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COLONIZATION OF *CHROMOLAENA* AND TOBACCO BY *PSEUDOMONAS PARAFULVA* ROS-1

RAYMOND ORIEBE ANYASI[1*], HARRISON IFEANYICHUKWU ATAGANA[2] AND RENE SUTHERLAND[3]

[1]*Department of Environmental Sciences, University of South Africa*
[2]*Institute for Science and Technology Education, University of South Africa*
[3]*Vegetable and Ornamental Plants Laboratory, Agricultural Research Council-Pretoria, South Africa*
**Corresponding author's email: ghnlwl@yahoo.com*

Abstract

This study sought to determine the effectiveness of colonization of bacterial endophytes following inoculation of the cells in plants. Different methods of inoculation including seed immersion, root immersion, and foliar spraying were studied on *Chromolaena odorata* and *Nicotiana tobacum* for 10, 20 and 30 days respectively. This was to ascertain whether *Pseudopodium parafulva* is an endophyte of both sets of plants. The foliar parts of the plants were assessed post inoculation (PI) for their colonization by the bacteria. Significant differences at $p<0.05$ of colonization were established by the different inoculation methods. Foliar spray demonstrated the highest colonization in both *Chromolaena* and tobacco plants, followed by root immersion. Leaf inoculation in tobacco plants demonstrated a positive colonization which was not significant. However, seed inoculation provided colonization in *Chromolaena* plant at 10, 20 and 30 days PI at a frequency lower than that of tobacco. With root immersion in *Chromolaena*, there was colonization at 10 days PI, no colonization at 20 days PI, but colonization re-appeared at 30 days PI. The 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 was an endophyte of tobacco and *Chromolaena* plants, and also that foliar spraying was an effective method of inoculating endophytes in both plants.

Key words: Bacteria endophytes, *Chromolaena* plant, Endophyte colonization, *Pseudomonas parafulva,* Tobacco.

Introduction

Pseudomonas parafulva Ros-1 is an endophyte that occurs in plants growing in areas with petroleum hydrocarbon contamination. This explains the implication of the bacteria in the phytoremediation of petroleum aromatic hydrocarbon (PAH) (Khan *et al*., 2013). *Pseudomonas parafulva* as an endophyte leaves in a plant without causing any negative symptoms in the plant (Liu *et al*., 2014). It is a Gram-negative *Gammaproteobacteria* belonging to the order *Pseudomonadales* (Uchino *et al*., 2001). The bacteria according to Uchino *et al*., (2001), 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 (De Oliveira-costa *et al*., 2012; Germain *et al*., 2006; Van Aken *et al*., 2004; Weyens *et al*., 2009a).

Currently, no study has reported on the use of *P. parafulva*; a strain of *Pseudomonas* as an endophyte, let alone as an endophyte in phytoremediation studies. However, as there are several endophytes which are yet to be identified, it is imperative to test for new endophytes that could be employed in various applications including as fuel, medicine, or in the environment and agriculture. Bacteria from the genus *Pseudomonas* are microorganisms that effectively decompose organic pollutants through cometabolism in natural water and soil environments; hence they have been used in phytoremediation applications (Galazka & Galazka, 2015). *Pseudomonas parafulva* from the collection of culture was chosen for the study based on its recurrence in a plant-endophyte profiling study done on plants growing in PAH-contaminated environment (Anyasi & Atagana, 2017). The high incidence of the endophyte strain generated the need for pilot testing to establish the effectiveness of the bacteria in plant colonization and consequently in the phytoremediation of PAHs.

