ISSN - 2277 - 0593

Journal of Natural Sciences, Engineering and Technology

IN VITRO AND *IN VIVO* INVESTIGATION OF PLANT GROWTH PROMOTING AND BIOPROTECTING RHIZOBACTERIA ON EARLY MAIZE PLANT GROWTH

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ABSTRACT

The use of soil microorganisms as biofertilizers and biocontrol agents in agriculture is important in maintaining the soil ecological balance and sustainability of agroecosystems. Rhizobacteria were isolated and identified from the rhizosphere of matured field grown with maize (*Zea mays* L.) at the field of Federal University of Agriculture, Abeokuta teaching and research farm. The effect of inoculation of maize seeds (Oba super 2) with rhizobacteria on growth promotion and bioprotection against *Fusarium moniliforme* were investigated in vitro and in pot experiment in a screen house. Eight treatments representative of at least all the different genera of the isolated rhizobacteria and an uninoculated control in both cases were used. Treatments were replicated 3 times in a completely randomised design. Data obtained were subjected to analysis of variance (ANOVA) and means separated using Fisher's LSD test ($P<0.05$). Fifty four rhizobacteria were isolated and identified consisting of *Bacillus cereus* (22%), *Pseudomonas aeruginosa* (2%), *Micrococcus acidophilus* (26%), *Proteus morganii* (11%), *Staphylococcus aureus* (9%), *Streptococcus faecium* (28%) and *Staphylococcus parasiticus* (2%). The in-vitro growth promotion study revealed that there was no significance difference among treatments in plant height and root mass. However, *Pseudomonas aeruginosa* and *Bacillus cereus* significantly increased the plant height length (0.13-0.21) and root mass (0.10-0.12) above control. The in-vitro biocontrol screening revealed that none of the treatments except *Pseudomonas aeruginosa* and *Bacillus cereus* showed inhibition (2.0-2.5mm) against *Fusarium moniliforme*. Results from the screen house study showed that treatment significantly increased the plant height and root mass except *Staphylococcus aureus* and *Staphylococcus parasiticus* and only *Bacillus cereus* and *Pseudomonas aeruginosa* increased only the root mass. *Bacillus cereus, Pseudomonas aeruginosa, Micrococcus acidophilus, Proteus morganii* greatly reduce the recovery of the pathogen (*Fusarium moniliforme*) from infected seeds whereas *Streptococcus faecalis* was slightly effective. It was observed from the study that only *Pseudomonas aeruginosa* and *Bacillus cereus* had traits of plant growth and bioprotection and as such they have prospects for use as plant growth promoting and as biocontrol.

Keywords: Rhizobacteria, In-vitro, In-vivo, Bioprotecting, Growth promoting

INTRODUCTION

Plant growth in agricultural soils is influenced by biotic and abiotic factors. Farmers use physical and chemical approaches to manage the soil. To improve crop yield, the application of microbial products is less common. Plant growth promoting rhizobacteria (PGPB) include bacteria that colonise the root of plants following inoculation into seeds and enhance plant growth. The impor-

tances of these rhizobacteria are for the maintenance root health, nutrient uptake and tolerance of environmental stress (Cook, 2002). The mechanism by which these PGPB increase crop performance is either suppression of plant disease (Bioprotection), improved nutrient acquisition (Biofertilization) and phytohormone production (Biostimulation) Ping and Bowland, 2004.

Application of PGPR for control of fungal pathogens in green house systems shows considerable promise (Paulitze abd Belanger, 2001). One approach for selection of organisms with the potential to control soil borne phytopathogens is to isolate from soil that are suppressive to that pathogen (Weller *et al.,* 2002).

Fusarium moniliforme is a major cause of stalk rot, ear rot, root rot and seedling blight of maize (Windels, 1994). Fungicide used as seed dressing protect the seeds but not the roots from fungal infection (Hebber *et al.,* 1992a) and they do not improve nutrient acquisition hence use of biological control using root associated antagonistic bacteria may provide a means of controlling diseases of crops and improving plant growth by improving nutrient acquisition and phytohormone.

The objective of this study was to isolate PGPB from maize and the effect of the isolated PGPB on seed borne pathogen (*Fusarium moniliforme*), growth stimulation and seedling emergence of maize

MATERIALS AND METHODS

Sample Collection

Five maize plants were selected at random from the maize field of the teaching and research farm of the Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. The soil adhering to the roots (root zone soil) were shaken into sterile bottle and transported to the laboratory immediately for processing.

Isolation of Rhizobacteria from the Rhizosphere of maize plants

Ten (10) grams of soil collected from the rhizobacteria of maize was weighed and placed inside sterile 250 ml Erlenmeyer flask containing 90 ml of sterile phosphate buffer, the flask was shaken well to homogenise the suspension. The samples were serially diluted and appropriate dilutions were plated. The petri dishes were then incubated at 28 ^{0}C in an incubator for 24 h thereafter, bacterial growth was observed and number of colonies per plates was recorded.

Isolation of Fusarium moniliforme from the diseased plant

Fusarium moniliforme was isolated from diseased plant of maize. The slant of *Fusarium moniliforme* was stored on Potato dextrose agar (PDA) at 4 ^{0}C in a refrigerator for further studies.

