoderma virides* and *Bacillus subtills*. The effect of *Bacillus subtilis* on fungi isolated from soil samples in Farm A showed highest inhibition in *Fusarium oxysporum* (5.41±0.08a) and lowest in *Aspergillus niger* (2.73±0.04c). In Farm B, *B substilis* recorded the highest inhibition in *Rhizopus stolonifer* (6.33±0.10a) and lowest in *Aspergillus terreus* (2.09±0.05c). Farm C had highest inhibition for *B substilis* in *Aspergillus flavus* (5.05±0.08a) and lowest in *Fusarium oxysporum* (2.04±0.02b), while Farm D recorded the highest inhibition in *Aspergillus flavus* (5.20±0.10a) and least in *Fusarium oxysporum* (1.30±0.04c). Result on the percentage inhibition of fungi isolates from the cultured soil specimen showed that for *Rhizopus stolonifer*, *T. virides* had the highest percentage inhibition (5.23±0.02a) on soil collected from farm C while the least percentage inhibition was seen for farm B (3.33±0.10b). *B. subtilis* and *T. virides* have antifungal properties which may have been associated with the biocontrol activity observed in this study.

Keywords: *Trichoderma Viride*; Bacillus subtilis; Bio-Fungicides; Pathogens; Control; Soil

1. Introduction

The soil harbors many forms of microorganisms, majorly bacteria and fungi (Singh, 2019). The soil microbial community shows multiple levels of biological organization which encourages genetic variability as well as evenness among communities (Zheng, 2019). Living portion of the soil body includes small animals and microorganisms but it is generally considered that its microorganisms play the most important role. Soil is the largest source of micro-organisms and numerous varieties of microorganisms are living on the earth soil (Mya, 2020). Plant diseases represent a significant threat to global food security and agricultural sustainability. Fungal pathogens can cause massive agricultural losses by infecting and damaging crops, resulting in reduced yield, poor quality and economic losses (Savary *et al*., 2019). Traditional approaches to managing phytopathogenic fungi include resistant crop varieties, cultural practices, and chemical fungicides. While the latter can effectively manage fungal diseases, their frequent use negatively impacts the

* Corresponding author: Achugbu Adaeze Nnedinma.

environment and raises concerns regarding safety and potential harm to human health. In recent years, a growing interest in sustainable alternatives has emerged. Biological control using living microorganisms to manage pests and diseases in agricultural systems is among the most promising solutions (Suzanne, 2023).

Microbes with biocontrol activity, such as biocontrol fungi, are natural antagonists, and respective research contributes to developing safer and more environmentally friendly fungicide options. Understanding their modes of action, advantages, and applications can harness the power of biocontrol fungi for sustainable disease management. The "fungus" can exist as a plant pathogen, but it is also one of the microorganisms that is frequently utilized as a biological control agent of plant pathogens (Suzanne, 2023). Over the past decades, diverse attempts have been made in order to fight plant diseases through the development of synthetic fungicides (Soylu *et al*., 2010). Thus, various synthetic chemicals have been used as antifungal agents to inhibit the growth of plant pathogenic fungi. However, the widespread use of these chemicals has several drawbacks, including handling hazards, pesticide residues, cost, and threats to human health and environment (Bajpaia and Kang, 2012). Moreover, the intensive use of these chemicals creates imbalances in the microbial community, which may be un-favourable to the activity of the beneficial organisms and may also lead to the development of resistant pathogen strains (Marei *et al*., 2012), increasing environmental degradation. Owing to the limitations of chemical control measures, it seems appropriate to seek a more suitable control method (Chang *et al.,* 2007). Biological control appears as the most promising strategy, environmentally safe and cost-effective method for controlling the agricultural phytopathogens (Chang *et al.,* 2007; Ongena and Jacques, 2008; Yan *et al*., 2011).