This study used selected plants occurring in South Africa (tobacco and Siam weed), which have been implicated in various studies for the remediation of various soil contaminants (Atagana, 2011b; Bizily *et al*., 2000; Rugh, 2001). The current researcher's previous study reported the ability of *Chromolaena odorata* to remediate PCB-contaminated soil. Meanwhile, various other studies have reported the use of such plant to remove metal as well as PAHs from soil (Atagana, 2011a/b; Singh *et al*., 2009; Tanhan *et al*., 2007). In the study by Atagana 2011(a), *Chromolaena odorata* was able to extract PAHs through the root to the stem and leaf after 90 days of exposure to used engine oil-contaminated soil. Tobacco plants, on the other hand, have been extensively used in phytoremediation studies, and in various endophyte-assisted phytoremediation studies aimed at remediating environmental contaminants (Abhilash *et al*., 2009; Novakova *et al*., 2010; Russo *et al*., 2015). In one of the studies, the plant demonstrated its effectiveness after been inoculated using three methods, viz: foliar spraying; seed immersion and root immersion (Russo *et al*., 2015). Meanwhile, a study comparing the effect of two plants in endophyte-enhanced phytoremediation requires that an assessment be done on their ability to inhabit endophytes. The aim of the present study on which this article is based was to compare the colonization rates of *Pseudomonas parafulva* Ros-1 in tobacco and *Chromolaena* plants using the foliar spraying, and root and seed inoculation methods.

Methodology: *P. parafulva* was isolated from Rye grass (Lolium) collected from petroleum hydrocarbon contaminated soil in South Africa. Interest in 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 two minutes, cleaned with distilled water for one minute and flooded with commercial bleach for one minute. The sterilized plant was finally washed three times using distilled water, to remove the chemical residues. 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 leaves and were ground using a 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 (Galazka & Galazka, 2015).

Identification of the endophyte strain was done using both molecular and morphological data. The 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 (Russo *et al*., 2015). PCR, as well as fragment purification and sequencing, were performed in accordance with the recommendations of Jain *et al*., (2012). Fragment similarities were compared to those of previously published data and examined with BLASTn 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 seven 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 x 10^8 mL^{-1} of cells of *P. parafulva* according to the recommendations of Gurulingappa *et al*., (2010), 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 a plant bed which had already developed by the research team. The seeds were surface sterilized according to the method proposed by Sauer & Burrough (1986). Thereafter they were immersed in 10 mL of *P. parafulva* cell suspension for 24 hrs, and 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 one cm depth. The set up was maintained in greenhouse at 25°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.

Inoculation by foliar spray: A sterilized plastic hand sprayer with a volume of 50 mL was used to spray the seedlings (three weeks growth) with about 2 mL of the cell suspension. The control experiment was equally sprayed with an equal volume of cell-free surfactant.

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

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

A total of 30 plants and 90 pieces of the plant were examined, and the data were expressed as colonization frequencies using the following formula:

$$\text{Colonization frequency} = \frac{\text{Number of plant pieces colonized}}{\text{Total number of plant pieces}} \text{x } 100 \text{ (Russo } \textit{et al}\text{., 2015)}$$

Growth index: In the root and leaf inoculated plants, the plant growth rate was measured by measuring a length of the stem (L) on the days of testing L_0, L_{10}, L_{20}, and L_{30} respectively. With the seed-inoculated plant, the growth rate was measured by studying germinated seeds (G). A control experiment was measured from the uninoculated set-ups. Growth indexes were then measured as $(_{L1}\text{-}L_0)/L_0$ 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.

Results and Discussion

Pseudomonas parafulva was not recorded in the entire control experiment. The inoculation techniques were, however, successful in establishing the bacterium 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 over the recorded days (Table 1).

In the tobacco plants, the technique that resulted in the highest colonization was leaf spraying which

demonstrated a 100% colonization of leaves ten days post-inoculation, which was reduced to 14 and 4.5% on day 20 and 30 PI respectively (Fig. 1A). The other two inoculation techniques (seed and root), ten days PI showed 21 and 4% respectively. Further PI showed 6 and 2% from 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 PI. With seed inoculation and root immersion, the highest colonization was observed at ten days with values of 13 and 6% respectively. Seed inoculation resulted in 3 and 1% colonization at 20 and 30 days PI respectively, while no bacteria cells were found in *Chromolaena* at 20 days PI using the root immersion method, but resurfaced at 30 days PI by 1 %.

