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A STRATEGY TO PROMOTE GROWTH OF CROP PLANT USING PLANT GROWTH PROMOTING FUNGI (PGPF) ISOLATED FROM THE ROOT OF CASUARINA JUNGHUHNIANA

PRIYOM BOSE[1] AND S.UMA GOWRIE[2]

[1]Research Scholar, Department of Plant Biology and Plant Biotechnology, Ethiraj College for Women (Autonomous), Chennai 600008, Tamil Nadu.
[2]Associate Professor, Department of Plant Biology and Plant Biotechnology, Ethiraj College for Women (Autonomous), Chennai 600008,Tamil Nadu.

ABSTRACT

Endophytes are microorganisms which reside asymptomatically within a healthy plant without causing any detectable negative effect to the host plant. Plant Growth Promoting Fungus enhances plant growth through the production of secondary metabolites like indole acetic acid, siderophore, phosphate solubilization, Hydrogen Cyanide (HCN) and prevents the plant by its host defence interaction. In the present study, six endophytic fungi were isolated from the root of *Casuarina junghuhniana* (Casuarinaceae) which is an exotic actinorhizal multipurpose tree. Each fungal isolates were screened for its production of plant growth promoting traits. Anti-fungal activity was also studied against the fungal phytopathogens. Out of all the isolates, CJK1 which is identified as *Aspergillus sp* had the best plant growth enhancement traits.The effect of the potential isolate was tested for its plant growth promoting traits using *Vigna radiata*. Both the cell suspension and cell free broth of *Aspergillus sp* was treated with surfaced sterilized seed of *Vigna radiata*. The growth parameters (root-shoot length) were studied and compared with uninoculated control. These results imply that the potential fungal endophyte, *Aspergillus sp,* acts as a promising bioinoculant in the agroforestry system for improvement of commercially important crop plants.

KEYWORDS: Endophyte, *Casuarina junghuhniana*, PGPF, antifungal.

S.UMA GOWRIE
Associate Professor, Department of Plant Biology and Plant Biotechnology,
Ethiraj College for Women (Autonomous),
Chennai 600008,Tamil Nadu

***Corresponding author**

INTRODUCTION

Endophytes are microorganisms which reside asymptomatically in a specific chemical environment of the host plant .[1] Often endophytes are found to share similar genetic and molecular machinery with that of its host plant for synthesis of plant specific bioactive compounds. In the recent past, endophytes have become a prominent source of various natural products.[2, 3] In higher plants, several endophytic fungi and bacteria are able to synthesise plant growth promoting agents like indole acetic acid (IAA).[4-6] Plant growth promoting fungus enhances growth of the plant by production of indole acetic acid, phosphate solubilisation and by producing siderophore.[7-9] Endophytic fungi residing within the plant, growing in adverse condition possesses plant growth promoting properties and their potential fungi can be utilised to increase the production of industrially important plants growing in the same environment.[10,11] Keeping in view of these facts, growth promoting properties of the fungal endophytes which was isolated from the root of *Casuarina junghuhniana* were studied. *Casuarina junghuhniana* is an actinorhizal plant belonging to the family Casuarinaceae. This tree species is known to be a pioneer species of deforested land such as rocky, undulated slopes and undistributed regions .[12] It is known to have high fertility and impressive growth rate at varied soil types and climatic conditions.[13] This tree crop plays an important role in land reclamation, vegetative shelter bed , bio shield and can improve soil fertility in tropical and sub-tropical region .[14] This tree crop is rich in various phytoconstituents like carbohydrates, alkaloid, phenolics, flavonoids, tannin, steroids and triterpenoids.[15,16]*C.junghuhniana* has become a much preferred crop for the farmers of South India because of its fast growing behaviour in both coastal and inland sites. This tree crop has high calorific value for wood therefore they are desired crop for biomass power plants. Further, it has good pulping traits which are essential for manufacture of papers. IFGTB (Institute of Forest Genetics and Tree Breeding) reported that *C.junghuhniana* has significant faster growth and high yield compared to local Casuarina (*Casuarina equisetifolia*).The objective of this study is to isolate fungus from the root of *Casuarina junghuhniana* and to screen the isolates for its growth promoting trait, i.e, production of IAA, siderophore, HCN, ammonia and phosphate solubilisation. Antifungal activity of the potential isolate was also assessed to evaluate the bioactive potential in the field of plant disease management. Further, the effect of the potential isolate on the germination and growth (root-shoot length) of seeds of *Vigna radiata* was studied.

