

Antioxidants in mangrove plants and endophytic fungal associations

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Abstract

The manner in which fungal endophytes activate host stress response systems is unknown; the occurrence of additional mechanisms involved in symbiotically-conferred stress tolerance in mangrove plants (the primary producers of estuarine food chain) is also unresolved. Here, we report antioxidant potentials of four different mangroves and their endophytic fungi. We elucidated the possible roles of antioxidants in symbiotic association between mangroves and their endophytic fungi. Four different mangrove species and the predominant endophytic fungus *Aspergillus flavus* were analyzed using various *in vitro* assay systems (such as, iron chelating capacity, reducing power, and hydroxyl radicals/hydrogen peroxide/DPPH radical scavenging and inhibition of lipid peroxidation using the β -carotene-linoleate model system). *In vitro* models clearly established the antioxidant potency of extracts of mangrove plants and their respective endophytic fungi, which aids in understanding the mutualistic associations of plant and endophyte against various biotic and abiotic stresses. Re-infection analysis of endophytic *A. flavus* in tobacco plants confirmed the endophytic status of the fungus and its enhancement effect on plant growth.

Keywords: antioxidant; endophytic fungi; free radical scavenging activity; mangroves; salt stress.

Introduction

Reactive oxygen species (ROS), including free radicals such as the superoxide anion (O^{2-}), the hydroxyl radical (OH^{\cdot}), and non-free radical species like singlet oxygen (1O_2) and hydrogen peroxide (H_2O_2), are often generated as byproducts of biological reactions or from exogenous factors (Yildirim et al. 2001). When plants are subjected to environmental stresses, such as high salinity, high light intensity, temperature extremes, drought, herbicide treatment or mineral deficiencies, the balance between the production of reactive oxygen species (ROS) and the quenching activity of antioxidants is upset, often resulting in oxidative damage. Plants with high levels of antioxidants, either constitutive or induced, have adequate resistance against oxidative damage. Mangrove plants are salt-tolerant (up to 500 mM NaCl) plants, which act as primary producers in the estuarine food chain; they produce novel metabolites unique to the environment with various important economic and environmental functions (Bandarnayake 2002). Most mangrove plant studies do not consider the fact that plants in natural ecosystems have symbiotic associations with fungi. These fungi are important to the structure, function, and health of plant communities and may be responsible for the adaptation of plants to environmental stresses (Clay and Holah 1999). In addition, mutualistic symbiosis with mycorrhizal and endophytic fungi can confer salt tolerance to plants and decrease yield losses in cultivated crops grown in saline soils (Baltruschat et al. 2008).

Endophytes are hidden within healthy host plants are a poorly investigated group of microorganisms, but they represent an abundant and dependable source of novel bioactive compounds with huge potential for exploitation in a wide variety of medical, agricultural, and industrial areas (Tan and Zou 2001). Although there have been a number of studies on the biological activities of endophytes, such as antiviral, anticancer, antidiabetic and antimicrobial effects, very little is known about their antioxidant activities (Strobel et al. 2002). Moreover, the only known anti-stress biochemicals produced by endophytic fungi are alkaloids that decrease plant herbivory (Rodriguez et al. 2004). Few studies have shown how endophytes activate host stress response systems to various abiotic stresses like temperature (Redman et al. 2002), salt (Baltruschat et al. 2008), drought (Swarthout et al. 2009), and biotic stresses caused by fungal pathogens (Tian et al. 2008). However, the mechanism by which endophytes activate host stress response systems are unknown, and the occurrence of additional mechanisms involved in symbiotically-conferred stress tolerance in mangrove plants has not been demonstrated. Thus, in the present study we analysed the antioxidant potential in four different

mangrove species and their dominant endophytic fungus *Aspergillus flavus* Link, employing various *in vitro* assay systems, (such as the β -carotene-linoleate model system, iron chelating capacity, reducing power, and hydroxyl radicals/hydrogen peroxide/DPPH radical scavenging) to determine the symbiotic association of host and endophyte acclimation under various biotic and abiotic stresses.

