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Research Article
Comparative Study of the Colonization of *Chromolaena* and Tobacco Plants by *Bacteria safensis* CS4 using Different Methods of Inoculation

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Abstract

Background and Objectives: This study entails the effectiveness of colonization of bacterial endophytes following inoculation of the cells in plants. **Methodology:** Different methods of inoculation including seed immersion, root immersion and foliar spray were studied on *Chromolaena odorata* and *Nicotiana tabacum* for 10, 20 and 30 days. This was to ascertain the colonization ability of the endophytic strain amongst the two set of plants. The foliar parts of the plants were assessed post inoculation for the presence of the bacteria strain, followed by the growth parameters in the plant. Significant differences at $p<0.05$ of colonization were established by the different inoculation methods. **Results:** Foliar spray demonstrated the highest colonization in both *Chromolaena* and tobacco plants followed by root immersion. Leaf inoculation in tobacco plant demonstrated a positive colonization which is not significant. However, seed inoculation provided colonization in *Chromolaena* plant at 10, 20 and 30 days post inoculation at a frequency lower than that of tobacco. With root immersion in *Chromolaena*, there was colonization at 10 days post inoculation, no colonization at 20 days post inoculation, but colonization re-appeared at 30 days (PI). Growth index measured demonstrated a positive relationship between the inoculation of the endophyte and the growth parameters which included stem length and germination rate. **Conclusion:** This study, therefore, showed that the bacteria strain *B. safensis* CS4 can effectively be horizontally transferred into tobacco and *Chromolaena* plants using different methods. Foliar spraying demonstrated the optimal colonization of the strain in the plant leaves.

Key words: Bacteria endophytes, *Bacillus safensis*, *Chromolaena*, tobacco, endophyte colonization, plants

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Bacteria endophytes which came to the limelight recently long after fungi endophytes were discovered, lives in the tissues of plants without causing any apparent harm to the host plant. All the plants in existence are reportedly endowed with some fractions of endophytes, which can be identified in the seeds, root, stems and leaves[1,2]. This inhabitation does not continue throughout the life cycle of the plants, hence should be moderated through the scientific means such that the applications of endophytes in biological systems could be engineered[3]. About a million species of endophytes have been identified ranging between fungi and bacteria with symptomless infections in the tissues of the healthy plant[4]. Endophytes are micro-organisms with tremendous properties, ranging from its influence as a source of secondary metabolites, their ability to produce bioactive products are antimicrobial/antifungal characters and their ability to secrete natural products[5]. This wide diversity and properties of the organism require a continuous identification and study of their biological activities for easy profiling. This is because there are the sets of microbes and the components of the biodiversity that have remained unexplored even at this present age of scientific understanding[6].

Endophytes are widely reported to have the ability to colonize its host naturally either vertically or horizontally. These are mediated by a whole lot of factors ranging from the biotic to the abiotic ones. Most importantly, the native composition of the soil and the host plant genotypes plays an important role in bacteria endophytes interests in colonization of their hosts[7]. According to the study of Bulgarelli *et al*.[8] and Wagner *et al*.[9], the type and composition of soil influenced the composition of the bacteria endophytes community found in the host root. This report tarries with the fact that the type of soil dictates the nature of the inhabiting bacteria, serving as the initial inocula for endophytes colonization of its hosts[8]. Presently, there has not been much understanding on the molecular mechanisms by which plants tend to select specific bacterial endophytes over another[10,11]. As such, following the interest from the molecular characterization of each genus, specific bacteria endophytes could be selected for a particular plant's colonization based on its characteristics. This could provide a driver for the engineering of its activities in the host plant and may suffice in enhancing the biological applications of bacterial endophytes.