MATERIALS AND METHODS

Source of endophytic fungi

The plant root material of the *C. junghuhniana* (2-4yrs) was collected from State Forest Research Institute, Kolapakkam, Chennai, Tamil Nadu. The root samples of the plant were collected in random manner at the plantation site, kept in a sterile polythene bags and stored in ice box. The root samples were transported to laboratory within 3 hrs of collection and stored at 4˚C until further isolation procedure was completed. Identification (authentication) of the plant samples were confirmed at Botanical Survey of India (BSI), Coimbatore, Tamil Nadu.

Isolation of endophytic fungi

The root samples were washed in running tap water for 10 minutes to remove soil particles and adhered debris. These were rewashed several times in distilled water. The root samples were cut into thin sections of about 0.5mm. The tissue segments were sterilized using the modified method of Fisher *et al*.[17] The sterilized tissue samples were individually inoculated in Potato Dextrose Agar (PDA) medium, purchased from Himedia, contained in Petri plates supplemented with Streptomycin (100 mg/L). These Petri plates were incubated at 37˚C for 3 days. After attaining visible growth, the fungal colonies were sub cultured in PDA plates and stored at 4˚C.

Identification of endophytic fungi

The identification of the endophytic fungus was done at genus level on the basis of the colony morphology, pigmentation, spores at the hyphal tip using standard manual.[18]

Fermentation and extraction

Two or three pieces of the grown culture were cut out from the culture plate and were inoculated in a 250ml Erlenmeyer flask containing 150ml Potato Dextrose Broth (PDB), purchased from Himedia, for 21 days at 30˚C. The fungal broth culture was filtered to remove the mycelium. The filtrate was extracted with ethyl acetate solvent (1:1 ratio) three times. The organic phase was evaporated to dryness and stored at 4˚ C for further studies. The crude culture filtrate extract was dissolved in DMSO to obtain different concentration.

Determination for the production of IAA

The fungal isolates were sub cultured on Potato Dextrose Agar (PDA) medium and was grown for the period of 5 days. After which, each of the isolates were inoculated in Potato Dextrose Broth (PDB) medium incubated for 7 days in a shaker at 150 rpm/min and was centrifuged at 7000 rpm for 20min. The supernatant was utilised and the pellet was discarded. Production of IAA by the fungal endophytes were determined by the method used by Bhagobati and Joshi .[19] In 1ml of the aliquot of the supernatant, 4ml of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% Perchloric acid) was added and was kept at room temperature for 20min. The presence of IAA was confirmed with the observation of colour change and absorbance at 535nm using UV spectrophotometer (UV 1650PC Shimadzu).[20] The concentration of IAA was determined by using IAA standard curve.

Determination for Siderophore production

Siderophore production was determined using Chrome Azurol S agar (CAS) medium .[21] Fungal isolates positive for the production of siderophore produces an orange halo around the fungal growth.

Determination for phosphate solubilisation
Each fungal isolates were inoculated in Pikovskaya (PVK) agar medium without rose bengal for the period of 3 days at 28˚C with continuous observation for colony diameter.[22] The phosphate solubilizing fungus was identified by its formation of clear zone around their colony.

