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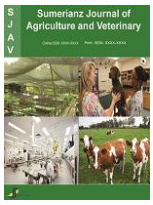

Original Article Open Access

Use of Bacterial Endophyte as a Control for White Aphid's Infestation in Tobacco Plant

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Abstract

This study entails the effectiveness of colonization of bacterial endophytes as a way of controlling the attack of aphids in tobacco, following inoculation of the cells in plants. *Pseudomonas parafulva* Ros-1 were inoculated using foliar spraying and the treated was studied against the un-inoculated control in *Nicotiana tabacum* for 60 days. This was to ascertain whether *P. parafulva* can inhibit the infestation of aphids on the plant. The foliar parts of the plants were assessed post inoculation for their colonization by the pest. Significant differences at $P < 0.05$ of colonization were established between the inoculated and the control. Un-inoculated control demonstrated the highest infestation in the tobacco plant for the period of the test. Leaf inoculation of an endophytic strain of *P. parafulva* provides succor to the treat of aphids on tobacco plants. Growth index measured demonstrated a positive relationship between the inoculation of the endophyte and the growth parameters which included stem length and germination rate. This study, therefore, showed that the bacteria strain P. Parafulva Ros-1 as an endophyte of tobacco could be used to curb the infestation of aphids.

Keywords: Bacteria endophytes; *Pseudomonas parafulva*; Tobacco; Endophyte colonization; Plants; White aphid; Pests.

1. Introduction

Pseudomonas parafulva Ros-1 strain is a bacteria endophyte occurring in plants growing in areas with high level of petroleum hydrocarbon contamination. This strain of bacteria is reported to have been used in the phytoremediation of petroleum aromatic hydrocarbon (PAH), [1]. *P. parafulva* as an endophyte lives in a plant without causing any negative symptoms in the plant [2]. It is a Gram-negative *Gammaproteobacteria* belonging to the order *Pseudomonadales* [3]. The bacteria according to Alcantara, *et al.* [3], was classified in group II, class I (Cluster 1) of the *Pseudomonas fulva* strain, on the bases of genetic recharacterization. *Pseudomonas species* and other bacterial endophytes have been tested in their ability for phytoremediation of organic contaminants, and in those studies have demonstrated their effectiveness in reducing or completely removing such contaminant from either soil or water [4-7].

Currently, no study has reported on the use of *P. parafulva*; a strain of *Pseudomonas* as an endophyte, let alone as a way of controlling pests. However, owing to the fact that there are several endophytes yet to be identified, it is imperative to test for new endophytes that could be employed in the various applications such as in fuel, medicine, environment, and most importantly agriculture. Bacteria from the genus *Pseudomonas* from literatures are microorganisms that effectively decompose organic pollutants through cometabolism in natural water and soil environment, hence have been used in phytoremediation applications [8]. *Pseudomonas parafulva* from the collection of culture was chosen for the study based on its reoccurrence in a plant-endophyte profiling study done on plants growing in the PAH-contaminated environment [9, 10]. The high incidence of the endophyte strain generated the need for its pilot testing to establish the effectiveness of the bacteria in plants colonization and consequently in the control of aphids since that pest is one that affects the growth of tobacco.

Aphids are small sap-sucking insects and members of the superfamily Aphidoidea. Common names include greenfly and blackfly, although individuals within a species can vary widely in colour. The group includes the fluffy white woolly aphids [11]. A typical life cycle involves flightless females giving living birth to female nymphs without the involvement of males. Maturing rapidly, females breed profusely so that the number of these insects multiplies quickly. Winged females may develop later in the season, allowing the insects to colonise new plants. In temperate regions, a phase of sexual reproduction occurs in the autumn, with the insects often overwintering as eggs [12].

The life cycle of some species involves an alternation between two species of host plants, for example between an annual crop and a woody plant. Some species feed on only one type of plant, while others are generalists, colonising many plant group. Aphids are among the most destructive insect pests on cultivated plants in temperate regions [12]. Control of aphids is not easy. Insecticides do not always produce reliable results, given resistance to several classes of insecticide and the fact that aphids often feed on the undersides of leaves. On a garden scale, water jets and soap sprays are quite effective.

