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Full Length Research Article

Study of Heavy Metal Tolerance and Plant Growth Promoting Ability of *Frankia* Species Isolated from Root Nodules of *Casuarina* in Palghar, Maharashtra

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Abstract

Actinorhizal plants such as *Casuarina* offer the dual benefit of increasing the nitrogen content of soils as well as bringing about phytoremediation of heavy metal contaminated soils. These plants and their bacterial symbionts (*Frankia* spp.) have been studied in many parts of India, particularly in the southern states. However, to the best of our knowledge, no such study has been conducted in Maharashtra. In this work, two *Frankia* spp. were isolated from the root nodules of *Casuarina* trees from Palghar region in Maharashtra. The isolates, named FDM and FTW, were found to be closely related to *Frankia asymbiotica* and *Frankia sp. Cal.*1, respectively. Both the isolates were found to be heavy metal tolerant (0.5 mM of Cd, 1 mM of Cu, 2 mM of Pb and Zn, and 0.5-1 mM of Ni). They were also found to enhance seed germination of *Casuarina equisetifolia*. To the best of our knowledge, the effect of *Frankia* on seed germination has not been reported in any previous study. The *Frankia* isolates FDM and FTW also enhanced the stem length of *Casuarina equisetifolia* plantlets by 184% and 52.28% respectively. Since the isolates show the dual advantages of heavy metal tolerance and plant growth promoting abilities, they have the potential to be used for promoting the growth of *Casuarina* plants, which in turn can be employed for phytoremediation of heavy metal-contaminated soils.

***Key words*:** Casuarina, *Frankia* spp., Heavy metals, Phytoremediation, Nitrogen fixation

Nitrogen-fixing actinorhizal trees such as *Casuarina* are widely planted in tropical and subtropical regions around the world. In agro and farm forestry, they are used as windbreaks and shelterbelts to protect agricultural fields. *C. equisetifolia* is the most widely planted and domesticated species of *Casuarina* in India [5].*Casuarina* farming is very popular in the coastal regions of India as it buffers the economy against crop failure and drought [4]. Coastal *Casuarina* plantations have the potential to protect agricultural lands from salt spray [1-2]. In addition, *Casuarina* farming also has several ecological and socioeconomic benefits. These trees are generally planted as pioneer species in degraded and dry sandy soils where they help reclaim the sites through atmospheric nitrogen fixation. The growth of *Casuarina* increases soil fertility through complex, pedogenetic processes which, in turn, can help the growth and establishment of other plant species [3].

One of the ecological advantages of *Casuarina* plants is that they can help reclaim metal-contaminated and salinized land [6-8]. The coastal regions of India are contaminated with heavy metals [9-12]. It is a well-known fact that the presence of alleviated levels of certain metals in soil can cause phytotoxicity, which in turn can reduce the productivity of soils and negatively affect the entire ecosystem. *Casuarina* plants have the ability to grow in saline as well as heavy metal laden soils at higher levels than their counterparts [13].This characteristic is attributed to their symbiosis with the actinomycete *Frankia* which can produce a variety of metallophores that help in the detoxification of potentially toxic elements [14]. Besides, *Frankia* has the ability to fix atmospheric nitrogen under both free-living and symbiotic associations. All these advantages make *Frankia* an important candidate as a bio-fertilizer to improve the growth of *Casuarina* plants, which in turn can be used for the reclamation of salinized and heavy metal contaminated lands [15].

Studies on actinorhizal plants and their symbionts have been widely reported from the coastal areas of Tamil Nadu and Andhra Pradesh in South India. To the best of our knowledge, no such study has been reported from in the state of Maharashtra. Thus, the present study was undertaken to isolate and identify heavy metal tolerant and plant growth-promoting species of *Frankia* from the Palghar region of Maharashtra, India.

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MATERIALS AND METHODS

Collection of root nodules and surface sterilization

Healthy root nodules of *C. equisetifolia* were collected from Suruchi beach, Palghar district, Maharashtra, India. (19.340973, 72.791050). The root nodules were collected in a clean zip-lock bag, stored in an ice pack container and transported to the laboratory the next day [17].The nodules were washed thoroughly in running tap water for approximately 5 minutes followed by washing with distilled water for 2 minutes. They were dissected into individual lobes and exposed to 6% sodium hypochlorite containing 0.1% Tween-80 for 10 minutes [18].They were then subjected to surface sterilization using 0.1% mercuric chloride for 10 minutes. Finally, the nodules were washed thoroughly with sterile distilled water three to four times to remove traces of mercuric chloride.