The statistics indicated that ten days PI 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 $p>0.0001$, but the interaction was highly significant at $p<0.0001$. At 30 days PI, none of the factors were significant ($p>0.0001$).

Growth index: The growth parameters of plants measured at intervals of 10, 20 and 30 days PI with uninoculated control in potting soil are reported in (Figs. 2 and 3). It was observed that the inoculation of bacterial endophyte P. parafulva exerted a positive growth effect on the plants inoculated by foliar spraying and root immersion compared to the uninoculated plants. Overall stem length was increased by 56% ($p<0.05$), with the highest growth observed at 30 days PI. The growth of plants in the control experiment, increased by 24%, but such growth was not significant at $p<0.05$. In the seed inoculation sample, there was a 98% average germination rate (99% for tobacco and 97% for *Chromolaena*) compared to the 64% germination rate in the control experiment (Fig. 4). Nevertheless, the growth index measured in the leaf, root, and seed inoculated plants seemed to be positively affected by the inoculation of the endophyte strain Ros-1, when compared to the uninoculated plants (Fig. 5).

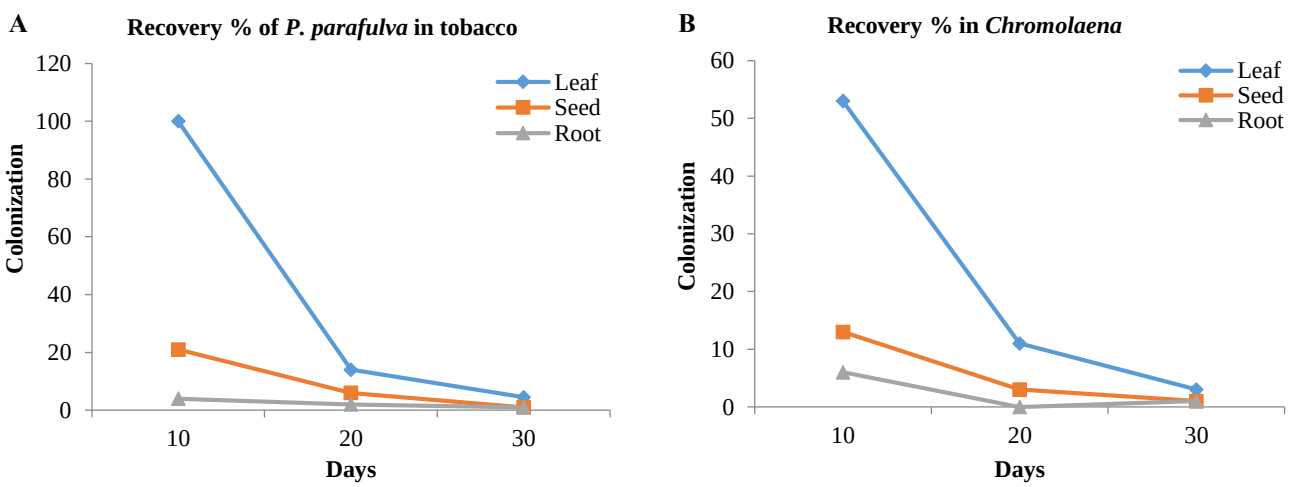

Fig. 1. Colonization frequencies of *P. parafulva* in leaves of plants using different inoculation techniques (foliar spraying, seed inoculation, and root immersion) in ten, 20 and 30 days PI. (A) Tobacco and (B) *Chromolaena*.