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This study used a selected South Africa tobacco, a plant which has been implicated in various studies for the remediation of various soil contaminants [13-16]. Author's previous study reported the ability of tobacco in the remediation of various pollutant-contaminated soils as was reported by lots of literature [17, 18]. Meanwhile, various other studies have reported the use of such plant in the removal of metal as well as PAHs from soil [19, 20]. In the study by Atagana [7], *Chromolaena odorata*, another Southern African plant was able to extract PAHs through the root to the stem and the leaf after 90 days of exposure to a used engine oil contaminated soil. Tobacco plants, on the other hand, have been extensively used in phytoremediation studies, just as it has been employed in various endophyte-assisted phytoremediation studies in the remediation of environmental contaminants [4, 7]. In one of the studies, the plant demonstrated the effectiveness to be inoculated using three methods viz: foliar spray; seed immersion and root immersion [6]. Meanwhile, a study involving the comparing of the effect of two plants in endophyte enhanced phytoremediation requires that such be studied on their ability to inhabit endophytes. Therefore, the aim of the study was to evaluate the effect of colonization of *Pseudomonas Parafulva* Ros-1 in tobacco on white aphids attack on the plant.

2. Methodology

P. parafulva 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 minutes, cleaned with distilled water for 1 minute and flooded with commercial bleach for I minute. 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 three 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 [9]

Identification of the endophyte strain was done using both molecular and morphological data. Extraction of DNA was done 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 [9]. The PCR, as well as the fragment purification and sequencing, were performed according to Jain, *et al.* [14]. Fragment similarities were compared with that of the previously published data and examined with BLAST in GenBank. The sequence generated was submitted to GenBank (accession number KX756323.1).

P. parafulva was obtained from cultures maintained on potato dextrose agar (PDA: Sigma Aldrich South Africa) for 7 days at 28 ^{o}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 x 10^{8} mL^{-1} of cells of *P. parafulva* according to Ryan, *et al.* [21], using a Neubauer hemocytometer.

2.1. Inoculation of Seed

Seeds of tobacco were purchased from Seeds of Africa in Cape Town, South Africa, while the seeds were surface sterilized according to Tanhan, *et al.* [22]. The seeds were then immersed in 10 mL of *P. parafulva* cell suspension for 24 hrs, and then allowed to air-dry in a sterile laminar flow cabinet for 45 minutes before being sown in 12 x 12 cm plastic pots containing potting soil at 1 cm depth. The set-up was maintained in the greenhouse at 25 ^{o}C following a photoperiod of 12-12 hrs light and day. A control experiment was set up using a bacterial cell-free solution containing 0.05 % Tween 20.

2.2. Inoculation by Foliar Spray

To do this, seeds were planted in 12 x 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 about 2 ml of the cell suspension. The control experiment was equally sprayed with an equal volume of the cell-free surfactant. The entire treated and control plants were allowed for 24 hours at 25 ^{o}C, and a photoperiod of 12 hours before replanting in the pots.

Watering of the plants in all the set-up was done using the manual watering system, making sure that the appreciable water is allowed into the pots. Each experiment and the control was replicated into 3 and done on three different dates. The experiment was allowed for one month. At the end of each growth period, the number of the pests on the leaves of the plants in the experimental and control set up were counted using the help of a magnifying glass. 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, this a way of measuring the presence of the bacteria endophytes. The presence or absence of *P. parafulva* growth was recorded after 10 days at 25 ^{o}C.

A total of 30 plants and 90 pieces of the plant were examined, and the data expressed as colonization frequencies with the formula below.

Colonization frequency = number of plant pieces colonized/total number of plant pieces x 100 [6].

2.3. Growth Index

In inoculated plants, 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. A control experiment was measured from the uninoculated setups.

Growth indexes were then measured as $(L_1-L_0)/L_0$ 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 ANOVA in excel.

3. Results and Discussion

Pseudomonas parafulva 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 were differences in the colonization frequencies amongst the pots of plants used over time. Meanwhile, the difference in the colonization frequencies did not significantly affected the infection of the pest in the plant species amongst the recorded days (Table 1).

In the plants, the 0-10 days resulted in highest infection of the leaf by the pest as it demonstrated a 100 % infection of the leaves 10 days post inoculation, which was reduced to 14 and 4.5 % on the 20 and 30 day PI respectively (Figure 1a). The un-inoculated control resulted in 100 % infection throughout the duration of the study.

