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Bacteria-Endophyte Enhanced Phytotreatment of Petroleum Hydrocarbon-Contaminated Soil by Nicotiana Tabacum

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Abstract - The study entails an isolation of Bacterial endophyte from plant growing around a crude oil sludge dam. Based on morphological characterisation, gram reactions and 16S rDNA sequence analysis, the isolate was identified as *Bacillus sefensis* strain CS4. Following the tests on the abiotic effects as well as the initial concentration of perylene on growth and degradation efficiency of the strain, a total degradation percentage of 87 % was observed from the initial concentration of 30 mg/l for one week. Analysis of the degradation products of perylene using GC-MS/MS, indicated a shift from the previously degradation product of other bacterial endophytes showing that the strain provided a new pathway for PAH degradation. In order to evaluate the influence of a polycyclic hydrocarbon transforming ability of the bacterial strain on the phytoremediation of petroleum aromatic hydrocarbon (PAH), *B sefensis* CS4 was inoculated into *Nicotiana tabacum* plants. The plants were grown for 16 weeks with or without PAH (500 mg/kg soil in each 1L pot) in non-sterile peat medium. Plants inoculated with the strain CS4 were much tolerant towards the phytotoxic effects of PAH, in terms of biomass index, leaves and stem dry weight. Although the presence of plants acted as the main effective treatment for PAH dissipation (52–69%), the inoculum with the strain leads to the highest PAH removal (up to 85%). Non-inoculated control planted in the contaminated soil was susceptible to the phytotoxicity of the contaminants in the parameters tested. The study therefore presented the strain as a suitable plant endophyte for enhanced phytotreatment of PAH.

***Keyword*:** Phytoremediation, Bacterial endophytes, Petroleum aromatic hydrocarbons, *Basillus safensis*, *Nicotiana tabacum*

1. Introduction

Bacillus safensis strain CS4 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., 2014). *B safensis* as an endophyte lives in a plant without causing any negative symptoms in the plant (Liu et al., 2014). It is a Gram-positive Gammaproteobacteria belonging to the order Bacillales (Uchino et al., 2001; Anyasi et al., 2019). The bacteria according to literature, was classified in group II, class I (Cluster 1) of the *Bacillus safensis* strain, on the bases of genetic recharacterization. Bacillus 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 (Van Aken et al., 2004; Weyens et al., 2009; Germain et al., 2010; De Oliveira et al., 2012).

Currently, no study has reported on the use of *B sefensis* CF4; a strain of *Bacillus* as an endophyte, let alone as an endophyte in phytoremediation studies. 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 agriculture. Bacteria from the genus *Bacillus* are microorganisms that effectively decompose organic pollutants through cometabolism in natural water and soil environment, hence have been used in phytoremediation applications (Galazka and Galazka, 2015). *B sefensis* 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 (Anyasi and Atagana, 2018). 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 phytoremediation of PAHs.

This study used selected plants in South Africa (tobacco). This plant has been implicated in various studies for the remediation of various soil contaminants (Atagana 2011b; Rugh, 2001, Bizily et al., 2000). Author's previous study reported the ability of *N. tabacum* in the remediation of PCB-contaminated soil (Anyasi, 2012). Meanwhile, various other studies have reported the use of such plant in the removal of metal as well as PAHs from soil (Tanhan et al., 2007; Atagana 2011a/b). In the study by Atagana 2011(a), *Chromolaena odorata* 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. Meanwhile, a study involving endophyte enhanced phytoremediation requires that the bacteria be inoculated into the plant before the degradation reaction. Therefore, the aim of the study was to inoculate *B sefensis* CS4 in tobacco plant and use the inoculated plant in the remediation of PAH-contaminated soil.

2. Methodology

B safensis CF4 was isolated from Rye grass (Lolium) collected from around a crude oil sludge dam 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 (Galazka and Galazka, 2015).