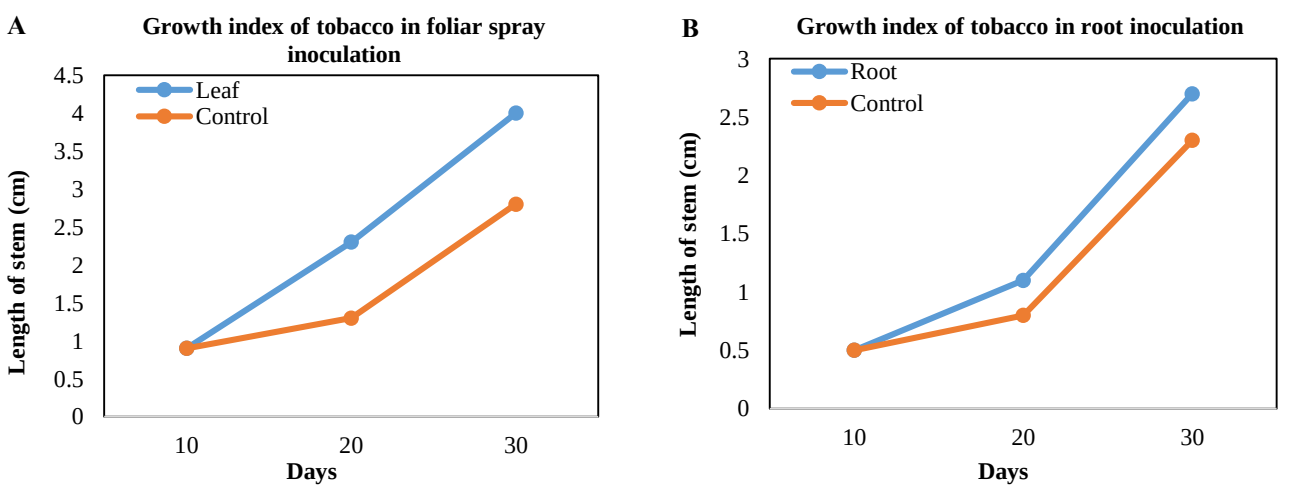

Fig. 2. Growth index of plants inoculated with endophyte *P. parafulva* Ros-1 at different inoculation methods for tobacco at 10, 20 and 30 days PI, compared to uninoculated control. (A) Leaf spray and (B) Root immersion.

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	<0.01	1	3.01	<0.07	1	3.68	<0.06
Techniques	2	54.21	<0.01	2	2.32	<0.01	2	2.04	<0.24
Species vs techniques	4	14.28	<0.01	4	35.56	<0.01	4	1.19	<0.98