Bacillus safensis is bacteria endophytes of plants growing in a PAH-contaminated soil. The genus is a Gram-positives pore-forming and rod bacteria originally isolated from a spacecraft in Florida and California states of the United States of America (USA)[12]. At present, very little is known concerning the bacteria endophyte genus in their application in phyto-remediation studies. Although there was a report of the isolation of the genus bacteria endophyte by Caraballo[13] in Cabo Rojo, Puerto Rico, in the study to identify bacteria endophytes from the leaf of certain *Coccoloba uvifera*. Still. No mentioning of the fact that the bacteria were studied in terms of understanding their colonization capabilities that could enable their use as a tool for soil reclamation was heard. However, to draw from the pool of hundreds of thousands of bacteria endophytes and need to understand their interaction in the tissues of the plant, draw the need for the study of those interactions[14]. In a build-up to this holistic study centered on the assessment of bacteria endophytes isolated from plants growing around petroleum hydrocarbon-contaminated soil, *Bacillus safensis* CS4 was part of the bacteria genus that has a high percentage of re-occurrence followed by its ability to degrade[2] selected PAHs. This ability of the endophyte strain to use PAH as their sole source of food and energy generated the need for its pilot testing to establish the effectiveness of the bacteria in plants colonization and consequently in phyto-remediation of PAHs.

In this study, Tobacco and *Chromolaena* were used as the test plants owing to the reason that the plants have featured in many phyto-remediation studies, hence has been reported as part of the plants with the ability to be used as reclamation plants for soils contaminated by organic and inorganic compounds[15,16]. For the two plants to be successfully used for an endophyte-enhanced phytoremediation study, it is ideal that their ability to host bacteria endophyte strains are tested since the contaminant degradation is meant to occur in-planta. Tobacco, although has been extensively used for the remediation of organics and its ability to host endophytes, such has not be demonstrated on *B. safensis* and to be used for degradation of HMW-PAHs. This study, therefore, aimed to provide the possibility of colonization of *Bacillus safensis* CS4 on Tobacco and *Chromolaena* plants for a subsequent use in a phytoremediation study.

MATERIALS AND METHODS

The *B. safensis* was isolated from Rye grass (Lolium) collected from petroleum hydrocarbon contaminated soil in South Africa. The interest for the strain was based on its high incidence amongst the plants sampled. A clean Rye grass was surface sterilized using 75% (v/v) ethanol for 2 min, cleaned with distilled water for 1 min and flooded with a

commercial bleach for 1 min. The sterilized plant was finally washed three times using distilled water to remove the residues of the chemicals. Confirmation of the success of the sterilization was done by inoculating the water from the final rinse on an LB agar medium. The sterilized plants were separated into roots, stem and leave and were ground using sterile mortar. The paste of the plant was streaked in bacteriological agar for 3 days. Single colonies were transferred into the nutrient agar and preserved. To verify the purity of the strains, a single colony was viewed under a high powered microscope[17].

Identification of the endophyte strain was done using both molecular and morphological data. The DNA was extracted using a commercial DNA extraction kit (Genelute DNA kit from Sigma-Aldrich). In molecular identification, PCR was used to amplify the internal transcribed spacer region of the ITS rDNA[18]. The PCR, as well as the fragment purification and sequencing were performed according to Jain *et al.*[19]. Fragment similarities were compared with that of the previously published data[19] and examined with BLASTn in GenBank. The sequence generated was submitted to GenBank (accession number KX756323.1).

The *B. safensis* was obtained from cultures maintained on potato dextrose agar (PDA: Sigma Aldrich South Africa) for 7 days at 28°C in the dark. The bacteria were harvested and placed in test tubes containing 0.05% (v/v) aqueous solution of Tween 20 (Merck South Africa). Suspensions were adjusted to 1×10^8 mL^{-1} of cells of *B. safensis* according to Gurulingappa *et al.*[20], using a Neubauer hemocytometer.

Inoculation of seed: Seeds of tobacco were purchased from Seeds of Africa in Cape Town, South Africa, while that of *Chromolaena* was collected from the plant's bed already developed by the research team. The seeds were surface sterilized according to Sauer and Burroughs[21]. The seeds were then immersed in 10 mL of *B. safensis* cell suspension for 24 h and were allowed to air-dry in a sterile laminar flow cabinet for 45 min before being sown in 12×12 cm plastic pots containing potting soil at 1 cm depth. The set up was maintained in the greenhouse at 25°C following a photoperiod of 12-12 h light and day. A control experiment was set up using a bacterial cell-free solution containing 0.05% Tween 20.

Inoculation by foliar spray: To do this, seeds were planted in 12×12 cm pots filled with the growth medium as mentioned above. A sterilized plastic hand sprayer of 50 mL volume was used. The seedlings of 3 weeks growth were each sprayed with about 2 mL of the cell suspension. The control experiment was equally sprayed with an equal volume of the cell-free surfactant.

