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Growth Promotion and Phytopathogen Inhibition Potentials of Lemon Grass (*Cymbopogon citratus*) Endophytic Bacteria

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Abstract

Fresh and apparently healthy leaves and roots of lemon grass were collected and surface - sterilized using 70% (v/v) Ethanol, 3% (v/v) sodium hypochlorite solution and sterile distilled water. Isolation of endophytic bacteria was achieved using culture technique while, characterization was done based on morphological, biochemical and microscopic characteristics. Growth promotion potentials of some selected isolates were tested using tomato and millet seeds. Similarly, antagonistic potentials against *Fusarium oxysporum* were evaluated. A total of 16 endophytic bacteria were isolated and identified as *Bacillus* spp (3 isolates), *Escherichia coli* (1 isolate), *Klebsiella pneumoniae* (3 isolates), *Micrococcus* spp (3 isolates), *Pseudomonas* spp (1 isolate), *Rhizobium* spp (2 isolates) and *Staphylococcus aureus* (3 isolates). Growth promotion test showed that, only *K. pneumoniae* significantly improved ($P < 0.05$) the germination time, germination percentage, shoot length and fresh weight of tomato seeds. None of the bacteria showed evidence of improving any of the parameters of germination of millet seeds. All the endophytic bacteria significantly inhibited ($P < 0.05$) the growth of *F. oxysporum. S. aureus* yielded the largest (21.30 mm) while, *Bacillus cereus* yielded the smallest (17.2 mm) zone of inhibition. Moreover, all the isolates especially *S. aureus* significantly inhibited ($P < 0.05$) the growth of *F. oxysporum*. In conclusion, Lemon grass harbours a variety of endophytic bacteria some of which showed potentials of enhancing the emergence and development of tomato seedling, and also have antagonistic activity against *F. oxysporum.*

Keywords: Endophytic bacteria, Lemon grass, *Fusarium oxysporum*, Growth promotion, Biocontrol.

1. Introduction

The recent surge in the need to exploit the health benefits that microbial inoculants may give to plants as well as, the desire to reduce the use of chemicals due to health and ecological concerns, has fuelled interests in studying an array of bacteria and fungi called "Endophytes". Hallmann *et al.* (1997) defined endophytic bacteria as all bacteria that can be detected inside surface-sterilized plant tissues or extracted from inside plants and having no visibly harmful effect on the host plants. This definition includes internal colonists with apparently neutral behaviour as well as symbionts. It also includes bacteria, which migrate back and forth between the surface and inside of the plant during their endophytic phase.

Bacterial endophytes are found in a variety of plants, ranging from herbaceous plants, such as maize and beet, to woody plants (Ryan *et al*., 2007). Bacteria belonging to the genera *Bacillus* and *Pseudomonas* are easy to culture, and the cultivation-dependent study has identified them as frequently occurring endophytes (Seghers *et al*., 2004). *Bacillus* sp. and *Enterobacter* sp. were found in maize (Surette *et al*., 2003; McInroy and Kloepper, 1995), *Klebsiella pneumoniae* in soybean (Kuklinsky-Sobral *et al*., 2004), *Rhizobium leguminosarum* in Rice (Yanni *et al*., 1997), *Rhizobium* in carrot and rice (Surette *et al*., 2003), *Escherichia coli* in Lettuce (Ingham *et al*., 2005). Indeed, numerous reports have shown that endophytic microorganisms can have the capacity to control plants (Sturz *et al*., 1997; Duijff *et al*., 1997; Krishnamurthy and Gnanamanickam, 1997), insects (Azevedo *et al*., 2000) and nematodes (Hallmann *et al.* 1997, 1998). In some cases, they can also accelerate seedling emergence, promote plant establishment under adverse conditions (Chanway, 1997) and enhance plant growth (Bent and Chanway, 1998).