Enrichment and isolation of Frankia

The surface sterilized nodules were suspended in saline and crushed using a sterile glass rod. The suspension was then homogenized using a vortex and inoculated in two enrichment media viz. Frankia Defined Minimal (FDM) medium and Frankia Tween medium (FTW) [19-20]. The carbon sources in FDM and FTW media are sodium succinate and Tween-80 respectively, both of which are selectively metabolized by *Frankia* spp. FTW medium helps in the selective enrichment of *Frankia* because it has ferric citrate that inhibits the growth of other bacteria. The enrichment flasks were incubated at 30 °C for two weeks along with uninoculated control flasks. The enriched broth was then isolated on sterile Qmod agar plates [21], which were incubated at 30 °C till visible colonies developed.

Identification of Frankia

Preliminary identification of *Frankia* was based on microscopic observation using the micro chamber agar spot technique [22], observation of colony morphology on Qmod agar plates, results of catalase test [23] and the ability to grow in nitrogen-free media such as Jensen's and Burk's medium [24]. Confirmatory identification was carried out using 16S rRNA sequence analysis, using *Frankia*-specific primers viz. 5′-TTGATGGAGAGTTTGATCCTGG-3′ and 5′-AGAAAGGAGGTGATCCAGC-3′ for gene amplification [25]. The PCR products were purified and sequenced using Genetic Analyzer, Applied Biosystems. The sequences obtained were compared with sequences in the National Center for Biotechnology Information (NCBI) using the BLAST program.

Effect of temperature on growth of the isolates

The effect of temperature on the growth of the *Frankia* isolates was determined by growing them in Qmod broth at 30 °C, 37 °C and 55 °C for 10 days, and comparing the turbidity with that of uninoculated controls.

Heavy metal tolerance of the isolates

The tolerance of the *Frankia* isolates to heavy metals such as $CdCl_2$, $CoCl_2$, $CuCl_2$, $Pb(NO_3)_2$, $NiCl_2$, $AgNO_3$ and $ZnSO_4$ was studied by spot inoculating them on Qmod agar plates containing 0.1, 0.5, 1.0, 2.0 and 5.0, millimolar (mM) concentrations of each heavy metal. The highest concentration of each heavy metal showing growth of the isolate was considered as the tolerance level for that metal [26].

Effect of bioinoculum on seed germination and plant growth

A pot study was carried out to determine whether a bioinoculum prepared from the *Frankia* isolates had any positive effect on seed germination and growth of *C. equisetifolia*. A 1:2 mixture of cocopeat and sieved sand was used as the potting medium. The mixture was sterilized by autoclaving at 121 °C and 15 psi for 20 minutes. Approximately 500 grams of this mixture was added to each of the three disinfected pots.

The seeds of *C. equisetifolia* were carefully plucked from mature fruits which had been dried in sunlight for two days (Fig 1). The seeds were surface sterilized by the same method as that used for the sterilization of the root nodules.

Fig 1 Fruits and seeds of *C. equisetifolia*

150 seeds were sown in each of the three pots. In order to prepare bioinoculum, the two *Frankia* isolates (FDM and FTW) were grown separately in 100 mL of sterile Qmod broth for one week. 50 mL of each broth culture was added to separate 'Test' pots (Pots 2 and 3) as a bio-inoculum. Pot 1 was maintained as a control; 50 ml of sterile Qmod broth was added to it. All the pots were kept for a period of one month to allow seed germination and plantlet development [27-28]. The moisture content in the pots was maintained by adding distilled water. The seed germination index was calculated as the ratio of the number of seeds germinated to the number of seeds potted. At the end of 1 month, the shoot length was measured from the surface of the potting medium to the apex of the plant.

Statistical analysis

A descriptive analysis of the data was performed. The *Z*-test was used to compare the germination index of the seeds in the control and test pots. The shoot lengths of the three treatments were checked for normal distribution using the D'Agostino's K-squared Test. The variances of the three groups were calculated using the Levene's test. Further a one-way ANOVA was performed to compare the effect of the treatments (isolates) on the shoot length. Post-hoc tests such as Tukey's HSD and unpaired t-tests were used to identify whether the treatment with the bioinoculum had a significant effect on the tested parameters. The statistical analysis was performed using VassarStats statistical computation website (http://vassarstats.net/) [29].