Materials and methods

Chemicals

Sodium bicarbonate, Folin–Ciocalteu reagent, Gallic acid, Sodium nitrite (NaNO_2), Aluminium chloride (AlCl_3), Sodium hydroxide (NaOH), Quercetin, 1, Ethanol, xylenol orange, Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT) were obtained from Merck, Mumbai, India. 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma- Aldrich (Steinheim Germany). H_2O_2 , HPLC grade methanol, Ammonium ferrous sulphate, deoxy ribose, Ferric chloride, EDTA, Ascorbic acid, Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Potassium hexacyanoferrate, Ferric chloride, HEPES, ferrous sulphate, Ferrozine, Chloroform, Tween 80 were obtained from SD Fine Chemicals, Mumbai, India. β -carotene and Linoleic acid were purchased from HiMedia, Mumbai, India. All other reagents were analytical grade.

Sampling of plant material and preparation of extract

Disease-free leaf and root samples of four mangrove plants *Avicennia officinalis* L., *Kandelia candel* (L.) Druce, *Excoecaria agallocha* L., *Rhizophora mucronata* Lam., which are medicinally important, were collected in sterile plastic bags from Chorao, Goa, India. The leaf samples were washed in running tap water and immediately processed. About one gram of fresh leaf material was cut into smaller pieces and immersed in 20 ml of 20 % methanol. The leaf sample was left in methanol for 18 h and the extract was filtered and stored for assays. The root samples were washed in seawater to remove mud particles and then lyophilized for 12 h. The lyophilized samples were powdered, and one gram of powdered sample was **taken**, mixed with 20 ml of 20% methanol and incubated in shaking water bath for 24 h at 35 °C. The mixture was centrifuged at maximum rpm and the supernatant was collected and stored.

Isolation of Endophytes

Leaves and roots of *Avicennia officianlis*, *Kandelia candel*, *Excoecaria agallocha* and *Rhizophora mucronata* were freshly collected and washed in seawater and dried. Leaf segments were cut equally with a sterilized cork borer from the mid portions of healthy leaves to include the midrib; roots were cut into pieces of similar size. The cut leaves and roots were surface sterilized (Kumaresan et al. 2002; Ravindran et al. 2010) by dipping into 70% ethanol for five seconds, followed by 4% NaOCl for 90 s and sterile water for 10 s, then inoculated on Malt Extract Agar (MEA) medium (Himedia, Mumbai, India) supplemented with antibiotic consisting of 10,000 units of sodium benzyl penicillin and 0.05 g of streptomycin sulfate per 100 ml of medium (prepared with filtered sterilized seawater) to inhibit bacterial growth. Petri dishes were incubated at 37 °C for one to two weeks. The fungi grown out from the tissues were isolated and transferred to MEA (Malt Extract Agar) slants under sterile conditions.

Preparation of endophytic fungal extract

Fungus grown in MEA broth for 6–7 days was separated into mycelial mat and culture filtrate by filtration through Whatman No 1 filter paper (9 cm) under vacuum. The mycelial mat thus obtained was further centrifuged at 4000 rpm for 10–15 min to separate the remaining culture filtrates. The final mycelial mat sample was stored at 4°C for use within 24 h. A mycelial mat of one gram was extracted with 20 ml of 20% methanol.

Identification of endophytic fungus using microscopic and morphological characters, and sequencing analysis

The endophytic fungal isolates from different mangrove plant leaves and roots were identified based on the colony morphology and microscopical features using standard taxonomic keys and monographs (Ellis 1971; Carmichael 1980). For ITS gene analyses, DNA was extracted from the fungal mycelium following the CTAB method (White et al. 1990). PCR amplification of the ITS region was achieved with ITS primers (White et al. 1990). Amplification was done under the following reaction conditions: initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. There was a final extension step of 10 min at 72°C with a 4°C soak at the end. The PCR products were purified with a MinElute PCR

Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Direct sequencing of the ITS rRNA gene from different endophytic *A. flavus* strains was performed with an automated multicapillary DNA sequencer (ABI Prism 3130xl Genetic analyzer, Applied Biosystems, Foster City, CA, USA). Sequence data were submitted and deposited in GenBank under accession numbers JQ269820; JQ269821; JQ269822; JQ269823; JQ269824; JQ269825; JQ269826; JQ269827.