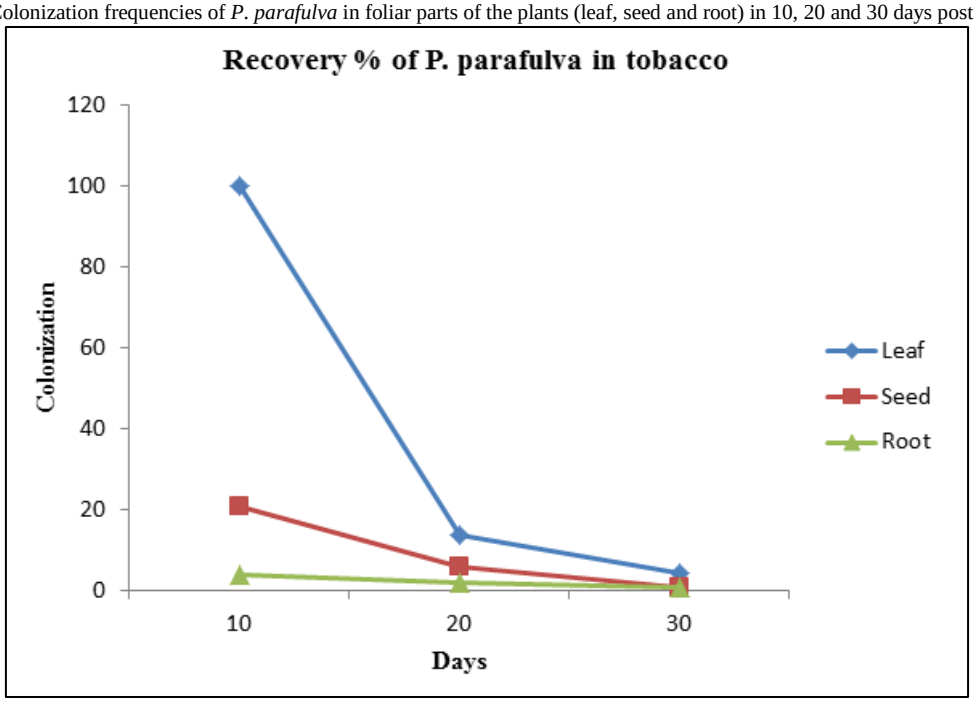
The statistics indicated that 10 days post inoculation demonstrated a highly significant effect on the bacterial inoculation technique factor, plants species factor and their interactions at $p < 0.0001$ (Table 1). At 20 days PI, the species and technique factors were not significant at $p > 0.001$, but the interaction was highly significant at $p < 0.001$. At 30 days PI, none of the factors were significant ($p > 0.0001$).

Table-1. Result of the factors tested using descriptive statistics

	10 days			**20 days**			**30 days**		
	df	***F***	***p***	***df***	***F***	***p***	***df***	***F***	***p***
Species	1	25.43	<0.001	1	3.01	<0.068	1	3.68	<0.006
Techniques	2	54.21	<0.001	2	2.32	<0.001	2	2.04	<0.236
Species vs techniques	4	14.28	<0.001	4	35.56	<0.001	4	1.19	<0.979

At $P < 0.05$ level of significance

Figure-1. Colonization frequencies of *P. parafulva* in foliar parts of the plants (leaf, seed and root) in 10, 20 and 30 days post inoculation



3.1. Growth Index

Growth parameters of plants measured at intervals of 10, 20 and 30 days post inoculation with uninoculated control in potting soil are reported in Figures 2 and 3. It is observed that inoculation of bacterial endophyte P. parafulva exerted a positive growth effect on the plants inoculated by foliar spray as compared to the uninoculated plants. Overall Stem 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 (Figure 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 Ros-1, as compared to the uninoculated plants.

Figure-2. Growth index of plants inoculated with endophyte *P. parafulva* Ros-1 at different inoculation methods in tobacco in 10, 20 and 30 days post inoculation, compared with uninoculated control