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 (Russo et al., 2015). The PCR, as well as the fragment purification and sequencing, were performed according to Jain et al. (2012). Fragment similarities were compared with that of the previously published data and examined with BLASTn in GenBank. *Bacillus safensis* CS4 was obtained from cultures maintained on potato dextrose agar (PDA: Sigma Aldrich South Africa) for 7 days at 28 oC in the dark. The bacteria were harvested and placed in test tubes containing 0.05 % (v/v) aqueous solution of Tween 20 (Merck). Suspensions were adjusted to 1 x 108 mL-1 of cells of B. sefensis according to Weyens et al. (2009a), using a Neubauer hemocytometer.

2.1. Artificial Contamination of Soil

50 kg of the sterile peat was contaminated with perylene dissolved in hexane to the tune of 500 mg/kg and was homogenised with a manual roto. The contaminated soil was analysed to ascertain the level of contamination as the initial concentration.

2.2. Inoculation of the Plant

Seeds of tobacco were planted in 12 x 12 cm plastic pots containing potting soil at 1 cm depth. The plants were allowed to grow for three weeks. The 3 weeks old plants were each sprayed about 2 ml of the cell suspension filled with surface sterilized broth, using a sterilized plastic hand sprayer of 50 ml volume. 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 ^{0}C, and a photoperiod of 12 hours before transplanting into another 1L pots containing 1 kg of non-sterile peat previously treated with 500 kg of the selected PAHs.

Watering of the plants in 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 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 ^{0}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.

2.3. Growth Index

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 L30 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 the control.

The data generated were analyzed using ANOVA in excel.

3. Results

Bacillus safensis CS4 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).