Total soluble phenolic content assay

Total soluble phenolic content was estimated for each extract following Ravindran and Naveenan (2011). Gallic acid was used as the phenolic standard. One-thousand microliters of sample and 2 ml of sodium bicarbonate were incubated at room temperature for 2 min followed by addition of 100 μ l of Folin-Ciocalteu reagent and incubation in darkness for 30 min. The samples were then vortexed, followed by measurement of absorbance at $\lambda = 725$ nm using a spectrophotometer. Gallic acid at a concentration of 1.0 mg ml⁻¹ was used as the standard; a standard curve was obtained from various made up concentrations.

Determination of total flavonoid content

Total flavonoid content of the extract was estimated by colorimetry following Zhishen et al. (1999). The extract (100 μ l) was mixed with 300 μ l of distilled water and 30 μ l of 5% NaNO₂. After 5 min, 30 μ l of 10% AlCl₃ were added and mixed well. The mixture was incubated for 5 min, and 0.2 ml of 1mM NaOH were added. Finally the volume was made up to 1.0 ml with distilled water H₂O and mixed well. Absorbance was measured at 510 nm. Quercetin was used to calculate the standard curve. A concentration versus absorbance curve was plotted and the slope value was determined.

DPPH Scavenging Assay

Sample (mangroves and endophytic fungi) extracts at different concentrations (50 – 200 μ g ml⁻¹) were used to determine the ability to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals following Yildirim et al. (2001). A DPPH radical solution (1 mM) in 95% ethanol was prepared. Eight-hundred microliters of DPPH solution were mixed with 200 μ l of sample extract, vortexed, and incubated for 30 min at room temperature in darkness. The control sample was 1.0 ml of 95% EtOH. After 30 min incubation, samples were poured into microcentrifuge tubes and centrifuged for 5 min at 13,000 rpm. Absorbance of each sample at $\lambda = 517$ nm was then measured. Butylated Hydroxy Anisole (BHA) and

Butylated Hydroxy Toluene (BHT) were used as reference compounds. Antioxidant activity was determined as percent (%) DPPH scavenging, calculated using the formula: [(control absorbance – extract absorbance) / (control absorbance) × 100].

Hydrogen peroxide Scavenging Assay

Hydrogen peroxide scavenging activity was determined following Long et al. (1999) with minor changes. Aliquots of different sample concentrations (50 – 200 $\mu\text{g ml}^{-1}$) and 50 mM of H_2O_2 were mixed in a ratio of 1:1 (v/v) and incubated for 30 min at room temperature. After 30 min, 90 μl of H_2O_2 -sample solution was mixed with 10 μl of HPLC-grade methanol and 0.9 ml of FOX reagent (Ferrous Oxidation Xylenol [FOX] prepared following Ravindran and Naveenan [2011] before the assay) was added. The reaction mixture was mixed well and incubated at room temperature for 30 min. The reaction mixture without the sample was used as a blank. The absorbance of the ferric xylenol orange complex was measured at 560 nm. Scavenging activity leads to the inhibition of ferric xylenol orange complex formation, and scavenging activity was calculated by the formula: % hydrogen peroxide scavenging = [(control absorbance - extract absorbance) / (control absorbance) × 100].

Hydroxyl Radical Scavenging Assay (HRSA)

This assay is based on quantification of degradation product of 2–deoxyribose by condensation with thiobarbituric acid (TBA) (Elizabeth and Rao 1990). In this assay, the hydroxyl radical is produced by the Fe^{3+} ascorbate EDTA H_2O_2 system, i.e., Fenton's reaction. The reaction took place in phosphate buffer (20 mM, pH 7.4) containing 2–deoxyribose (2.8 mM), FeCl_3 (100 μM), H_2O_2 (1 mM), EDTA (100 μM) and various concentrations (50–200 $\mu\text{g ml}^{-1}$) of the test sample and reference compound (mannitol). Ascorbic acid (100 μM) was added last and the mixture was incubated at 37°C for 1 h. The reaction volume of the above mixture was 1.0 ml. After incubation, 1.0 ml of 2.8% TCA and 1.0 ml of 1% aqueous TBA were added and the mixture was incubated at 90°C for 15 min to develop color. Absorbance was measured at 532 nm after cooling. Percentage scavenging was determined as: % scavenging = [1 – (sample absorbance / blank absorbance) × 100]