Cymbopogon citratus, commonly known as the Lemon grass, is a tropical herb that is popular in south East Asia and Africa. The plant has a plenty of medicinal uses, prominent among which is its application as antihelmintic, aphrodisiac, appetizer and laxative. It is used in Ayurvedic medicine in the treatment of epilepsy, leprosy and bronchitis (Parrotta, 2001).

Strobel *et al*. (2004) reported that, close to 300,000 different plant species exist on the earth each of which hosts one or more endophytes. Only a fraction of these plants have been fully explored relative to their endophytic biology. In view of the medicinal and other uses of *C. citratus*, a study on its endophytic microorganisms would be of great impact. In an earlier study, Deshmukh *et al.* (2010) reported 24 different fungal species belonging to 21 genera isolated from the leaves and rhizomes of *C.*

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citratus. To the best of our knowledge, no previous studies have been done regarding the endophytic bacteria of the same plant, hence the need for this study. The current study, therefore, aims at evaluating the plant growth promotion and biocontrol potentials of endophytic bacteria isolated from *C. citratus*.

2. Materials and Methods

2.1. Sample Collection

For the isolation of endophytic bacteria, fresh and apparently healthy leaves and roots of *C. citratus* were collected using a sterile scissors, during the rainy season from the Botanical Garden of the Department of Biological Sciences Bayero University Kano Nigeria. All samples were immediately transported in sterile bags to the Microbiology laboratory of Bayero University Kano for analysis.

2.2. Sample Pre-Treatment and Surface Sterilization

Upon the arrival of the samples at the laboratory, they were processed immediately without any delay as follows: The leaves and roots of the plant were washed separately under running tap water to remove adhering soil particles, and the majority of microbial surface epiphytes. The samples were then subjected to surface sterilization procedure as follows: An initial wash in sterile distilled water to remove adhering soil particles, 1 minute immersion in 70% (v/v) ethanol, followed by a 2 minute immersion in 3% (v/v) sodium hypochlorite and finally, a three times rinse in sterile distilled water (Hallman *et al*., 1997).

2.3. Isolation of Endophytic Bacterial Isolates

To target a wide range of endophytes, five different isolation media were used, i.e., Yeast extract sucrose agar (Yeast extract 4.0 g; Sucrose 20.0 g; KH_2PO_4 1.0 g; $MgSO_4$ 0.5 g; Agar 15.0 g in 1.0 L distilled water, pH adjusted to 6.2 ± 0.2 and autoclaved at 121 ^oC for 15 minutes) which is selective for the isolation of *Rhizobium* species, Nutrient agar (Oxoid), MacConkey agar (Oxoid), Nutrient broth (Oxoid), yeast extract agar (Sigma-Aldrich) and Brain heart infusion agar (Oxoid).

The isolation followed the protocol of Sheng *et al*. (2008) with some modifications. Each of the collected *C. citratus* samples was aseptically homogenized in a sterile blender (Panasonic MS-337N) and a three-fold serial dilution was carried out after which, 1 mL aliquot from each dilution was inoculated in triplicates on the various growth media using pour plating method. The cultures were then placed in an incubator (Gallenkamp series) at room temperature for 48 hours. Individual colonies were picked and streaked on fresh culture media for purification to generate pure cultures. Control cultures of the surface-sterilized but unhomogenized leaves of the plant were also prepared and incubated at similar conditions with the test culture plates.

2.4. Morphological and Biochemical Characterization of the Bacterial Isolates

Cell morphology of the pure cultures obtained was determined by the Gram staining method (Bartholomew, 1962). Biochemical tests, such as catalase, coagulase, oxidase, indole, methyl red, Voges-Proskauer urease activity, citrate utilization, cellulose hydrolysis, starch hydrolysis and triple sugar iron tests were done according to the procedures described by Cappuccino and Sherman (2000). Endospore staining and capsule staining were also carried out.