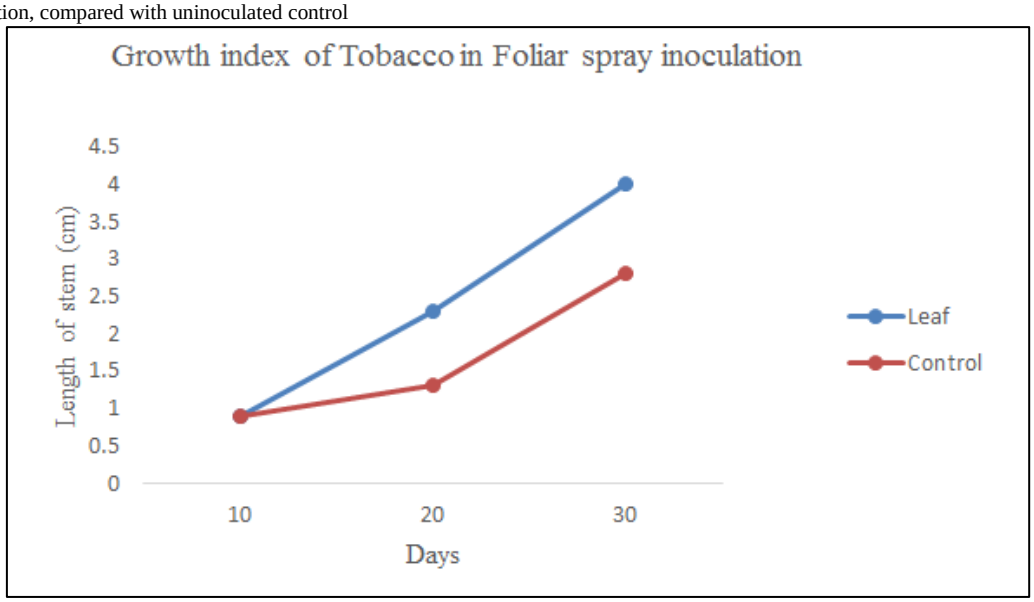


Figure-3. Growth index of plants inoculated with endophyte *P. parafulva* Ros-1 at different inoculation methods in *Chromolaena* in 10, 20 and 30 days post inoculation, compared with uninoculated control. (a) Leaf spray and (b) Root immersion

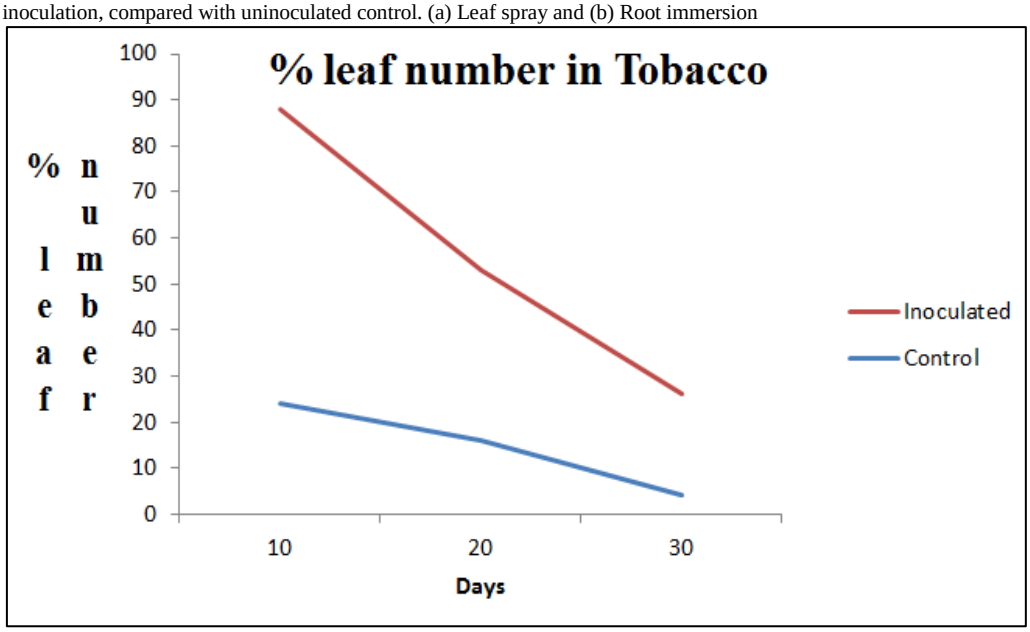
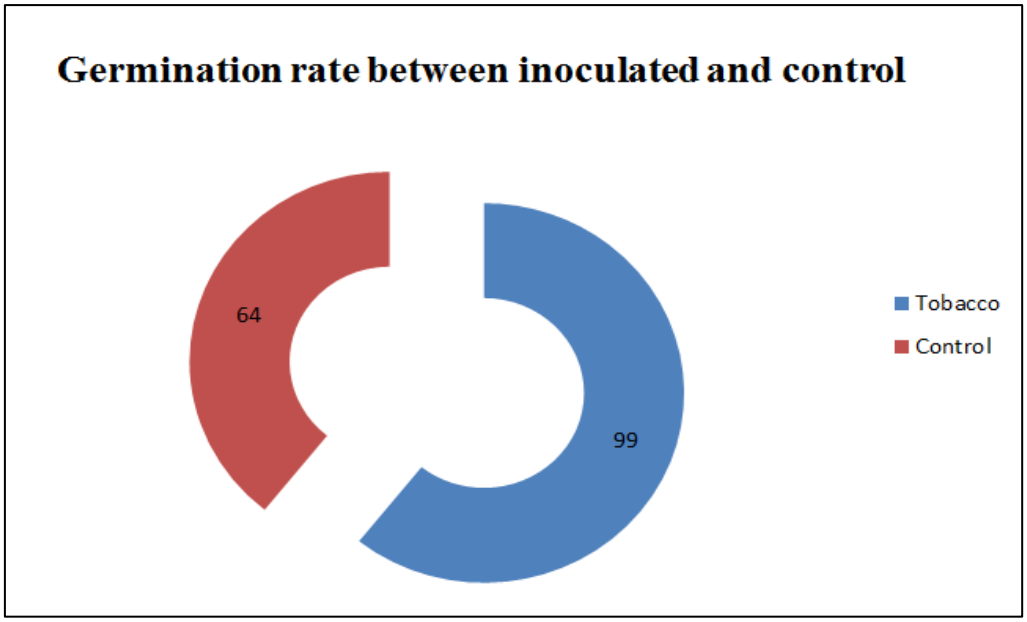