Determination for the production of HCN
Each isolates was inoculated on PDA medium containing 4.4g glycine/l. Isolate positive for HCN production were noted by change of colour of the inoculated filter paper from yellow to orange brown .[23]

Determination for the production of ammonia
Fungal isolates were inoculated in 10ml PDB and was incubated for the period of 5 days at 30˚C in rotatory shaker. 0.5ml of Nesseler's reagent was added in each culture tubes. Development of yellow to brown colour determines the production of ammonia.[24]

Effect of the fungal isolate on seed germination and plant growth promotion
The fungal isolate showing maximum plant growth promoting traits would be selected to test its effect on seed germination and plant growth (root-shoot length) on the seed of *Vigna radiata.* The seed of *Vigna radiata* was surfaced sterilized using 1% NaOCl for 1min and was then thoroughly washed in distilled water and dried in sterilized filter paper. 50 sterilized seeds were then soaked for 24hr in selected endophytic fungal suspension. After 24hrs, the treated seeds were transferred to a sterilized petri plate containing sheets of moistened filter paper. Another 50 sterilized seeds were treated with cell free broth culture of selected isolate (the fungal endophyte was grown in PDB medium for 5 days) .[19, 25] Control was maintained by treating the sterilized seeds with sterile water.[26] The germination percentage and the root-shoot length was studied after 24hrs incubation and continuously observed for five days from the initial day of incubation.

Antifungal activity
The fungal phytopathogens *Curvularia sp* (2030), *Alternaria sp* (2101) and *Fusarium sp* (4894) were obtained from MTCC, revived and maintained on PDA slants for further analysis. Fungal spore suspension (100 µl) was swapped on their respective PDA plate and wells (7mm) were bored on the agar surface using sterilized cork borer. Each well was loaded with 50µg and100µg concentration of the crude broth extract. Ethyl acetate solvent was used as negative control and Gentamycin (100 µg) was used as positive control. Triplicates were maintained for all the samples. The plates were incubated for 5 days at 28˚C and the zone of inhibition was observed and measured.

Statistical Analysis
The data presented are the means of three replicates. Promotion of growth (root-shoot length) of *Vigna radiata* was determined by statistical significance using Student's *t*- test.

RESULTS AND DISCUSSION

Plant root system is a rich habitat for microorganisms which are often explored to obtain beneficial microbes useful for the formulation of bioinoculants for promotion of growth and yield of commercially important plant.[27] The microbial community within the root system depends on the root type, plant species and the soil condition on which the host plant is grown .[28] *Casuarina junghuhniana* is a multipurpose tree crop which has a unique ability to sustain in nutrient deficient soil and extreme climatic conditions. In the present study, six different endophytic fungi were screened for its growth promoting traits. The isolates were designated as CJK1, CJK2, CJK4, CJK6, CJK7 and CJK8 (Table 1). Plant growth promoting fungi (PGPF) enhances plant growth by its production of IAA, phosphate solubilisation, siderophore production, production of HCN and ammonia thereby promoting an increase in yield of important crop plant .[29]

Table 1
Plant Growth Promoting traits of fungal root endophyte of Casuarina junghuhniana

Fungal Isolate	Plant Growth Promoting Factors				
	IAA	Siderophore	Phosphate Solubilization	HCN	Ammonia
CJK1	+	+	+	+	+
CJK2	+	+	+	-	+
CJK4	+	-	+	+	+
CJK6	-	-	+	+	+
CJK7	+	+	-	+	+
CJK8	-	+	+	+	+

Among all the fungal isolates, four isolates showed positive result in the production of indole acetic acid (Table 1). The concentration of IAA produced by CJK1is 40µg/ml, CJK2 is 47.3µg/ml, CJK4 is 36.7µg/ml and CJK7 is 54µg/ml. IAA is a phytohormone which is considered to be most important form of auxin .[30] IAA acts as a signal molecule in the process of the development of plant including organogenesis and various cellular responses like cell differentiation, cell division, expansion and genetic regulation.[31]Many endophytic microorganisms are able to produce IAA in the presence of precursor L tryptophan .[32]In the present study, four endophytes were able to produce IAA even in the absence of tryptophan in their growing medium. In earlier studies, production of IAA by endophytic fungi namely *Fusarium sp* and *Aspergillus sp* has also been reported .[30]The production of siderophore was screened using chrome azurol S agar. In four of the isolates growing in CAS agar medium (Table1), orange halo appeared around these fungal colonies due to the chelation of iron, indicated the presence of siderophore. Siderophore plays an important role as a biocontrol