Biological control of plant diseases is the suppression of populations of plant pathogens by living organisms (Heimpel and Mills, 2017). Amongst beneficial microorganisms, isolates can be selected which are highly effective against pathogens and can be multiplied on artificial media. Application of such selected and mass-produced antagonists in high densities once or several times during a growing season is called “augmentative biological control” (Eilenberg *et al*., 2001; Heimpel and Mills, 2017; van Lenteren *et al.,* 2018). In some cases, antimicrobial metabolites produced by selected microbial organisms are included in the product, and some products even contain only antimicrobial metabolites without living cells of the antagonist (Glare *et al*., 2012). Biofungicide is the general name given to microorganisms (microbial pesticides) and naturally occurring compounds that possess the ability to control plant diseases (biochemical pesticides) (Roger and Keinath, 2010). There are many fungi which are used as biofungicides like; *Aspergillus* spp, *Trichoderma* spp, *Bacillus* spp, *Pseudomonas chlororaphis* etc. *Trichoderma* spp. is the only group of fungi that have received much attention as a bio-control agent for plant pathogens including *Botrytis* spp., *Sclerotinia sclerotiorum, Cladosporium Colletotri cumacutatum* and *Fusarium oxysporum* (Elad, 2000; Patel and Saraf, 2017). Research on *Trichoderma* spp. as a biocontrol agent begun about four decades ago and several isolates of *Trichoderma harzianum* have since been tested for their antagonistic activities on *B. cinerea* over the years (Bogumił *et al*., 2013; Soliman *et al*., 2015). Microbial biological control agents use a great variety of mechanisms to protect plants from pathogens. Biocontrol fungi are among the naturally occurring microorganisms and can suppress plant diseases. They live in the plant rhizosphere, competing with pathogens for nutrients and space, and may colonise the plant roots. Several of these natural antagonists can produce substances that inhibit the growth and development of plant pathogens. They may also induce systemic resistance in plants, enhancing their defence against diseases (Butt *et al*., 2001). Among the fungal antagonists being researched and used as microbial fungicides, *Trichoderma* spp. are the most prominent. These biocontrol fungi are effective against a broad range of fungal plant pathogens, including *Fusarium, Rhizoctonia, Botrytis* and many more, and are considered opportunistic plant symbionts (Harman et al., 2004). With more research efforts being channeled into realizing the full potential of biofungicides. This study was aimed at evaluating the effect of bio-fungicide in the control of fungi isolated from selected farm soils across Nnamdi Azikiwe University, Awka.

2. Materials and methods

2.1. Study Area

The study was carried out at the Department of Botany Laboratory, Nnamdi Azikiwe University, Awka.

2.2. Collection of Soil Samples

Soil samples were collected from 4 different farm sites across Nnamdi Azikiwe University main campus viz: farm site behind Bioscience auditorium (site A), farm site beside Unizik security block (site B), farm site beside GTB building (site C) and farm site opposite Elmeda hostel (site D). Soil samples were collected with sterile hand trowel at 0-15cm depth. The soil samples were kept in sterile airtight polyethene bags and labelled properly for analysis.

2.3. Collection of Antagonistic Microorganism (Bio-fungicide)

2.3.1. Trichoderma virides

A leguminous plant (*Phaseolus vulgaris*) was planted into 2 separate containers using loamy soil (Figure 1) and cow dung (Figure2) as substrate. The plant was then allowed to sprout. After germination, a growth media was prepared by measuring 6.5g of SDA into 100ml of conical flask and then dissolved with water. The mixture was autoclaved for 20 minutes at 121°C. After autoclaving, the media broth was poured into a Petri dish and allowed to cool. Using a sterile forceps, some root hairs were picked from the sprouted plant from each substrate (for loamy soil and cow dung) and placed on the SDA culture media. The sealed petri dish was incubated for 72hours and checked for presence of *Trichoderma* spp. The presence of *Trichoderma* spp was identified with reference to the Manual of Fungal Atlases (Barnett and Hunter, 2000; Watanabe, 2002; Ellis *et al*., 2007).