2.5. Evaluation of Plant Growth Promoting Effects of the Endophytic Bacteria on Tomato and Millet Seeds

A total of nine isolates were randomly selected and tested using Petri plate trials in order to evaluate their growth promotion effects on tomato and millet seedlings. A loopful growth of each bacterial isolate was inoculated in 10 mL of Luria-Bertani (LB) broth **(**HIMEDIA**)** in a test tube, and incubated for 24hrs. Tomato and millet seeds were obtained from the Department of Crop Protection, Bayero University Kano. The seeds were surface-sterilized by immersing in 70% ethanol (1 minute) and 2% sodium hypochlorite (2 minutes) and then rinsed thoroughly in sterile distilled water. The surface-sterilized seeds were added to the inoculated LB medium (ten per test tube), and incubated for 24 hrs to allow bacterial penetration. Another set of ten surface sterilized seeds of tomato and millet each, were inoculated in sterile LB broth for 24 hrs in order to serve as negative control. The culture fluid was then aseptically decanted and the treated seeds from the test tubes were then planted in Petri dishes layered with moistened cotton wool. Seedlings were grown at room temperature with regular watering. After 10 days of nursing, growth parameters, such as height, fresh weight, number of leaves of the seedlings, and time of germination of the seeds, were both measured. The test was conducted in triplicates as adopted by Ji *et al*. (2014).

2.6. Evaluation of Antagonistic Effect of the Endophytic Bacteria against F. Oxysporum

Fusarium oxysporum, a soil-borne fungal pathogen of plants was collected from the culture collections of the Plant Biology Department of Bayero University Kano. The identity of the fungus was authenticated by sub-culturing on potato dextrose agar (BIOMARK Laboratories). The culture was incubated at room temperature for five days. Morphological characteristics and reverse pigmentation of the fungus on PDA were noted and recorded. A sterile needle was used to pick a small portion of the mycelium of the test fungus, and this was transferred on to a drop of lacto phenol cotton blue on a clean glass slide. The preparation was then carefully emulsified so as to disperse the inoculum. A cover slip was placed carefully and finally; the preparation was viewed under the microscope using × 100 oil immersion objectives. Features, such as the nature of hyphae, spore types and spore attachment, were observed and recorded. Final authentication was done by making reference to Benson (1998). A needle-full mycelial mat of freshly cultured *F. oxysporum* was picked using a straight wire loop, and placed on one side of a Petri dish containing PDA and the fresh culture of the endophytic bacterial isolate was streaked on the other side of the plate. A minimum of 35 mm separation was maintained between the organisms. The PDA plates were incubated at 28^o C for 7 days. The antagonistic effects of the bacterial endophytes against the fungus were confirmed by inhibition zones

formed between the bacterial endophytes and the fungus. A PDA plate inoculated with *F. oxysporum* only, served as the control. The test was carried out in triplicates (Ji *et al*., 2014).

2.7. Statistical Analysis

All data obtained (in triplicates) were tested for statistical significance using the Statistical Package for Social Science (SPSS) version 21.0. General linear model multivariate analysis was used to test the data obtained from the germination tests of tomato and millet seeds and means were separated using Least Significant Difference (LSD). Data from the antagonistic tests of the endophytic bacteria on *F. oxysporum* were tested using one-way ANOVA. Means were separated using LSD. All analyses were carried out at 5% level of significance.

3. Results

3.1. Occurrence and Morphological Characteristics of Endophytic Bacteria of Lemon Grass

The various endophytic bacteria and their frequency of occurrence are represented in Table1. A total of 16 endophytic bacteria were isolated. Among these, 10 (62.5%) were isolated from the roots, while the remaining 6 (37.5%) were isolated from the leaves of the plant. The bacteria belong to the genera *Bacillus*, *Escherichia*, *Klebsiella*, *Micrococcus*, *Pseudomonas*, *Rhizobium* and *Staphylococcus*.