RESULTS AND DISCUSSION

Screening, enrichment and isolation

Root nodules of *C. equisetifolia* were used for the isolation of *Frankia.* The gram staining of root nodule suspension showed the presence of gram-positive filamentous structures typical of actinomycetes morphology (Fig 2A-B).

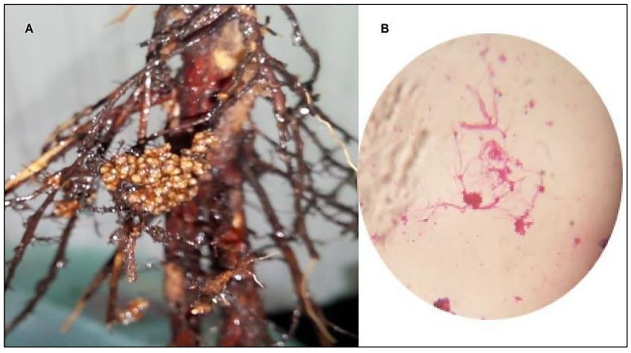

Fig 2 (A) Root nodules of *C. equistelifolia*; (B) Gram staining of root nodule suspension showing filamentous structures typical of actinomycetes

Enrichment of *Frankia* from the root nodule suspension gave visible growth in FDM and FTW media after 10 and 13 days of incubation respectively (Fig 3). Subsequent enrichment in Qmod broth showed visible growth after one week of incubation.

Fig 3 FDM and FTW broth after 13 days of inoculation with root nodule suspension

Isolation of the enriched broth on sterile Qmod agar plates gave two different isolates after incubation for 2 weeks. The isolates were named as FDM and FTW on the basis of the medium in which they were enriched. Both FDM and FTW produced circular to oval colonies with an irregular edge. The colonies of FDM were chalky white and leathery while those of FTW were yellow-centered and tough to remove from the agar surface. These characteristics match those reported for *Frankia* by Lechevalier and Lechevalier in 1984 [30].

Identification of the isolates by microscopic, biochemical and molecular techniques

The micro chamber agar spot assay technique revealed that both FDM and FTW had the typical filamentous structure of *Frankia* spp (Fig 4). Gram staining of the isolates showed the presence of Gram-positive filaments.

Both the isolates were catalase positive and showed growth in the form of visible turbidity in nitrogen-free Jensen's medium and Burk's medium after 10 days of incubation. Spot inoculation on Burk's agar medium resulted in visible growth after 12 days of incubation, as opposed to *E. coli* which failed to grow on the medium. These results indicate that both FDM and FTW isolates have the ability to fix atmospheric nitrogen in the free-living state. The members of the genus *Frankia* are well known for their ability to fix atmospheric nitrogen under free-living conditions and inside the root nodules as a symbiotic association [31].

Both FDM and FTW were identified as *Frankia* spp. by 16S rRNA sequence analysis. FDM was closely related to *Frankia asymbiotica* while FTW was related to *Frankia sp. Cal.*1. The sequence data of both FDM and FTW were submitted to GenBank, NCBI. The sequences have been accepted and provided with GenBank accession numbers OQ152027 and OQ152031, respectively.

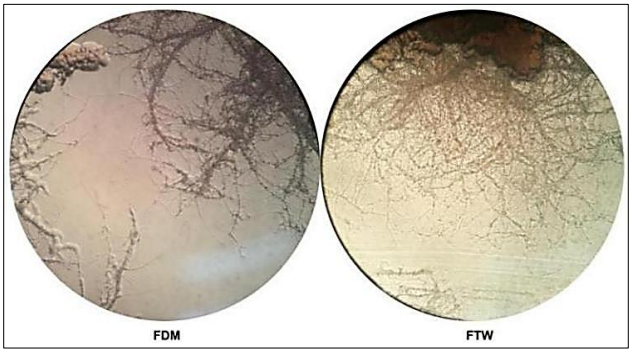

Fig 4 Filamentous morphology of FDM and FTW observed under 40X magnification

Effect of temperature

The effect of temperature on the growth of FDM and FTW was studied. Luxuriant growth was observed at 30 °C as indicated by turbidity in the growth medium along with biomass scum formation on the surface of the medium; moderate growth was observed at 37 °C and scanty growth at 55 °C with no surface growth.