2.3.2. Bacillus subtilis

One gram (1g) each of loamy soil and cow dung was measured into test tubes containing 5ml of sterile distilled water. The test tubes were covered with a foil and placed in a water bath at 80 °C for 10 mins, after which it was brought out and allowed to cool. The cooled samples were poured into a Petri dish using a micropipette and then agar broth was poured over it and allowed to gel. The Petri dishes were sealed using a masking tape and placed in the incubator at 37 °C for 48 hours. After 48 hours, presence of *Bacillus* spp. was identified with reference to the Manual of Fungal Atlases (Barnett and Hunter, 2000; Watanabe, 2002; Ellis *et al.,* 2007). *Trichoderma* and *Bacillus* spps. were sub-cultured to obtain pure cultures. The isolates were maintained on nutrient broth at 40C in the refrigerator until when required.

2.4. Isolation of Fungal Pathogen from Soil Samples

2.4.1. Preparation of Media

Potato Dextrose Agar (PDA) was used for isolation of fungal pathogens from the soil. The media was prepared according to the manufacturer's instruction. The media was autoclaved at 121°C for 20 minutes after which they were dispensed in Petri dishes and sealed to prevent contamination.

Isolation of fungi was done by agar dilution Figure method. One gram of each soil sample was added separately to 99 ml of sterile distilled water and shaken until it became homogeneous suspension. Next, 1ml of the suspension at 10^{-2} was added to test tubes containing 9 ml sterile distilled water and then shaken until a homogeneous suspension was obtained at 10^{-3} dilution. Further dilutions were made to 10^{-7}. To already molten media, Streptomysin 0.1 g /1L media of PDA and neomycin 0.01 g / 1 L of PDA was added to prevent the growth of bacteria. 10 ml of the PDA was dispensed into Petri dishes after which 1ml of the suspension from each serial dilution was added into the petri dishes before it solidified. The culture was incubated at room temperature for 7 days.

2.5. Identification of Fungal Isolates from Soil Samples

Isolated fungi were identified based on their micro and macro-morphological characteristics using standard taxonomic key used previously by Samson *et al*. (2010).

2.6. Determination of Fungicidal Activity Using the Agar Well Diffusion Method

The agar diffusion method as described by Esimone *et al*. (1998) and Osadebe and Ukwueze (2004) was adopted for this study. Standardized Nutrient broth culture of the test isolate containing approximately 107cells/ml organisms was used. 0.1 ml of the broth culture was introduced into sterile Petri dishes and 15 ml of molten nutrient agar poured into the Petri dishes. The contents were thoroughly mixed and allowed to solidify. Three holes each measuring 5.0 mm in diameter were made in each of the solid agar Figures using a sterile cork borer; the isolated fungi were inoculated into the holes separately using the inoculation loop. The inoculums were later sprayed with the antagonistic microorganism *T. virides* and *B. subtilis* separately; the preparations were labeled and incubated; observations were made for 7 days. At the end of the 7th day, the Figures were collected and the zones of growth inhibition were measured. The extent of inhibition was expressed in terms of the diameter of the inhibition zone as measured with a transparent metre rule. The effects of the biofungicide on the fungal pathogens were recorded.

2.7. Data analysis/experimental design

Data obtained from the study were subjected to Analysis of Variance (ANOVA) at 5% significance level. The experimental design for this study was a Randomized Complete Design with three replicates.

Figure 1 *Phaseolus vulgaris* in loamy soil

Figure 2 *Phaseolus vulgaris* in cow dung

3. Result

3.1. Identification of Fungal Isolates on Soil Samples Analyzed

Table 1 showed the several fungal species isolated. This was based on physical observation of the growth of the fungi on the soil specimen and the structures that were observed under the microscope to reveal the type of hyphal growth, fruiting bodies and if present, the types of resting spores.