Table 1. Distribution of Endophytic Bacterial Genera in the Roots and Leaves of Lemon Grass

Bacterial isolates	Root	Leaves
Bacillus	2	1
Escherichia	1	0
Klebsiella	2	1
Micrococcus	2	1
Pseudomonas	0	1
Rhizobium	2	0
Staphylococcus	1	2
Total	10 (62.5%)	6 (37.5%)

3.2. Growth Promotion Potentials of the Endophytic Bacteria

This was carried out to evaluate the potentials of the isolates in enhancing tomato and millet seeds germination. The effects of the bacteria on the germination of tomato seeds are presented in Table 2. Statistical analysis of the result showed significant difference between the mean values of all the germination parameters when tested jointly ($P < 0.05$). A separate ANOVA conducted between subjects showed significant difference ($P < 0.05$) between the mean values of germination time, germination percentage, length of shoot, fresh weight. No significant difference ($P > 0.05$) was observed between the mean values of the number of leaves. Multiple comparison tests showed that, only the mean germination time of *K. pneumoniae* (2.0 days), and *E. coli* (3.3 days) were shorter than the corresponding value yielded by the control (3.7 days). However, it is only the mean germination time of *K. pneumoniae*-treated seeds that was statistically different ($P < 0.05$) from all others including the control. Similarly, the germination percentage of 100 and 96.7 were recorded for *K. pneumoniae*, and *E. coli*-treated seeds, respectively. As with germination time, only the germination percentage of *K. pneumoniae*-treated seeds was statistically greater ($P < 0.05$) than that of all others, including the control. For shoot length, only *K. pneumoniae*-treated seeds (4.80 cm) yielded better than the control (4.20 cm). The values were also found to be statistically different ($P < 0.05$). The mean fresh weight yielded by *K. pneumoniae*-treated seeds (0.050 g) and *S. aureus* (0.040 g) were greater than the value yielded by the control (0.033 g). However, only the mean fresh weight of *K. pneumoniae*-treated seeds was statistically different ($P < 0.05$) from that of the control.

The result of the germination test of millet seeds, as presented in Table (3), show the control yielding the mean germination time, mean germination percentage, number of leaves and shoot length of 2.6 days, 45.3%, 1 leaf, and 4.0 cm, respectively. None among the endophytic bacteria-treated seeds yielded better results in all the parameters tested. However, the mean fresh weight results showed yields of 0.040, 0.033 and 0.033 g from *E. coli*, *K. pneumoniae*, *and Micrococcus* spp treated seeds, respectively, and these were higher than the fresh weight of 0.030 g yielded by the control. However, the values were not significantly different ($P < 0.05$) from one another and the control.

Table 2. Effects of Endophytic Bacteria on Tomato Seeds Germination

Endophytic Bacterium	Germination Time (Days)	Germination Percentage	Number of Leaves	Length of Shoot(cm)	Average Fresh Weight(g)
Bacillus subtilis	5.5 ± 0.29	90 ± 0.00	2 ± 0.00	3.4 ± 0.31	0.030 ± 0.00
Bacillus cereus	4.0 ± 0.00	46.7 ± 3.33	2 ± 0.33	4.2 ± 0.10	0.030 ± 0.00
Escherichia coli	3.3 ± 0.33	96.7 ± 3.33	2 ± 0.00	3.1 ± 0.03	0.030 ± 0.00
Klebsiella pneumoniae	2.0 ± 0.00	100 ± 0.00	2 ± 0.33	4.8 ± 0.42	0.050 ± 0.00
Micrococcus spp	6.3 ± 0.33	53.3 ± 3.33	2 ± 0.00	3.1 ± 0.35	0.020 ± 0.00
Micrococcus luteus	7.0 ± 0.33	53.3 ± 3.33	2 ± 0.00	4.2 ± 0.09	0.031 ± 0 .00
Rhizobium spp	4.3 ± 0.33	26.7 ± 3.33	1 ± 0.00	2.7 ± 0.15	0.022 ± 0.00
Staphylococcus aureus	6.3 ± 0.33	63.3 ± 3.33	2 ± 0.33	4.2 ± 0.20	0.040 ± 0.00
Control	3.7 ± 0.33	93.3 ± 6.67	2 ± 0.33	4.2 ± 0.17	0.033 ± 0.00