Figure-4. Rate of germination of plant between inoculated with bacteria endophyte strain Ros-1 by seed immersion in 7 days post-inoculation, with uninoculated control



3.2. A Linear Relationship Between Inoculation and Uninoculated Control

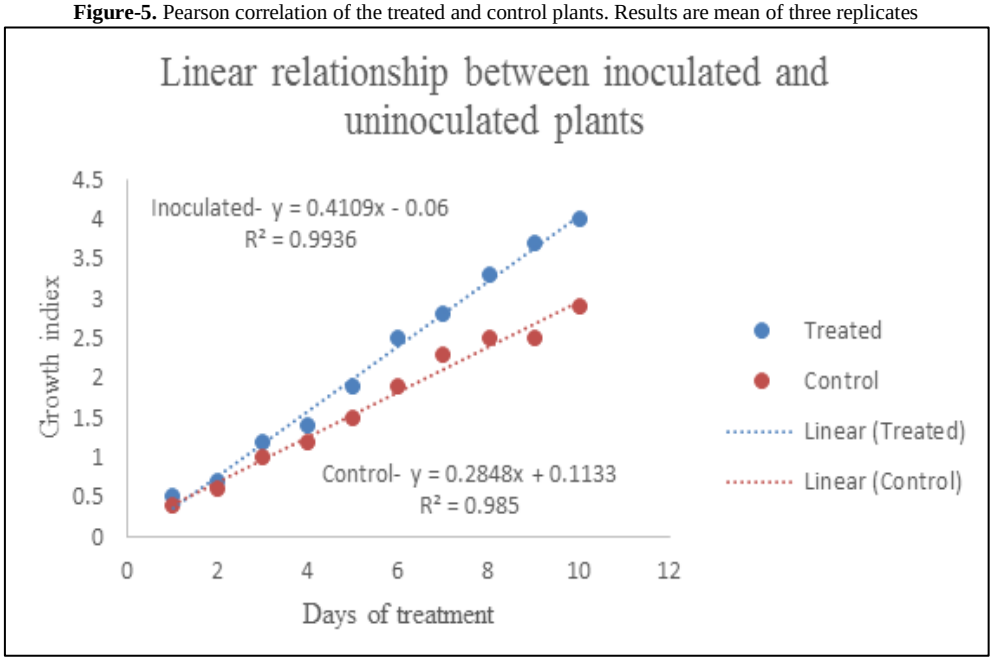
Statistical analysis of the study generated a linear model for the inoculated samples as

$y = 0.4109x - 0.06$ (Eqn 1)

While that of the uninoculated control plants as

$y = 0.2848x + 0.1133$ (Eqn 2)

Figure-5. Pearson correlation of the treated and control plants. Results are mean of three replicates