Table 1 Identification of Fungi Isolates on Soil Samples Analyzed

Macroscopy	Microscopy	Organism
Black colony, powdery with diffused hyphae in media	Smooth-walled stipe, conidiophores Radiate and terminate in vesicle.	*Aspergillus niger*
Light green and powdery colonies	Rough and coarse aerial hyphae Present with simple sporangiophore Which are shaped globose	*Aspergillus flavus*
Whitish colony which later turns brownish	Brown-black, globose sporangia, rhizoids also present with zygospores	*Rhizopus stolonifer*
Rapid growing colony of cinnamon brown Surface	Uncoloured, smooth-walled, vesicle Is biseriate-spherical with globose Shaped conidia	*Aspergillus terreus*
Peach-violet coloured colony with profuse Growth	Microsporangia are relatively slender and thin walled with three to four Septa	*Fusarium oxysporum*

3.2. Percentage Inhibition of *Bacillus subtilis* on Fungi Isolated from Soil Sample

Table 2 Percentage Inhibition of *Bacillus subtilis* on Fungi Isolated from Soil Sample

Fungi Isolates	***B. subtilis***			
	Farm A	**Farm B**	**Farm C**	**Farm D**
Rhizopus stolonifer	4.41±0.08b	6.33±0.10a	3.23±0.02c	3.01±0.04d
Aspergillus flavus	4.38±0.04b	4.50±0.08b	5.05±0.08a	5.20±0.10a
Aspergillus niger	2.73±0.04c	2.23±0.04c	3.45±0.01a	2.84±0.00b
Aspergillus terreus	4.78±0.04a	2.09±0.05c	2.80±0.08b	2.66±0.04b
Fusarium oxysporum	5.41±0.08a	2.41±0.01b	2.04±0.02b	1.30±0.04c

Results show values of mean of triplicate analysis ± STD. Figures with different alphabets on the same column/row are significantly different ($P<0.05$)

Table 2 recorded the effect of *Bacillus subtilis* on fungi isolated from soil samples in Farm A, B, C and D. Farm A showed highest inhibition in *Fusarium oxysporum* (5.41 ± 0.08^{a}) and lowest in *Aspergillus niger* (2.73 ± 0.04^{c}). In Farm B, *B substilis*

recorded the highest inhibition in *Rhizopus stolonifer* (6.33±0.10[a]) and lowest in *Aspergillus terreus* (2.09±0.05[c]). Farm C had highest inhibition for *B substilis* in *Aspergillus flavus* (5.05±0.08[a]) and lowest in *Fusarium oxysporum* (2.04±0.02[b]), while Farm D recorded the highest inhibition in *Aspergillus flavus* (5.20±0.10[a]) and least in *Fusarium oxysporum* (1.30±0.04[c]).

3.3. Percentage Inhibition of Fungi Pathogens Isolated from Soil Sample by *T. virides*

Result on the percentage inhibition of fungi isolates from the cultured soil specimen in Table 3 showed that for *Rhizopus stolonifer*, *T. virides* had the highest percentage inhibition (5.23±0.02[a]) on soil collected from farm C while the least percentage inhibition was seen for farm B (3.33±0.10[b]). *Aspergillus flavus* had the highest percentage inhibition (6.20±0.10[a]) in soil collected from farm D while the least percentage inhibition for *A. flavus* was seen at farm A(3.38±0.04[d]). *Aspergillus terreus* and *Fusarium oxysporum* had the highest percentage inhibition of (2.74±0.06[a]) and (4.41±0.08[a]) respectively for farm A while the least percentage inhibition the fungal organisms were (1.89±0.05[c]) and (2.30±0.04[c]) in farm B and D respectively. *Aspergillus niger* had the highest percentage inhibition (3.53±0.04[a]) at farm A, while the least percentage inhibition was (2.30±0.01[b]) at farm C. There was significant difference in the dilution treatments of the antagonistic microorganism and between the fungi isolates from the soil samples ($p<0.05$).