Inoculation of plant root: Using the root immersion method, the same 3 weeks old plants as in above was removed from the pots and rinsed using sterile distilled water 3 times. The ends of the clean-clear roots were cut off to give a better opening for absorption before it was put into individual test tubes with 2 mL of a bacterial cell suspension. The roots of the plants in the control experiment were also submerged in 0.05% of the surfactant alone. The entire treated and control plants were allowed for 24 h at 25°C and a photoperiod of 12 h before replanting in the pots.

Watering of the plants in all the set up were done using the manual watering system, making sure that the appreciable water is allowed into the pots. Each experiment and the control were replicated into 3 and done on 3 different dates. The experiment was allowed for the number of days depending on the allotted period. At the end of each growth period, the leaves of the plants in the experimental and control set up were harvested and were dried in the air on a sterile laminar flow, making sure that dead tissues were not included. About three leaves from each set up were used with 1 cm piece of each leaf being cultured in Petri dishes containing PDA with 0.1% stick antibiotics consisting of 0.02 g each of penicillin, streptomycin and tetracycline. The presence or absence of *B. safensis* growth was recorded after 10 days at 25°C.

A total of 30 plants and 90 pieces of the plant were examined and the data expressed as colonization frequencies with the equation[18]:

$$\text{Colonization frequency} = \frac{\text{No. of plant pieces colonized}}{\text{Total no. of plant pieces}}\times100$$

Growth index: In root and leaf inoculated plants, plant growth rate was measured by means of a length of the stem (L) measured on the days of testing as L_0, L_{10}, L_{20} and L_{30}, respectively. With the seed inoculated plant, the growth rate was measured by means of germination of the seeds (G). A control experiment was measured from the uninoculated setups. Growth indexes were then measured as:

$$\frac{L_1 - L_0}{L_0}$$

for the length of stem and percentage of germination (presence of stem) in inoculated plants compared to uninoculated controls in seed germination.

The data generated were analyzed using a one-way ANOVA in excel and Spearman correlation used to express

the significance. Linear regression was used to demonstrate the relationship between the treated and the control.

RESULTS

Bacillus safensis was not recorded in the entire control experiment, but the inoculation techniques were successful in establishing the bacteria as an endophyte in the sample plants, although there was a difference in the colonization frequencies based on the techniques used over time. Meanwhile, the inoculation method significantly affected the colonization of leaves in the two plants species amongst the recorded days (Table 1).

In tobacco plants, the technique that resulted in higher colonization was the leaf spray which demonstrated a 100% colonization of leaves 10 days post inoculation, this was reduced to 14 and 4.5% on the 20 and 30 day (PI), respectively (Fig. 1a). The other two inoculation techniques (seed and root) inoculations, 10 days post inoculation showed 21 and 4%, respectively. The 20 and 30 days post inoculation, 6 and 2% resulted in day 20 (PI), while there was an equal colonization frequency at 1% on day 30.

For *Chromolaena* plants, foliar spraying resulted in the highest colonization with the following values; 53% at 10 days (PI), 11 and 3% for 20 and 30 days (PI). With seed inoculation and root immersion, highest colonization was observed at 10 days with values 13 and 6%, respectively. Seed inoculation resulted in 3 and 1% colonization at 20 and 30 days (PI), respectively, while no bacteria cell was found in *Chromolaena* at 20 days post inoculation using root immersion method, but resurfaced at 30 days post inoculation by 1%.

The statistics indicated that 10 days post inoculation demonstrated a highly significant effect on the bacterial inoculation technique factor, plants species factor and their interactions at $p<0.01$ (Table 1). At 20 days (PI), the species and technique factors were not significant at $p>0.01$, but the interaction was highly significant at $p<0.01$. At 30 days (PI), none of the factors were significant ($p>0.01$).