At $p<0.05$ level of significance

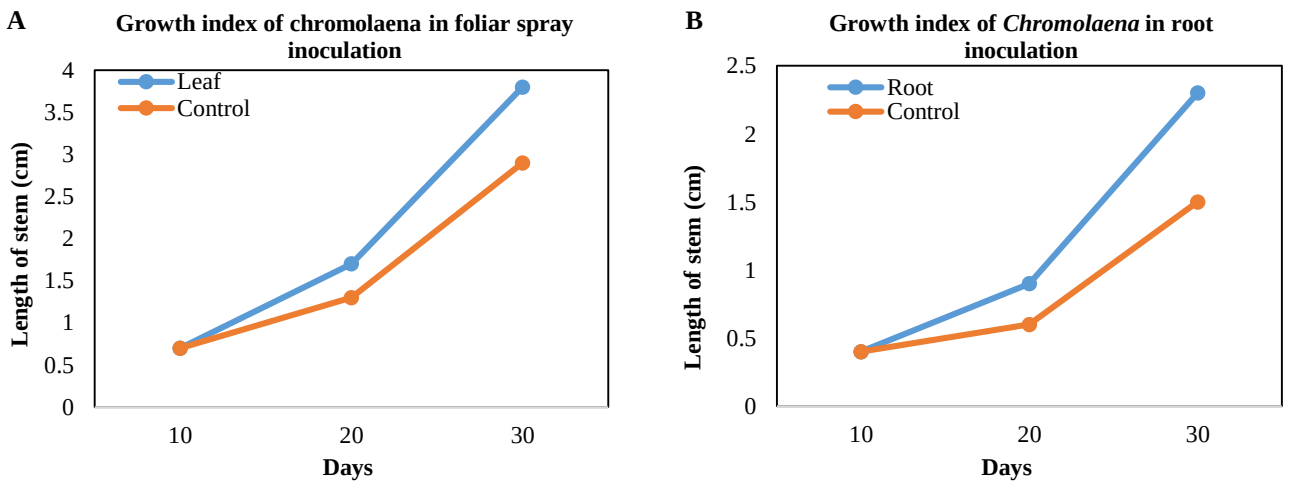

Fig. 3. Growth index of plants inoculated with endophyte *P. parafulva* Ros-1 at different inoculation methods in *Chromolaena* at 10, 20 and 30 days PI, compared with uninoculated control. (A) Leaf spray and (B) Root immersion.

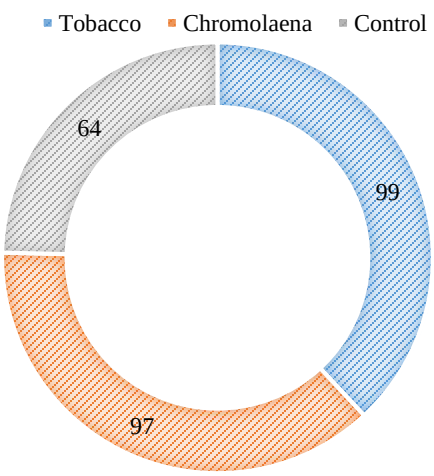

Fig. 4. Rate of germination of plant seeds inoculated with bacteria endophyte strain Ros-1 by seed immersion in seven days PI, with uninoculated control.

Linear relationship between inoculation and uninoculated control: A 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 was

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

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 shown 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.

The findings of this research study showed that the endophyte *Pseudomonas parafulva* strain Ros-1 could be successfully inoculated in tobacco and *Chromolaena* using different methods that included foliar spraying, root and seed immersion. The extant literature demonstrates the ability to inoculate *Pseudomonas species* into plants (willows and grass Khan *et al*., (2014); cocks foot see Galazka & Galazka (2015). Other studies have also shown the possibility of transferring endophytes from one plant to another through inoculation. For example, in the study of Doty *et al*., (2009) diazotrophic endophytes were used. Brownbridge *et al*., (2012) used *B. bassiana* strain LPSC 1067, which was inoculated into pine; this was also confirmed by Russo *et al*., (2015). *Burkholderia fungorium* DBTI was inoculated in hybrid poplar. Other plants such as corn, opium, cocoa, banana, coffee, etc. have been used to grow endophytes in different studies (Reddy *et al*., 2009; Tafera & Vidal, 2009). All these studies reported success in respect of the inoculation methods employed.

The colonization of plants by *P. parafulva* seems to be dependent on the methods of colonization, the type of isolate and the plants in question. As demonstrated in the literature, apart from leaf inoculation as shown by the results of this study, the direct injection of endophytes resulted in a greater colonization frequency, while foliar dipping also showed such recovery of the endophytes as well (Ownley *et al*., 2008; Pasada *et al*., 2007). The colonization of fungi in plants has shown successes in various endophyte studies, to buttress the ability to recover endophytes in plants PI (Akello *et al*., 2007; Russo *et al*., 2015). The presence of endophytes has also been shown to favor the growth of the plant, as a positive relationship between inoculation and the growth index measured. This

result therefore confirms the reports that endophytes favor the growth of plants as they induce biological activities in plant tissues, and bin so doing enhance the synthesis of phytohormones that play a role in growth promotion and root elongation (Gao *et al*., 2015; Weyens *et al*., 2009a/b). Endophytes have also been reported to enhance nutrient cycling in plants thereby supporting biomass increase in plant tissue (Ryan *et al*., 2008).