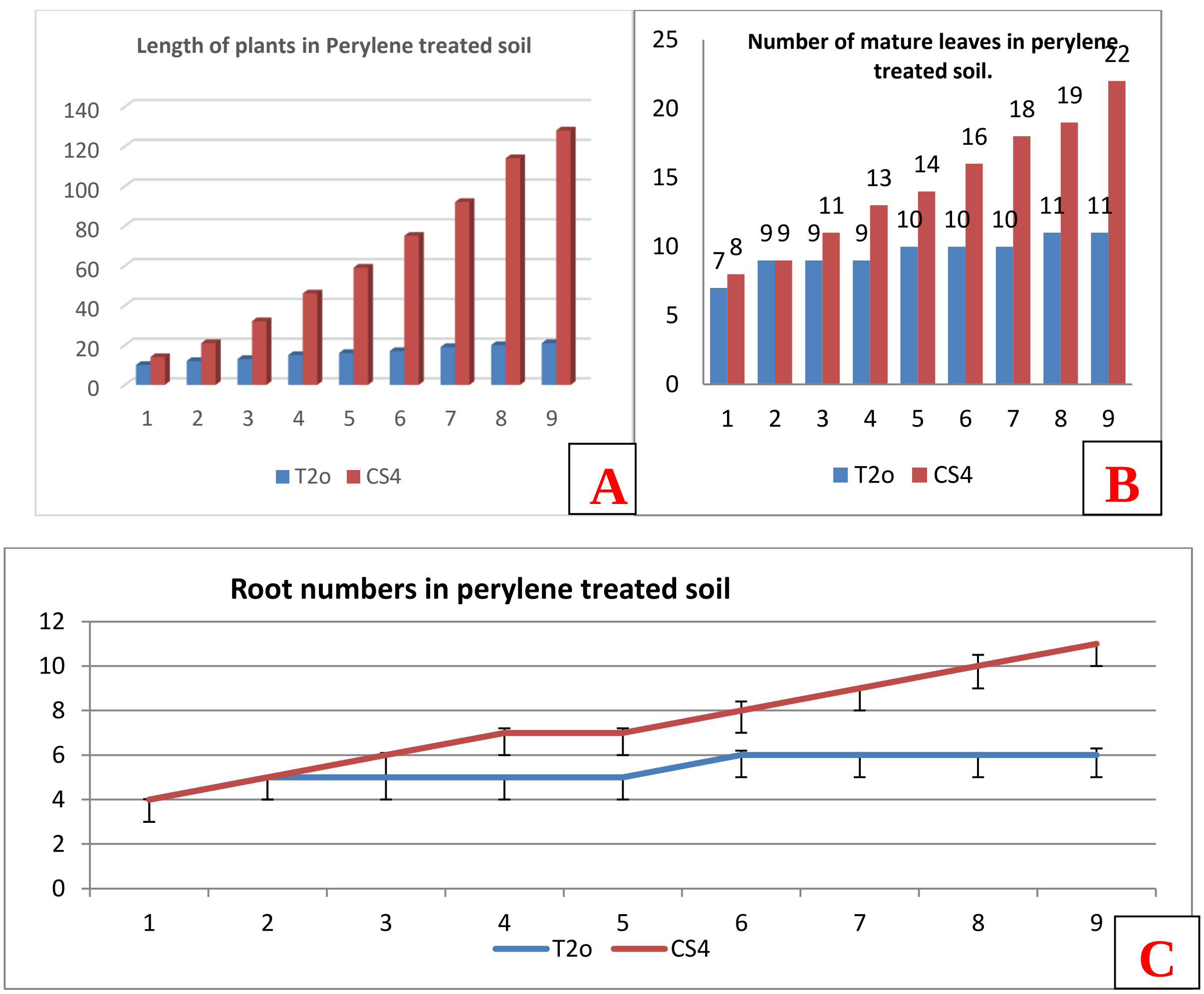

Figure 1: Growth parameters of *N. tabacum* (A-C) measured after 16-wks growing in the sterile peat substrate in the presence of perylene with *B. safensis* CS4/Tween 20: A=Length of plant; B=Number of mature leaves; C=Root numbers.