Growth index: Growth parameters of plants measured at intervals of 10, 20 and 30 days post inoculation with uninoculated control in potting soil were reported in Fig. 2 and 3. It was observed that inoculation of bacterial endophyte *B. safensis* exerted a positive growth effect on the plants inoculated by foliar spray and root immersion as compared to the uninoculated plants. Overall stem

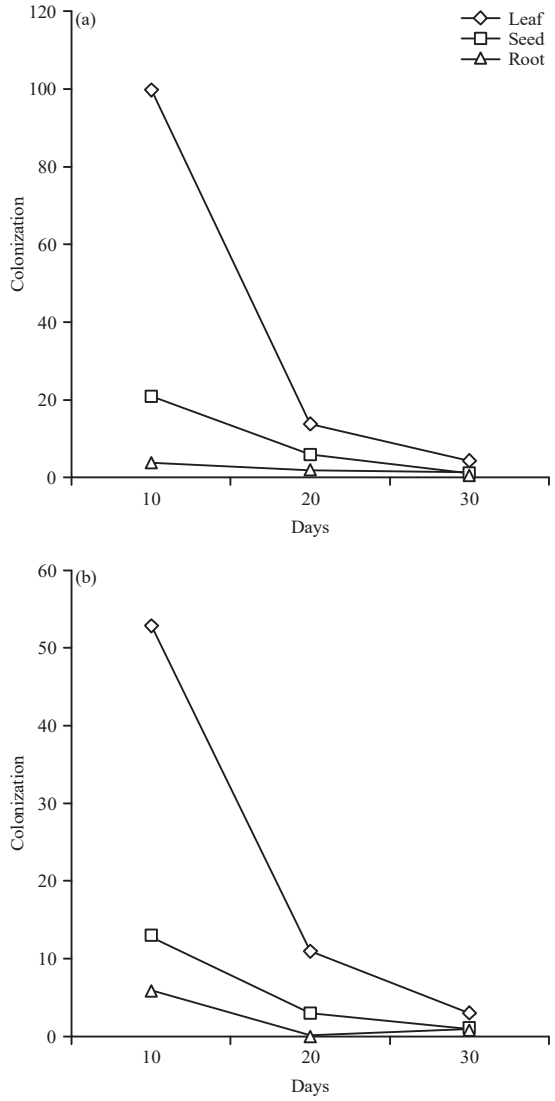

Fig. 1(a-b): Colonization frequencies of *B. safensis* in leaves of plants at different inoculation techniques (foliar spray, seed inoculation and root immersion) in 10, 20 and 30 days post inoculation, (a) Tobacco and (b) *Chromolaena*

Table 1: Result of the factors tested using descriptive statistics

	10 days			20 days			30 days		
Parameters	df	F-value	p-value	df	F-value	p-value	df	F-value	p-value
Species	1	25.43	<0.01	1	3.01	<0.0679	1	3.68	<0.06
Techniques	2	54.21	<0.01	2	2.32	<0.01	2	2.04	<0.2357
Species vs. techniques	4	14.28	<0.01	4	35.56	<0.01	4	1.19	<0.9789

At $p<0.05$ level of significance

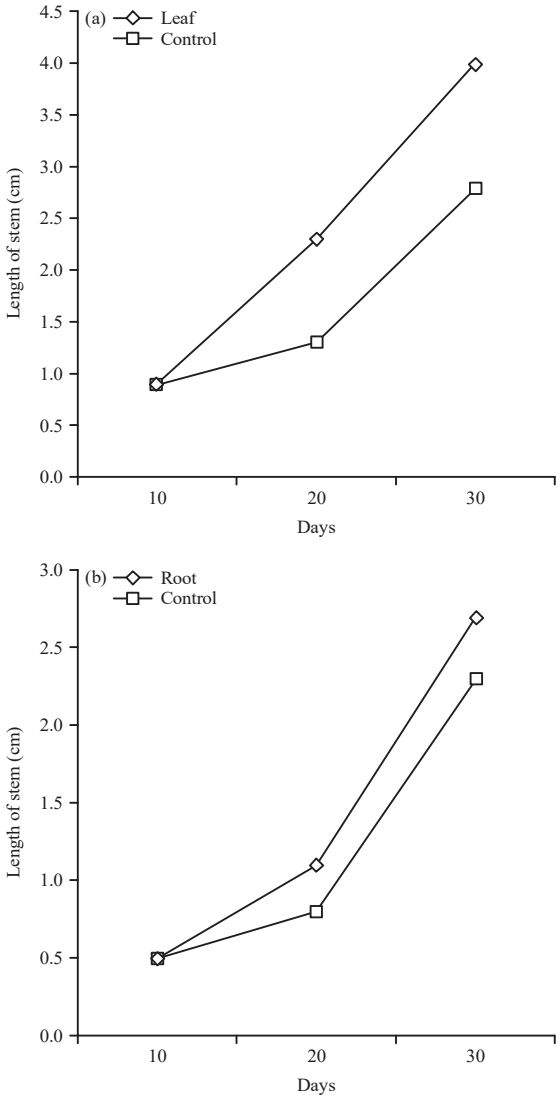

Fig. 2(a-b): Growth index of plants inoculated with endophyte *B. safensis* CS4 at different inoculation methods in tobacco in 10, 20 and 30 days post inoculation, compared with uninoculated control, (a) Leaf spray and (b) Root immersion