agent and thereby in suppression of diseases in host plant.[33]Siderophores have a greater affinity for iron and thus reduces its availability which is essential for the growth of phytopathogens , hence, such character indirectly serves as a plant growth promoting factor .[34]*Aspergillus niger* and *Aspergillus flavus* are reported to produce siderophore in both solid and liquid medium .[30]Phosphorus is an important macro element which is essential for the growth of plants. Therefore, plant growth is restricted in phosphorus deficient condition. Fungus are reported to be more promising in phosphate solubilisation in comparison to bacteria .[35]In the present study, five fungal isolates were able to solubilize phosphate which was determined by the presence of clear zone around the fungus grown in Pikovskaya agar medium containing tricalcium phosphate (Table1). *Penicillum sp* and *Aspergillus sp* are reported to be the most important genera which are used for the solubilisation of phosphates .[36,37]HCN production was determined by observing the change in colour of the filter paper from yellow to brown after the period of incubation. Five of the isolates showed positive result in the production of HCN (Table1). HCN is a low molecular weight metabolite which has a characteristic antagonistic property .[33]The production of HCN so far is reported mainly in bacterial endophytes .[38] *Pseudomonas aeruginosa* has showed positive result in production of HCN .[39]In the present study, all the isolates were able to produce ammonia (Table1).The production of ammonia was determined by the development of yellow to brown colour after its reaction with Nesseler's reagent. *Fusarium moniliforme* is a fungal endophyte which showed similar result for the production of ammonia .[30]It was reported that ammonia might have positive effect in plant root growth. [40]Fungal isolate CJK1, identified as *Aspergillus sp,* was found to give positive result for all growth promoting characteristics (Table 1, Figure 1: a-e). Hence, this potential fungus was selected to test its effect on the germination and growth (root-shoot length) using seeds of *Vigna radiata. Aspergillus sp* was identified through its colony morphology and microscopic study (Figure 2a & 2 b).The identified endophyte, *Aspergillus sp*, will be sequenced using 18S rRNA, and the sequence will be submitted to NCBI database to confirm its identity (species level).

Figure1
a) Test for IAA production
b) Orange halo for siderophore production
c) Clear zone for phosphate solubilization
d) Test for HCN production
e) Test for ammonia production

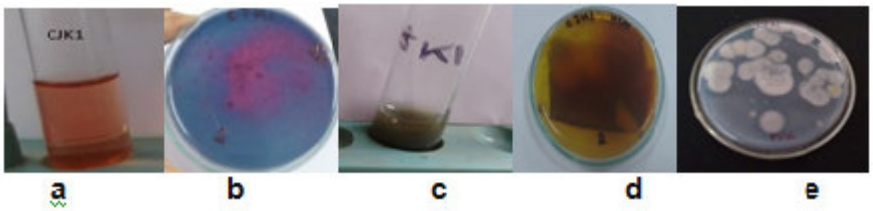
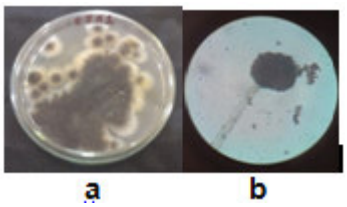


Figure 2
a) Colony morphology of CJK1
b) Microscopic structure of CJK1



It was observed that the seeds treated with cell free broth culture gave maximum result in comparison to the seeds given fungal suspension treatment. In germination test, cell free broth culture showed 100% germination, however, seeds treated with fungal suspension either got infected or germination was inhibited after 24hrs treatment. Control showed 87% germination after 24hrs. Figure 3 shows the elongation of shoot (mm) of the seeds treated with the cell free broth culture against the control seed which was treated with sterilized water. After 72hrs, emergence of lateral root and leaf was observed on the seeds treated with cell free broth culture, no such growth was found in seeds treated with sterilized water (Figure 4).This result concludes that CJK1 may be used as a promising bioinoculant as it has a significant effect on the growth of *Vigna radiata* ($p < 0.05$) when compared with the control.