Table 3 Percentage Inhibition of Fungi Pathogens Isolated from Soil Sample by *T. virides*

Fungi Isolates	***T. virides***			
	Site A	**Site B**	**Site C**	**Site D**
Rhizopus stolonifer	3.41±0.08b	3.33±0.10b	5.23±0.02a	5.01±0.04a
Aspergillus flavus	3.38±0.04d	4.00±0.08c	5.22±0.06b	6.20±0.10a
Aspergillus niger	3.53±0.04a	3.30±0.04a	2.30±0.01b	2.64±0.00b
Aspergillus terreus	2.74±0.06a	1.89±0.05c	2.10±0.08b	2.26±0.04b
Fusarium oxysporum	4.41±0.08a	3.41±0.01b	3.04±0.02b	2.30±0.04c

Results show values of mean of triplicate analysis ± STD. Figures with different alphabets on the same column/row are significantly different ($P<0.05$)

4. Discussion

In this study, *Rhizopus stolonifer, Aspergillus flavus, Aspergillus niger, Aspergillus terreus* and *Fusarium oxysporum* were fungi associated with soil sampled in this study. This agrees with previous researchers that the soil harbors many forms of microorganisms, majorly bacteria and fungi (Singh, 2019). The fungi isolated from the soil samples which were inoculated with *B. subtilis* and *T. virides* showed obvious changes which may be due to the inhibition of the various fungi. This observation is similar to those of other researchers; Ferreira *et al.* (1991); Schmid *et al*. (1997); Okigbo (2002) all used *B. subtilis* and/or *T. virides* on grapevine to control *Eutypalata*. More so, Litchi (*Litchi chinensis*) fruit treated with a culture solution of *B. subtilis* and *T. virides* were able to maintain a high quality for a longer period of time, with only 4.2% of rotten fruits being recorded (Jiang *et al*., 2003). Fungi have been effectively controlled by artificially applying antagonistic microorganisms (Oparaocha and Okigbo, 2003). *Bacillus subtilis* and *Trichoderma virides* have been observed to exhibit anti-fungal activity against many plant pathogenic fungi (Kim *et al*., 1995; Okigbo, 2002). These studies have proved that antagonistic microorganism such as *T. virides* and *B. subtilis* could be introduced onto the surface of plants, thus, can limit rot in stored products.

Results from this study showed rapid colonization of sites by *T. viride s*and *B. subtilis* before the development of fungal hyphae and on the other hand, the antagonism of these bacteria could have been the cause of the suppression. Reduction of rot in tubers inoculated *in vivo* with *Trichoderma viride* has also been reported (Okigbo and Ikediegwu, 2000). Antagonism by *T. virides* and *B. subtilis* was high in *R. stolonifer* and *A. flavus*; this could be attributed to the fact that the sporulation and germination of spores of *R. stolonifer* and *A. flavus* took longer time than the other fungi tested. Ekundayo and Haskin, (1969) observed that most fungi species require continuous light intensity for faster sporulation, hence, this delay may allow more time for the antagonistic microorganism to establish itself fully.

According to Ferreira *et al*. (1991), *B. subtilis* and *T. virides* has been implicated in the production of antibiotics. They have antifungal activities which may have been associated with the biocontrol activity observed in this study. Eshita *et al*., (1995), also stated that *B. subtilis* and *T. virides* has shown antagonism against *Monilia fructicola* by the production of an antibiotic and an antifungal substance identified as iturin from *B. subtilis* and *T. virides*.

5. Conclusion

The use of antagonistic microorganism such as *B. substilis* and *T. virides* as a biocontrol agent against fungal pathogens may be an economically viable way of suppressing postharvest rot. This procedure holds a future as an effective way to control post-harvest diseases since the long-lasting and highly efficient activity of a single, simple application of a bacterium to control rot is cost effective for developing countries where peasant farmers lack the finance to support prolonged spraying schedule with sophisticated equipment.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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