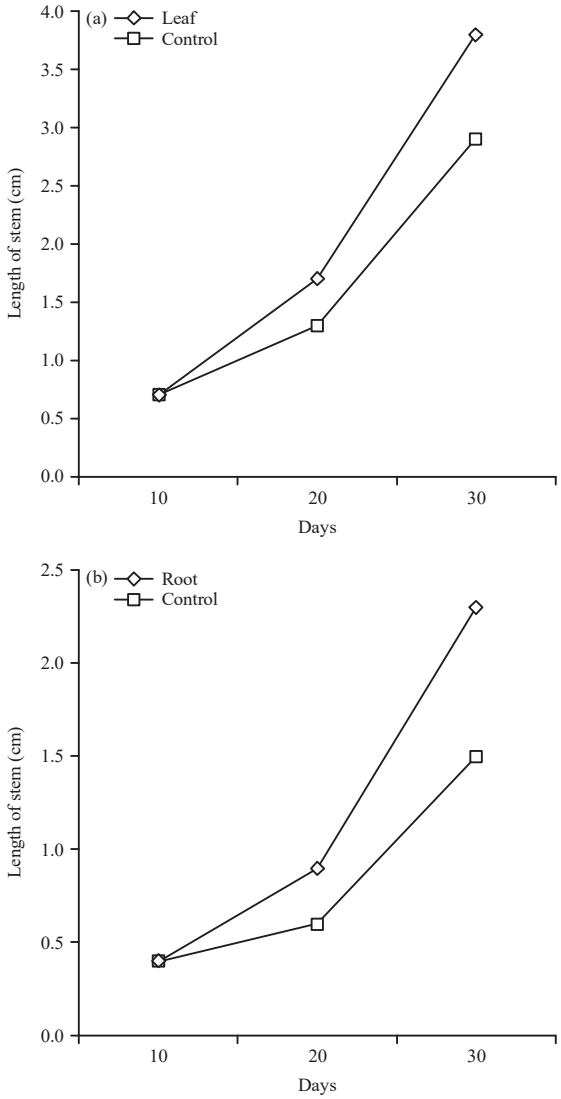

Fig. 3(a-b): Growth index of plants inoculated with endophyte *B. safensis* CS4 at different inoculation methods in *Chromolaena* in 10, 20 and 30 days post inoculation compared with uninoculated control, (a) Leaf spray and (b) Root immersion

length increased by 56% ($p<0.05$), with the highest growth observed at 30 days post inoculation. The growth of plants in control experiment, although increased (24%) but such growth was not significant at $p<0.05$. In the seed inoculation, there was 98% average germination rate (99% for tobacco and 97% for *Chromolaena*) compared to 64% germination in the control experiment (Fig. 4). Nevertheless, the growth index measured in the leaf, root and seed inoculated plants seem to be positively affected by the inoculation of the endophyte strain CS4 as compared to the uninoculated plants.

A linear relationship between inoculation and uninoculated control: Statistical analysis of the study generated a linear model for the inoculated samples as:

$$y = 0.4109x-0.06 \quad (1)$$

While that of the uninoculated control plants as:

$$y = 0.2848x+0.1133 \quad (2)$$

This was depicted from Fig. 5. It showed a positive relationship in both the treated and the control.