The results reported here corresponds with the findings of various endophytic studies focussing on the ability to colonize certain endophytes (of bacteria or fungi origin) into plants. Tefera & Vidal's (2009) study on corn and sorghum plants inoculated with a fungus endophyte, reported a positive colonization. The ability to recover *P. parafulva* in the plants tested in the present study was shown to be decreased over time. The result indicated that as the length of time (number of days was increased; the recovery potential of the inoculated strain was reduced across all three inoculation techniques. This may have been caused by 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 ascertain what those factors are. It is evident that *P. parafulva* Ros-1 favours leaf inoculation as 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 (Peng *et al*., 2013). The present study also showed that it was possible to inoculate semi-hardwood with endophytes as the high recovery frequencies were recorded in *Chromolaena* demonstrate. Future studies should endeavor to look at the length of colonization amongst plants within strains of bacteria.

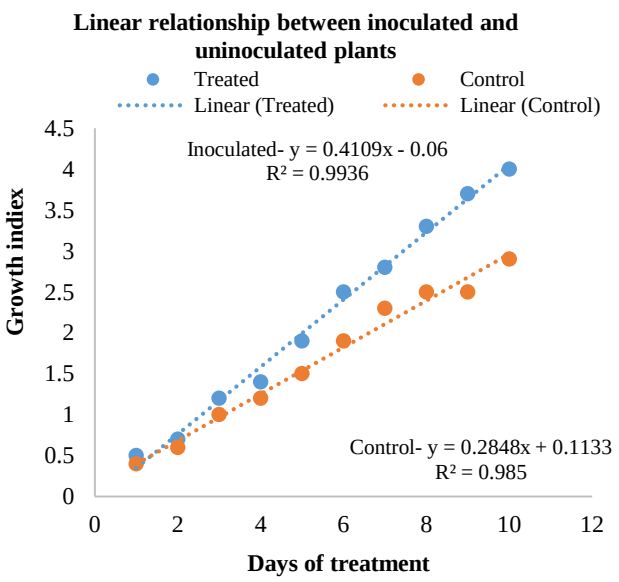

Fig. 5. According to the Pearson correlation, there were significant and positive correlations (r = 0.45, p = 0.003) (the significant level at $p<0.05$) in the growth indices between the inoculated plants and the uninoculated controls.

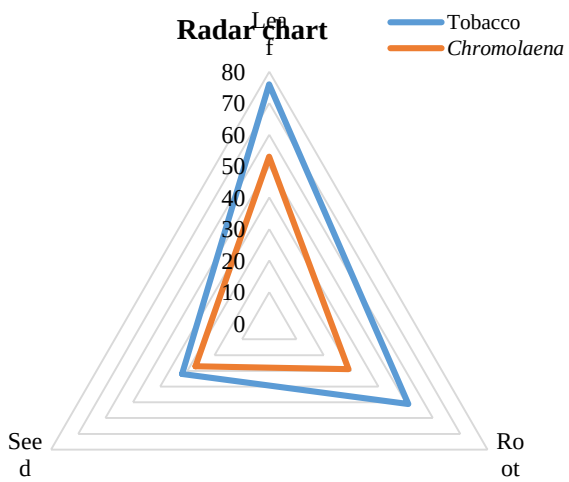

Fig. 6. Radar chart on the response of inoculation of *P. parafulva* on the leaf, root and seed of tobacco and *Chromolaena*. Results are mean of three replicates.

Conclusion

This research study demonstrated that the bacteria endophyte *Pseudomonas parafulva* Ros-1 strain was an endophyte of tobacco and *Chromolaena* plants, and can be transmitted through foliar spraying, as well as root and seed inoculation techniques. Colonization of the endophyte was recovered after 10, 20 and 30 days PI of the bacteria in the leaf of the plant by means of leaf spraying, seed, and root immersion except in the 20 days PI following root immersion. The measured growth index indicated a positive relationship between endophyte inoculation and plant growth. This study, therefore, indicated that *P. parafulva* Ros-1 could be inoculated into plants through different means.

Recommendations

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

The conditions necessary for endophyte colonization should be established within plants. Also, the interactions between organisms within plant tissues should be unraveled to estimate their impacts in endophyte colonization.

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