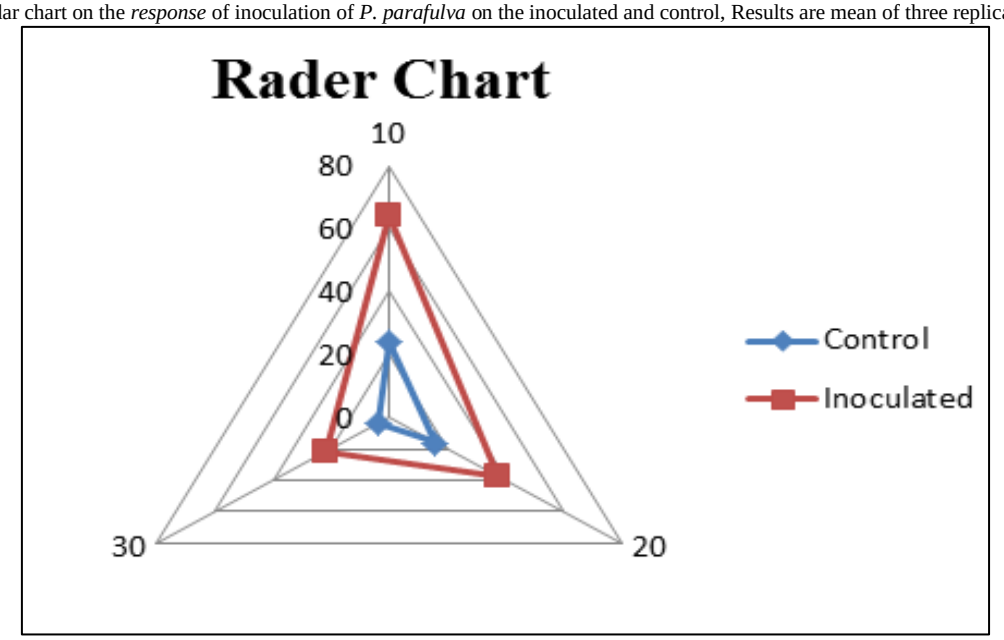


According to the Pearson correlation, there were significant and positive correlations ($r = 0.45$, $p = 0.003$) (the significant level at $P < 0.05$) in the growth indices between the inoculated plants and the uninoculated controls.

3.3. 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 using a radar plot as reported in Figure 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.

Figure-6. Radar chart on the *response* of inoculation of *P. parafulva* on the inoculated and control, Results are mean of three replicates



This research study observed that the endophyte *Pseudomonas parafulva* strain Ros-1 could be successfully inoculated in tobacco through different methods that included foliar spray, root and seed immersion. Past literature has demonstrated the ability to inoculate *Pseudomonas species* into plants (willows and grass by Khan, *et al.* [15]; cocks foot by Russo, *et al.* [23]. Other studies have also shown the possibility of transferring endophytes from one plant to the other through inoculation. For example, in the study of Tanhan, *et al.* [22], where diazotrophic endophytes were used. Tefera and Vidal [24], used *B. bassiana* strain LPSC 1067, which was inoculated into pine,

this was also confirmed by Russo, *et al.* [23]. *Burkholderia fungorium* DBTI was inoculated in hybrid poplar [25]. Other plants such as corn, opium, cocoa, banana, coffee, etc have been used to grow endophytes in different research studies [15, 25]. All these studies demonstrated successes in their inoculation methods employed.

The colonization of plants by *P. parafulva* is shown by this study to reduce the attack of pests which in this case is the white aphids. 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 [26, 27]. Colonization of fungi in plants has also shown successes in various endophyte studies, to buttress the ability to recover endophytes in plants after inoculation [4, 6]. The presence of endophytes also shows 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 [28, 29]. Endophytes have also been reported to enhance nutrient cycling in plants hence supporting biomass increase in plant tissues [17].

The results of this study are in agreement with various endophytic studies focussing on the ability to colonize certain endophytes of bacteria or fungi origin into plants. Tefera and Vidal [24], carried a study on corn and sorghum plants inoculated with a fungus endophyte and reported a positive colonization. The ability to recover *P. parafulva* 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 *P. parafulva* Ros-1 favours leaf inoculation as was demonstrated by the radar plot, this shows that the result is in consonance with literatures that has supported leaf inoculation as the best method of endophyte inoculation [27]. 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. The entire study is of importance as they have not been any known study involving the use of inoculated endophytes to curtail the influx of pest on the agricultural crops; hence the study should be expanded in the field.

4. Conclusion

This research study was able to demonstrate that bacteria endophyte *Pseudomonas parafulva* Ros-1 strain is an endophyte of tobacco plants, and 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 as well as the prevention of pest attack. This study, therefore, indicated that *P. parafulva* Ros-1 can be inoculated into plants through different means. And be used to curtail the infection of aphids

Recommendations

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.

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