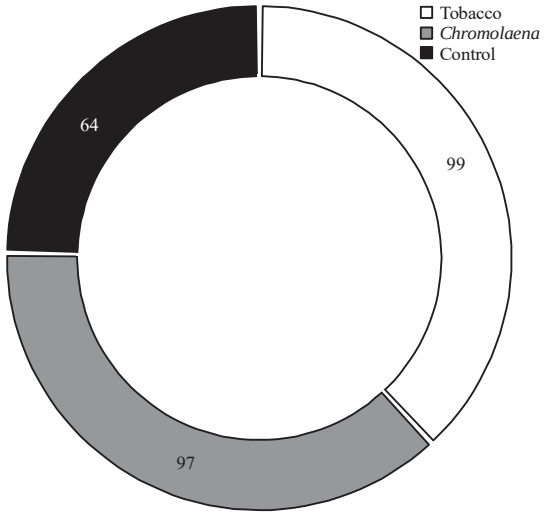

Fig. 4: Rate of germination of plant seeds inoculated with bacteria endophyte strain CS4 by seed immersion in 7 days post inoculation with uninoculated control

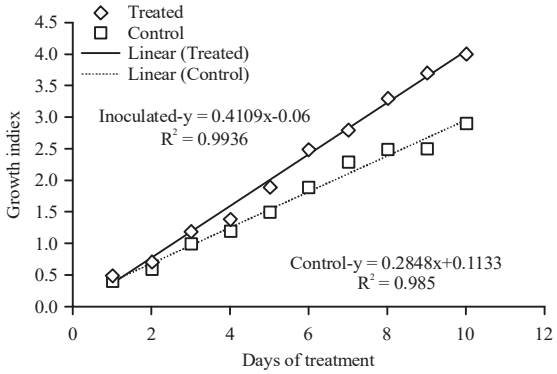

Fig. 5: Linear regression analysis between *B. safensis* CS4 inoculated plant and control

According to the Pearson correlation, there were significant and positive correlations (r = 0.45, p = 0.03) (the significant level at p 0.05) in the growth indices between the inoculated plants and the uninoculated controls

Response of colonization: Following the response of inoculation from the rate of colonization, there was higher response in foliar spray method than the root and seed immersion, this was demonstrated by using a radar plot as reported in Fig. 6. Tobacco plant yielded a higher colonization index in all the methods than *Chromolaena*. The entire response is in this order: Leaf spray>Root immersion>Seed immersion.

DISCUSSION

This research study observed that the endophyte *B. safensis* strain CS4 could be successfully inoculated into

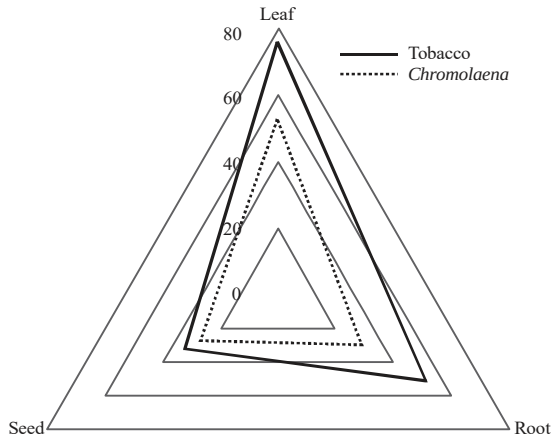

Fig. 6: Radar chart on the response of inoculation of *B. safensis* CS4 on the leaf, root and seed of tobacco and *Chromolaena*

Results are mean of three replicates

tobacco and *Chromolaena* through different methods that included foliar spray, root and seed immersion. Past literature has demonstrated the ability to inoculate *Bacillus* species into plants (willows and grass by Khan *et al.*[22], cocks foot by Galazka and Galazka[17]. Other studies have also shown the possibility of transferring endophytes from one plant to the other through inoculation. For example, in the study of Doty *et al.*[14], where diazotrophic endophytes were used. Brownbridge *et al.*[23] used *B. bassiana* strain LPSC 1067, which was inoculated into pine, this was also confirmed by Russo *et al.*[18]. *Burkholderia fungorium* DBTI was inoculated in hybrid poplar[24]. Other plants such as corn, opium, cocoa, banana, coffee, etc. have been used to grow endophytes in different research studies[25,26]. All these studies demonstrated successes in their inoculation methods employed.