Heavy metal tolerance

The heavy metal tolerance of FDM and FTW is represented in (Table 1, Fig 5).

Table 1 Heavy metal tolerance of FDM and FTW isolates

Heavy metal	Tolerance. (mM)	
	FDM	FTW
Pb	2	2
Zn	2	2
Cu	1	1
Ni	1	0.5
Cd	0.5	0.5
Ag	<0.1	<0.1
Co	<0.1	<0.1

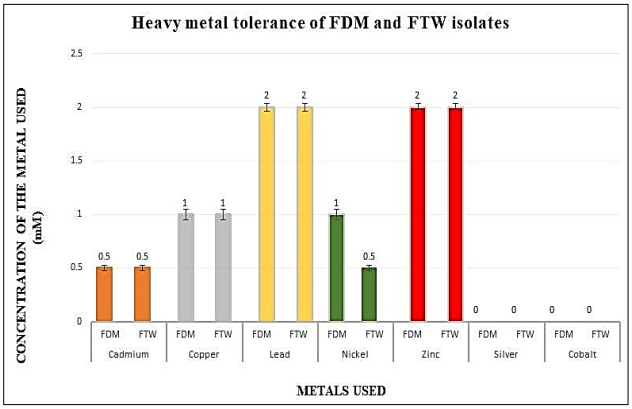

Fig 5 Heavy metal tolerance of FDM and FTW isolates

The Zn, Pb and Cu tolerance of FDM and FTW were found to be much higher than those reported by Abdel-lateif *et al*. [32]. Of the nine *Frankia* strains studied by them, eight strains could tolerate 0.5 mM Zn, two strains could tolerate 0.1 mM Pb, and eight strains could tolerate 0.1 mM Cu. However, three of their isolates could tolerate 0.1 mM Co. Richards *et al*. 2002, have studied the sensitivity of 12 *Frankia* strains to heavy metals by a growth inhibition assay. All the 12 strains were sensitive to <0.5 mM of Ag, Cd, and Ni, but were less sensitive to Pb (6 to 8 mM) and Cr (1.0 to 1.75 mM). Furthermore, 8 strains were sensitive to 0.1 mM Cu, but three strains designated DC12, EuI1c, and CN_3 were resistant to 2 to 5 mM of Cu and one strain designated CN3 could tolerate as high as 20 mM Cu. They suggested that the mechanism of Pb and Cu resistance may involve sequestration or binding mechanisms [26]. Michael *et al*. (2019) have carried out metallophore profiling of *Frankia* spp. to understand metal management in the rhizosphere. In their comprehensive study, they have highlighted that *Frankia* produces a variety of metallophores to manage metal stress and metal uptake. This metallophore-based management of metals contributes to the fitness of both symbionts, as well as provides essential elements to maintain the performance of their nitrogen-fixing symbiosis [14].

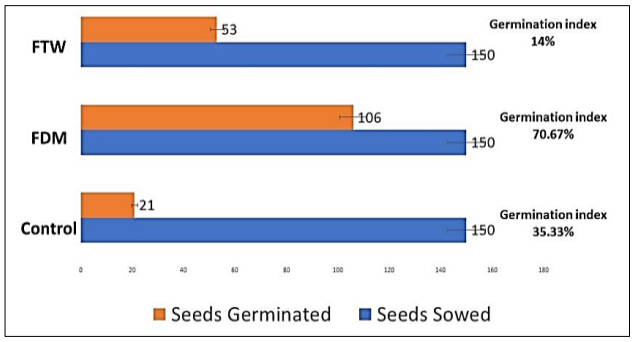

Fig 6 Comparison of seed germination index of treated v/s untreated plants

Fig 7 Germination of of *C. equisetifolia* seeds 10 days after sowing

Descriptive statistical analysis of the seed germination results was carried out. All three pairwise two proportion *Z*-tests (one-tailed) between the germination indices of the three groups revealed significant differences (alpha = 0.01). In particular the results showed that the germination index of both FDM treated and FTW treated seeds were significantly higher than that of the untreated control seeds. Also the germination index of the FDM treated seeds was significantly higher than that of the FTW treated seeds. To the best of our knowledge, no study has been reported on the effect of Frankia on seed germination. Our study shows that *Frankia* spp. can enhance the germination of *C. equisetifolia* seeds.