Results are values of three replicates ± the S.E (Standard error)

Table 3. Effects of the Endophytic Bacteria on Millet Seeds Germination

Endophytic Bacterium	Germination Time (Days)	Germination Percentage	Number of Leaves	Length of Shoot(cm)	Fresh Weight(g)
Bacillus subtilis	8.4 ± 0.18	23.6 ± 0.89	1.0 ± 0.00	3.6 ± 0.03	0.020 ± 0.02
Bacillus cereus	8.3 ± 0.10	24.0 ± 0.58	1.0 ± 0.00	2.9 ± 0.03	0.030 ± 0.00
Escherichia coli	5.4 ± 0.10	34.3 ± 1.20	1.0 ± 0.00	3.7 ± 0.05	0.040 ± 0.00
Klebsiella pneumoniae	6.3 ± 0.10	34.0 ± 1.00	1.0 ± 0.00	3.7 ± 0.03	0.033 ± 0.00
Micrococcus spp	7.03 ± 0.03	34.0 ± 2.10	1.0 ± 0.00	3.1 ± 0.01	0.033 ± 0.00
Micrococcus luteus	3.5 ± 0.00	21.3 ± 1.33	1.0 ± 0.00	1.2 ± 0.00	0.010 ± 0.02
Rhizobium spp	5.4 ± 0.09	40.0 ± 0.00	1.0 ± 0.00	2.8 ± 0.06	0.030 ± 0.00
Staphylococcus aureus	6.3 ± 0.08	40.0 ± 0.00	1.0 ± 0.00	1.2 ± 0.06	0.030 ± 0.00
Control	2.6 ± 0.07	45.3 ± 0.88	1.0 ± 0.00	4.0 ± 0.03	0.030 ± 0.00

Results are values of three replicates ± the S.E (Standard error)

3.3. Antagonistic Effects of the Endophytic Bacteria against F. oxysporum

The selected endophytic bacteria showed varying degree of inhibitory activity against the phytopathogen *F. oxysporum*. The result, as presented in Table 4, shows that all the means were statistically greater ($P < 0.05$) than the control, indicating the ability of the test endophytic bacteria in the inhibition of *F. oxysporum.* There was a significant difference ($P < 0.05$) between all the mean values of zone of inhibition. *S. aureus* and *Bacillus subtilis* yielded the highest zone of inhibition of 21.3 and 20.2 mm, respectively. However, there was a significant difference ($P < 0.05$) between the sizes of zone of inhibition yielded by the two bacteria. On the other hand, *Bacillus cereus* which produced a zone of 17.2 mm has the lowest inhibitory activity.

Table 4. Antagonistic Effects of Some Endophytic Bacteria against *F. oxysporum*

Endophytic Bacterium	Mean Zone of Inhibition (mm)
Bacillus subtilis	20.2 ± 0.17
Bacillus cereus	17.2 ± 0.12
Escherichia coli	18.5 ± 0.20
Klebsiella pneumoniae	19.2 ± 0.15
Micrococcus spp 1	18.1 ± 0.10
Micrococcus luteus	18.2 ± 0.12
Staphylococcus aureus	21.3 ± 0.21
Control	12.7 ± 0.15

Results are values of three replicates ± the S.E (Standard error)

Qualitative detection of enzymes, such as cellulase, catalase, amylase, urease and oxidase, was carried out and the distribution of some of the enzymes among the test bacteria is represented in Table 5.