The colonization of plants by *B. safensis* CS4 is shown by this study to be dependent on the methods of colonization, the type of isolate and the plants in question. It has been demonstrated by literature that apart from leaf inoculation just as was shown by the results of this study, direct injection of endophytes has resulted in a greater colonization frequency, also foliar dipping have shown such recovery of the endophytes as well[27,28]. Colonization of fungi in plants has also shown successes in various endophyte studies, to buttress the ability to recover endophytes in plants after inoculation[18,29]. The presence of endophytes also showed to favor the growth of the plant as there was a positive relationship observed between inoculation and growth index measured. This result is therefore in agreement with the reports that endophytes favour the growth of plants as they induce biological activities in the plant tissues and by so

doing enhance the synthesis of phytohormones that plays a part in growth promotion and root elongation[30-32]. Endophytes have also been reported to enhance nutrient cycling in plants hence supporting biomass increase in plant tissues[5].

The results of this study are in agreement with various endophytic studies focusing on the ability to colonize certain endophytes of bacteria or fungi origin into plants. Tefera and Vidal[25] carried a study on corn and sorghum plants inoculated with a fungus endophyte and reported a positive colonization. The ability to recover *B. safensis* in the plants tested in this present study was shown to be decreased with time. From the result, it showed that as the length of days was increased, the recovery potential of the inoculated strain was reduced in all the inoculation techniques. This may have been caused by the competition with other organisms not tested in the set-up, which may have initiated competition amongst themselves. Also, surface sterilization may not have had much surface area to contend with based on the method of inoculation and host responses. This means that host responses should be studied in other endophyte colonization studies to be able to ascertain what those factors are. It is evident that *B. safensis* CS4 favours leaf inoculation as was demonstrated by the radar plot, this showed that the result is in favour with literatures that has supported leaf inoculation as the best method of endophyte inoculation[33]. The study also showed that it is possible to inoculate semi-hardwood with endophytes as the high recovery frequencies recorded in *Chromolaena* was able to demonstrate this. Future studies should endeavor to look at the length of colonization amongst plants within strains of bacteria.

It is detailed that the native soil composition and the genotype of an endophyte host plant are important factors in the recruitment of bacterial endophytes. A study of the root endophytes of a certain plant grown in soils of different characteristics sums up the fact that soil types has an influence in the inhibition of endophytes in plant's systems[7]. Environmental conditions which included the nutrition of the soil, moisture, temperature, the genotype as well as the age of the host plant have a direct influence on the rate and type of endophyte colonization in the cells of a plant[9]. Other factors that tend to moderate the rate of endophyte's colonization of a plant include various plant management regimes such as; fertilizer applications as well as other agronomic activities. It is therefore indicated by literature that the selection of endophytes by plants may be functionally driven rather than being driven by phylogeny[7]. Interestingly, molecular mechanisms by which plants select specific bacterial endophytes over others remain largely unknown and has received little attention of late. For this reason, it is important that efforts that is geared towards the studies that assesses the molecular mechanisms of endophyte colonization is implemented[34].

CONCLUSION AND RECOMMENDATIONS

This research study was able to demonstrate that bacteria endophyte *Bacillus safensis* strain CS4 could possibly inhabit the tissues of tobacco and *Chromolaena* plants where they can get involve in the normal biological activities of the plant. The bacteria can be transmitted through foliar, root and seed inoculation techniques. There was colonization of the endophyte recovered after 10, 20 and 30 days post inoculation of the bacteria in the leaf of the plant by means of leaf spray, seed and root immersion except in the 20 days post inoculation following root immersion. Growth index measured indicated a positive relationship between endophyte inoculation and plant growth. This study, therefore, indicated that *Bacillus safensis* can be inoculated into plants through different means.

A time relation study should be carried out to ascertain the appropriate time it takes for optimum colonization of endophytes is established.

The conditions necessary for endophyte colonization should be established within plants.

The interactions between organisms within plant tissues should be unraveled so as to be able to estimate their impacts in endophyte colonization.

SIGNIFICANCE STATEMENT

This study discovered that the complexities of bacteria endophytes has the ability to influence their colonization in plants tissues based on the methods of inoculation applied. In addition, such property could vary amongst different species of plant. The inoculation of tobacco and *Chromolaena* by *Bacillus safensis* strain CS4 provided a positive colonization in the foliar parts of the plants, hence could be found in other part of the plants. This method of recruiting of this specific endophyte by plants has been able to unravel the mechanism involved in the process, an information that has not been available before now. This study helps the researcher to know that endophytes plays a significant role in the bioactivities of their host plant and this can be modelled to achieve the ultimate aim in endophytes application.

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