Identification of isolated rhizobacteria

The identification of isolated rhizobacteria was done based on colonial morphology, and biochemical characterisation according to the protocol found in Bergey's manual of determinative bacteriology (Holt *et al.,* 1994).

In vitro assay for plant growth promotion

In vitro assay for growth promotion was done as described by Luz (2001). The maize dried seeds (Oba Super 2) were surface sterilised by dipping the seeds in 1 % v/v sodium hypochlorite (NaOCl) for 3 min in a 500 ml conical flask and rinsed several times with sterile distilled water. Colonies of each rhizobacteria were grown on nutrient agar

for 24 h at 24 ^{o}C. Bacterial cells were removed from the surface of the culture medium with a sterile brush and placed on sterile distilled water. The concentration of each rhizobacteria was approximately 10^{7} cfu/ml. A suspension was then applied by dipping the seeds for 3 min and allowing them to dry at room temperature for 24 h. Non treated seeds were embedded in sterile distilled water for 3 min and allow to dry. For laboratory experiment each treatment was replicated 3 times at (30 grains, ten grains per plate). The seeds were dipped in 24 h nutrient broth culture in conical flask containing each of the seven different species of the isolated Rhizobacteria respectively for 3 min and allowed to dry on sterile filter paper at room temperature. Fifty seeds were then transferred into previously sterilised petri dishes containing Whatmann no. 1 filter paper and moistened with sterile distilled water, kept in a growth chamber at room temperature for 7 days. The seedlings were examined for plant height; root mass, number of germinated seeds (% seed germination).

In vitro assay for bioprotection

The plant protection assay was carried out according to Bacon et al (2001) and Hebbar *et al*. (1992c). Five millilitre plug of *Fusarium moniliforme* was taken from the outer margin of a 5 day old culture and placed at the centre of petri dish streaked with 24 h old culture of each Rhizobacteria test strain on a PDA plate. The plates were incubated at 30 ^{o}C for 5 days thereafter the plates were observed for growth and inhibition zones were recorded.

Screen house assay for plant growth promotion (In vivo experiment)

The growth promotion experiment was done in the growth chamber using the same treatment as its laboratory counterpart. Treatments were applied on non infected seeds of maize. The experimental design was completely randomised with 3 replicates of 10 seeds sown spaced 10 cm apart in an autoclaved soil in aluminium trays. Plant height and root mass was evaluated 30 days after planting. Data were subjected to analysis of variance and means were separated by Fisher's LSD test ($P< 0.05$).

Screen house assay for Bioprotection (In vivo experiment)

The screen house assay for bioprotection followed the same method as the screen house assay for plant growth promotion except that the maize seeds were dipped for 3 min in the suspension of rhizobacteria and were allowed to dry at room temperature for 24 h. Thereafter the seeds were also dipped in the *Fusarium moniliforme* suspension for 3 min and also allowed to dry before sowing the seeds in sterilised soil in aluminium trays. The experimental design was also completely randomised design (CRD) with 3 replicates of 10 seeds per tray. At the end of 30 days plant height, root mass and percentage fungus recovery were recorded.

RESULTS AND DISCUSSION

The following rhizobacteria were isolated from the rhizosphere of maize plant. They are Bacillus *cereus, Pseudomonas aeruginosa, Micrococcus acidophilus, Proteus morganii, Staphylococcus aureus, Streptococcus faecalis* and *Staphylococcus parasiticus.* Five of the rhizobacteria species are gram positive and two species are gram negative. The frequency of occurrence of the isolated bacteria showed that *Streptococcus faecalis* has highest population (28%) followed by *Micrococcus acidophilus* (26%) and the least population was recorded in Staphylococcus *parasiticus (*2%) (Table 1). This contrast the finding of Lambert *et al.* (1987) who studied

the ecology of maize rhizosphere and reported that fluorescent *Pseudomonas* represented the highest bacterial population. This may be due to abiotic factors, environmental condition, soil type and rhizocompetence of the organisms. It could also be due to biotic factors such as niche exclusion, agricultural practices, isolation technique and media used (Bennet and Lynch, 1981 and Hebber *et al.,* 1992a).

Table 1: Frequency of occurrence of isolated Rhizobacterial species

Isolated bacterial species	Frequency of occurrence (%)
Bacillus cereus	22
Pseudomonas aeruginosa	2
Micrococcus acidophilus	26
Proteus morganii	11
Staphylococcus aureus	9
Streptococcus faecalis	28
Staphylococcus parasiticus	2

The *in vitro* growth promotion experiment indicated that all the treatment had 100% seed germination. *Pseudomonas aeruginosa* and *Bacillus cereus* increased plant height and root mass when compared with the control although there were no significant differences among the treatments in the plant height and root mass of the maize seedling (Table 2). The reason for no significant differences among treatments in plant height and root mass of the maize seedling may be probably because the maize seedlings were incubated for 7 days. This finding is in line with the findings of Glick *et al.* (1995) where canola seeds inoculated with *Pseudomonas* spp under gnotobiotic condition *in vitro* in growth pouches increased the root length of the seedlings.