Table 5. Distribution of some enzymes among the test bacteria

Isolate	Catalase	Cellulase	Urease	Amylase
Bacillus subtilis	+	+	-	+
Bacillus cereus	+	+	-	+
Escherichia coli	+	-	-	+
Klebsiella pneumoniae	+	-	+	-
Staphylococcus aureus	+	-	+	+
Micrococcus luteus	+	+	+	+
Micrococcus spp	+	+	-	+
Rhizobium spp	+	+	-	+

+: Positive, **-:** Negative

4. Discussion

The result showed that the roots of *C. citratus* contain higher population of endophytic bacteria more than the leaves. This is most probably due to the fact that, the roots are the primary sites of infection as opined by Kobayashi and Palumbo (2000) and Hallmann *et al*. (1997). Similarly, Rosenblueth and Martinez-Romero (2004) found that, in most plants, the number of bacterial endophytes is higher in the roots than the above-ground tissues. Moreover, most endophytic bacteria are soil-borne and, therefore, colonize the roots region first and subsequently spread to other parts of the plants. Interestingly, opposite pattern of distribution was observed among the endophytic fungi that colonize same plant as reported by Deshmukh *et al*. (2010) who, in a study of fungal endophytes of *C. citratus* in two sites in India, reported 53% and 50% compared with 25% and 23% of fungi isolated from the leaves and rhizomes of the two sites, respectively. Furthermore, the isolates obtained in the present study are similar to the common endophytic bacteria isolated from different plants by different workers

at different times as reported by Ryan *et al*. (2007) as well as Rosenblueth and Martinez-Romero (2006).

The result shows that *K. pneumoniae* has potentials of promoting the growth of tomato seeds by ways of either shortening the length of germination period, improving the chances of seed germination, raising the length of shoot, improving weight gain or both. The mechanisms through which endophytes promote plant growth are many. These include: improved cycling of nutrients and minerals, phytoremediation (Ryan *et al*., 2007), phosphate solubilisation activity (Verma *et al*., 2001; Wakelin *et al*., 2004), Indole acetic acid production (Lee *et al*., 2004), production of a siderophore (Costa and Loper, 1994), and supply of essential vitamins to host plants among others (Pirttila *et al*., 2004).

All the tested bacteria showed antagonistic activity against the plant pathogen, *F. oxysporum* and, the activity was highest in *S. aureus* followed by *B. subtilis*. The result shows some agreement with the work of Ji *et al*. (2014) who reported the antagonistic activity of 12 endophytic diazotrophic bacteria isolated from Korean rice cultivars on mycelial growth of all the isolates of *F. oxysporum* tested. They further reported 4 species of both *Bacillus* and related genus *Paenibacillus* among the seven species with the highest antagonistic activity. The result also agrees with the work of Kim *et al*. (2008) who reported the antagonistic effects of 7 out of 20 *Bacillus* spp isolated from manure and cotton waste composts against soil borne fungi, *F. oxysporum*, *Rhizoctonia solani*, *Phytophthora casici* and *Sclerotinia sclerotium*. This in-vitro antagonistic effect of the endophytic bacteria against *F. oxysporum* is best explained by the mechanism of antibiosis. Several studies have indicated the ability of endophytic bacteria to exude compounds with antibiotic properties and biocontrol potentials. Notable among these include compounds, such as oligomycin A, kanosamine, zwittermicin A, and xanthobaccin produced by *Bacillus* spp (Compant *et al*., 2005). This further proves the potential application of these bacteria more especially *S. aureus* and *B. subtilis* as biocontrol agents of plant diseases and also potential sources of natural bioactive compounds.

The growth promotion and pathogen inhibition of the test bacteria might also be associated with the enzymes produced by the test bacteria. The bacteria were found to possess a variety of enzymes, such as catalase, cellulase and urease. Kuhad *et al*. (2011) reported the application of cellulase in plant pathogen and disease control, as well as plant growth and flower production. Catalase was reported to reduce the toxicity of hydrogen peroxide in plants (Felton *et al*., 1991), while urease when combined with nitrification inhibitors prevents loss of Nitrogen and improves yield (Freney, 1997).

5. Conclusion

The present study has shown that the internal tissues of *C. citratus* harbour a diverse range of endophytic bacteria that offer benefits to other plants in terms of growth promotion and pathogen inhibition. However, qualitative assay procedures that screen the useful bacteria for the production of useful enzymes, bioactive compounds and metabolites may reveal the answers for the potentials of these endophytic bacteria not only in growth promotion and biocontrol but possibly other areas.

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