Effect of Frankia bio-inoculum on the shoot length of C. equisetifolia plantlets

The mean shoot length of the untreated control plants was 2.85 cm, while that of the FDM treated and FTW treated plants were 8.10 cm and 4.34 cm respectively (Table 2, Fig 8). Thus, inoculation of *Frankia* FDM and FTW bioinoculum improved the growth of *C. equisetifolia* seedlings by 184% and 52.28% respectively as compared to the uninoculated control plants.

Table 2 Effect of *Frankia* bio-inoculum treatment on shoot length of *C. equisetifolia*

Parameter	Untreated Control	FDM Bioinoculum treatment	FTW Bioinoculum treatment
Mean shoot length (cm)	2.85	8.10	4.34
Variance of shoot length	1.64	1.42	1.59
Sample size	21	106	53

Fig 8 Comparison of shoot lengths of *C. equisetifolia* plantlets after one month

The shoot lengths of the three samples were found to be normally distributed using the D'Agostino's K-squared Test (at alpha= 0.01). Variances of the three groups had no significant difference (Levene's Test). A one-way ANOVA with the three groups (2 degrees of freedom) and a total sample size of 180 (177 degrees of freedom) had an observed F value of 176.91 with F (2, 177) = 4.605, p = 0.01. It revealed that there was a statistically significant difference in the shoot lengths between at least two treatments. Unpaired t-tests revealed statistically significant mean differences between the shoot lengths of the FDM treated plants v/s Control plants (t = -15.05, p <0.0001), FTW treated plants v/s Control plants (t = -3.57, p < .0001) and FDM treated plants v/s FTW treated plants (t = -15.124, p <0.0001). Tukey's HSD Test for multiple comparisons found that the mean value of shoot lengths was significantly different between all pairs of treatments. The p-value corresponding to the F-statistic of one-way ANOVA is lower than 0.01 which strongly suggests that one or more pairs of treatments are significantly different. From the above results, we can say that

on an average, the shoot lengths were significantly higher for the treated plants as compared to the untreated control plants. Also, FDM treatment resulted in a significantly higher shoot length as compared to FTW treatment.

Our results support the findings of Muthukumar and Udaiyan, 2010, who have reported 40% improved growth of *C. equisetifolia* seedlings with *Frankia* bioinoculum application as compared to uninoculated control [35]. The effect of four different *Frankia* spp. on the growth of *C. cunninghamiana* were studied at the nursery, Forestry Research Centre, Zimbabwe. The *Frankia* strains were inoculated separately to one month old *C. cunninghamiana* plantlets potted in nitrogen deficient soil. Plant heights were measured after fourteen months. Three Frankia strains increased the plant height in the range of 50% to 70%, while the fourth Frankia strain increased plant height almost three times in comparison to the uninoculated controls [36]. The increased growth of *C. equisetifolia* plants in presence of *Frankia* can be attributed to a range of plant growth-promoting substances produced by *Frankia* in addition to its nitrogen fixation. Marappa *et al*. (2020) have reported the production of various plant growth enhancement mediators by *Frankia* spp. such as IAA, methyl 4-hydroxybenzoate, dodecanoic acid, and some novel flavonoids [18].

CONCLUSION

The two *Frankia* strains used in our study, viz. FDM and FTW, were found to be heavy metal tolerant as well as possess plant growth promoting abilities. Both the strains significantly enhanced the seed germination index (FDM – 70% and FTW – 14%) as well as the average stem length (FDM - 184% and FTW - 52.28%) of *C. equisetifolia* plantlets. Thus, we conclude that both the strains have the potential to be used as bio inoculum to enhance the seed germination and growth of *C. equisetifolia*, though FDM shows greater promise. The results merit further investigation on the plant growth promoting abilities of the two strains, separately as well as in combination. The symbiotic association between the *Frankia* strains and *C. equisetifolia* can be used for the phytoremediation of heavy metal-laden soils which are common in coastal India. Further, the *Frankia* strains can be studied for their ability to remove heavy metals from wastewater.

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