Microorganisms that grow in the rhizosphere are ideal for use as biocontrol agents since the rhizosphere provides the frontline defence zone for roots against attack by pathogens (Nahad, 2008). Bioprotection by antagonism against phytopathogen microorganisms employed by plant growth promoting and bioprotecting rhizobacteria had been reported to be effective in the control of different phytopathogens (Colbert *et al.,* 1993; Cattelan *et al.,* 1999; Bacon et *al.,* 2001; Luz, 2001).

Table 2: In-vitro effect of inoculation of maize seeds with Rhizobacteria on % seed germination, plant height and root mass of maize seedling

Treatments	% seed germination	Plant height (cm)	Root mass(g)
Bacillus cereus	100	5.83	1.13
Pseudomonas aeruginosa	100	5.91	1.15
Micrococcus acidophilus	100	3.20	0.65
Proteus morganii	100	5.22	0.84
Staphylococcus aureus	100	5.03	1.43
Streptococcus faecium	100	5.06	0.86
Staphylococcus parasiticus	100	5.22	1.17
Control	100	5.70	1.03
LSD (P<0.05)		3.32	0.28

The *in vitro* antagonism carried out in this study showed that only *Pseudomonas aeruginosa* and *Bacillus cereus* recorded inhibition of 2.0-2.5 cm against *Fusarium moniliforme* under laboratory condition (Table 3). The inability of other rhizobacteria spp to inhibit growth of *Fusarium moniliforme in vitro* could be due to the fact that they lack PGPR traits such as biofertilisation, phytostimulation and suppression of plant diseases. It could also be that the rhizobacteria were not producing sufficient inhibitory substances and fungal cell wall lysine enzymes (Palumbo *et al.*, 2005).

Table 3: *In vitro* antagonistic effect of Rhizobacteria against *Fusarium moniliforme*

Bacteria	Zone of inhibition (mm)
Bacillus cereus	2.5
Pseudomonas aeruginosa	2.0
Micrococcus acidophilus	_
Proteus morganii	_
Staphylococcus aureus	_
Streptococcus faecium	_
Staphylococcus parasiticus	_
Control	_

The growth promoting screen house experiment also recorded 100% seed germination for all the treatments (Table 4). The plant height was significantly increased above the control except *Staphylococcus aureus* and *Staphylococcus parasiticus* but *Pseudomonas aeruginosa* recorded the highest plant height. *Pseudomonas aeruginosa, Bacillus cereus, Micrococcus acidophilus, Proteus morganii* and *Streptococcus faecalis* significantly increased the root mass above control. The reason for increased in plant height and root mass in *Pseudomonas aeruginosa, Bacillus cereus, Micrococcus acidophilus, Proteus morganii* and *Streptococcus faecalis* above control may be as a result of soil mineralisation and phytohormone production described by several plant growth promoting rhizobacteria (Kloepper, 1993). Data from screen house indicate that non treated seeds (control), Staphylococcus aureus and Staphylococcus parasiticus were severely contaminated showing 100% pathogen recovery. *Pseudomonas aeruginosa, Bacillus cereus, Micrococcus acidophilus* and, *Proteus morganii* greatly reduced pathogen recovery (0-30%) from infected seeds. Whereas *Streptococcus faecalis* was found slightly effective (Table 5).The mechanisms of biocontrol as reported by Luz (2001) is antibiosis, competition, niche exclusion, pathogen adherence, inactivation of fungus propagule stimulant present in the seed exudates. This result agreed with one of the mechanisms of biocontrol (antibiosis) as reported by Luz (2001).

Table 4: Effect of Rhizobacteria on % seed germination, plant height and root mass of maize 30 days after planting in the screen house

Treatment	% seed germination	Plant Height(cm)	Root Mass (g)
Bacillus cereus	100	98.20	6.67
Pseudomonas aeruginosa	100	83.05	6.27
Micrococcus acidophilus	100	77.10	4.90
Proteus morganii	100	76.60	4.97
Staphylococcus aureus	100	68.50	4.50
Streptococcus faecalis	100	78.30	5.04
Staphylococcus parasiticus	100	69.10	4.27
Control	100	75.90	4.67
LSD (P<0.05)		11.39	2.00

Table 5: Antagonistic effect of Rhizobacteria against *Fusarium moniliforme* on the percent (%) seed germination, plant height and root mass of maize 28 days after planting in the screen house

Treatment	Pathogen recovery from seeds (%)	Plant height (cm)	Root mass(g)
Bacillus cereus	0	77.06	5.43
Pseudomonas aeruginosa	0	78.82	5.60
Micrococcus acidophilus	3.0	76.83	4.90
Proteus morganii	3.0	75.06	4.10
Staphylococcus aureus	100	61.37	4.18
Streptococcus faecium	90	69.37	4.70
Staphylococcus parasiticus	100	60.30	4.60
Control	100	61.73	4.93
LSD (P<0.05)		13.53	1.91

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(Manuscript received: 11th June, 2012; accepted: 27th June, 2012).