Figure 3
Growth of Vigna radiata seedlings for a period of 72hrs

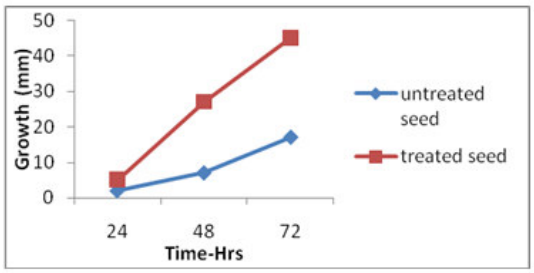
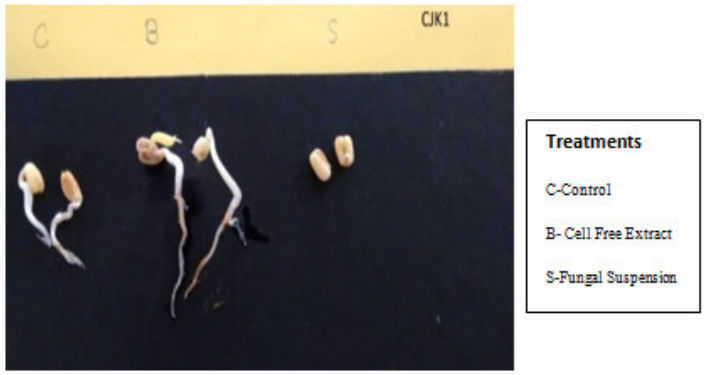


Figure 4
Growth of seeds of Vigna radiata treated with culture filtrate and spore suspension ofAspergillus sp CJK1 after 72 hrs



Antifungal activity

FIGURE 5
CJK1showing maximum zone of inhibition against Fusarium sp

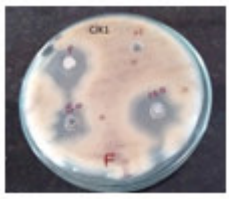
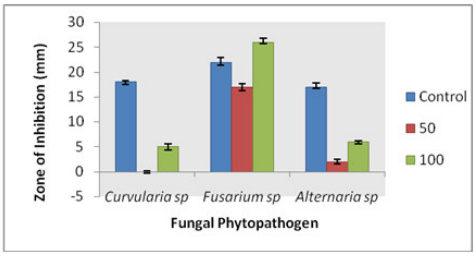


FIGURE 6
Antifungal Activity of CJK1



It was observed that ethyl acetate extract of CJK1 could inhibit the growth of all the fungal phytopathogen (Figure 5). However, maximum zone of inhibition over control was found against fungal phytopathogen *Fusarium sp* (Figure 6). It was reported that PGPF are non pathogenic in nature which acts as an potential biocontrol agent against fungal phytopathogen .[41]*Casuarina sp* is reported to be rich in terpenoid, steroid, flavonoid, phenols and tannin.[42]Bioactive constituents in ethyl acetate extract of the isolated fungal endophytes are also reported to have promising biologically active secondary metabolites which in turn is responsible for the effective antimicrobial activity.[43]The growing need for antifungal agent is due to an increase in resistance for the existing antibiotics.

CONCLUSION

In the present study, fungal root endophytes of *Casuarina junghuhniana* was isolated and screened for its growth promoting characteristics namely production of IAA, siderophore, HCN ,ammonia and phosphate solubilisation.CJK1 isolate identified as *Aspergillus sp* was found to be the best fungal endophyte with leading bioactive potential.Cell free broth culture of CJK1 showed promising growth on *Vigna radiata* over control. Antifungal activity of CJK1. was found maximum for the phytopathogen *Fusarium sp.*This implies that CJK1 can be used as a potential bioinoculant for plant growth improvement and as a bioprotectant in disease management of commercially important medicinal/vegetable/crop plant in cost effective manner. Further, this can be effectively utilised for the plants grown in agro forestry system.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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