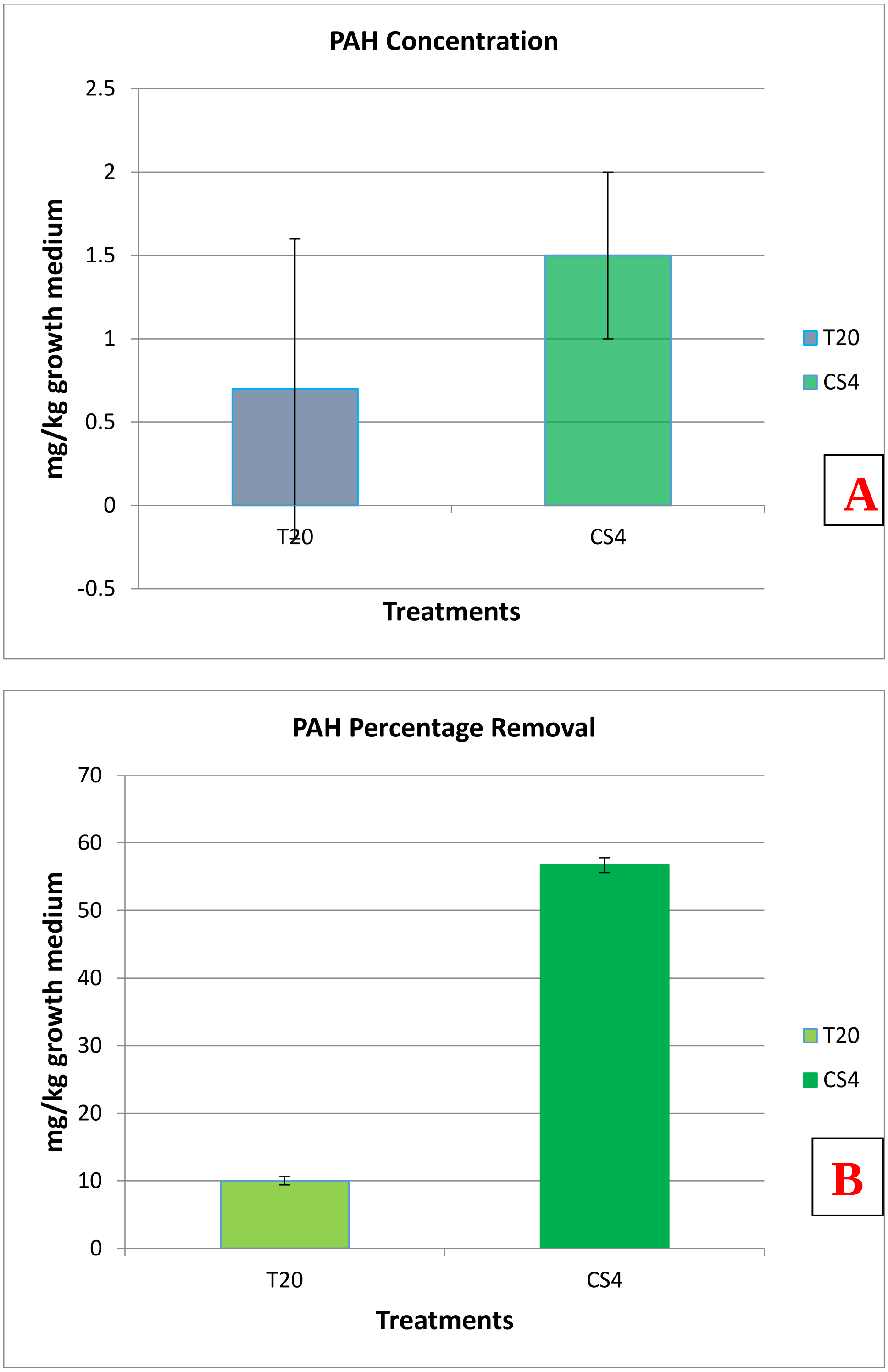

Figure 2: PAH concentration measured by GC–MS and percentage removal of PAH (A and B) in surfactant-inoculated (T20) and *B safensis* CS4-inoculated plant grown sand–peat substrate at the end of the 16-wk pot experiment. Error bars show the standard deviation. Mean values marked by the same letter are not significantly different at $P < 0.05$.

Growth parameters of tobacco plants measured after 16 wk of growth with PAHs in the growth substrate as well as in the absence (control) and in the presence of the bacterial inoculum (*Bacillus safensis* CS4) are reported in Fig. 1. As can be seen, PAH amendment exerted negative effects on non-inoculated plants in terms of both leaf number and stem length. In fact, a decrease of about 65% ($P < 0.01$), 54% ($P < 0.05$) and 60% ($P < 0.01$) was measured in the leaf number, stem length and root number respectively, in PAH treated plants comparing with the untreated ones. On the other hand, although the inoculation with *B. safensis* CS4 caused a strong change in growth parameters measured in plants grown in PAH-contaminated growth medium in respect to the non-inoculated ones, growth stability should be ensured once plant is grown in the presence of PAHs.

4. Discussion

The relationships between plants and their associated microorganisms are different and complex. Rhizospheric bacteria refer to bacteria living on the surface or around the roots, in contrast to epiphytic bacteria living on the leaves [11,24]. On the other hand, endophytic bacteria can be defined as bacteria colonizing the internal tissues of plants without causing negative effects on their host [18,25]. In the last decade, endophytic microorganisms have been taken into account for applications in assisted phytoremediation protocols. In fact, several studies have focused on the use of constructed plant-associated bacteria. On the other hand, catabolic genes and plant growth promoting mechanisms occurring in natural endophytic bacteria are also used for bioremediation purposes [26].

This study demonstrates that a bacterial isolate, originally identified for it's PAH degrading capacity; Bacillus safensis CS4, can infest *N. tabacum* plant and consequently improve the phytoremediation efficiency of PAHs. Germaine et al. [8] already reported the infection of nonendophytic, naphthalene-degrader strain *Pseudomonas putida* G7 in pea plant. However, the authors reported a weak phytoprotection effect of this inoculum towards plants exposed to naphthalene. This slight effect was due to the low root colonization of G7 strain (about 101 CFU g-1). Conversely, in the present study *B. safensis* CS4 was shown to have the capability of reaching a large population size both in PAH-treated and untreated *N. tabacum* roots, higher than that observed in the case of *P. putida* G7 [7-8,26].

5. Conclusion

Bacillus safensis CS4 according to this present study is found to ensure rapid and effective degradation of perylene within the plant in this case *N. tabacum* despite the unavailability of existing literatures using the plant together with the strain on bacteria. This means that the use of endophytes of this clad to enhance the phytoremediation of petroleum hydrocarbon is encouraged.

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