Reducing power determination

The reducing power of samples was determined following Oyaizu (1986). Different concentrations (50–200 $\mu\text{g ml}^{-1}$) of samples (0.5 ml) were mixed with 0.5 ml of phosphate buffer (0.2

M, pH 6.6) and 0.5 ml of potassium hexacyanoferrate (0.1 %). Mixtures were incubated at 50 °C for 20 min in water bath. After incubation, 0.5 ml of 10 % TCA were added to terminate the reaction and the upper portion of the reaction mixture (1ml) was mixed with 1ml of distilled water and 0.1 ml of FeCl₃ (0.01%). The solution was incubated for 10 min at room temperature and the absorbance was measured at 700 nm using a spectrophotometer. Increased absorbance indicated increased reducing power. BHT was used as the positive control.

Metal chelating assay

Metal chelating activity was estimated following Dinis et al. (1994) with slight modification. The reaction was performed in HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 20 mM, pH 7.2). Various concentrations (50–200 µg ml⁻¹) of samples were mixed with solution of 12.5 µM ferrous sulphate (FeSO₄) solution. The reaction was initiated by the addition of 75 µM ferrozine, the mixture was shaken vigorously and incubated for 20 min at room temperature. After incubation, absorbance was measured at 562 nm. EDTA was used as the reference compound and percentage chelating capacity was calculated as: % chelating activity = [(A₀ – A₁)/A₀] × 100

where, A₀ = absorbance of the blank, A₁ = absorbance of the sample

Antioxidant assay using the β – carotene – linoleate model system

The antioxidant activity of the extracts as inhibition of lipid peroxidation was evaluated by the β-carotene–linoleate model system based on the procedure described by Miller (1993). β-carotene–linoleate emulsion was prepared freshly (a solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two ml of this solution were pipetted into a 100 ml round-bottom flask. After the chloroform was removed at 40 °C under vacuum, 40 mg of linoleic acid, 400 mg of Tween 80 emulsifier and 100 ml of distilled water were added to the flask with vigorous shaking). Aliquots of 2.8 ml of emulsion were transferred into assay tubes containing various concentration of extract (0.2 ml). As soon as the emulsion was added, the initial absorbance was measured at 470 nm for each reaction and the tubes were incubated at 50 °C in a water bath for 2 h. The absorbance after incubation was measured. A blank lacking of β-carotene was prepared for background subtraction. The same procedure was used for synthetic antioxidant BHT (reference compound) at various concentrations

(50–200 $\mu\text{g ml}^{-1}$). The antioxidant activity was calculated using the following equation: antioxidant activity = (β -carotene content after 2 h / β -carotene content at initial stage) \times 100

Re-infection, isolation and identification of endophyte *Aspergillus flavus* from tobacco plants

Tobacco seeds (*Nicotiana benthamina* Domin.) were purchased from the Tobacco Research Center, Vedachanthur, Tamil Nadu, India. The seeds were first washed with sterile distilled water 3 times and then sterilized (with 2% NaOCl for 5 min). The seeds were then germinated in Petri dishes or flasks containing Murashige-Skoog MS medium (Murahige and Skoog 1962) supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar in temperature controlled (25 °C) growth chambers under a 16-h light/8-h dark cycle for 2-3 weeks. kanamycin (80 $\mu\text{g ml}^{-1}$ (w/v)) was added to the medium. Endophyte infection/inoculation was performed by spraying 5 ml of water containing 10^6 spores ml^{-1} of endophytic fungi onto the leaves of every plantlet. The leaves were tested for the occurrence of the endophyte by the procedure (Isolation of endophytes) described above after 25 days of artificial infection. In addition, the ITS region of the isolated endophyte was sequenced as above and the GenBank accession number was obtained (JQ269828). The effects of the endophyte on plant growth were measured as a proportion of dry weight (oven dried to constant weight at 90°C).

Statistical analysis

All data are given as the mean \pm SD of triplicates ($n = 3$). The EC_{50} values were calculated from the linear regression equation determined by XY scatter plots in Excel. Significant ($p \leq 0.05$) coefficients of determination (r^2) were calculated for the relationships between pairs of variables using MS Excel (Redmond, USA).

Results

Molecular identification of different isolates of endophytic *Aspergillus flavus* from mangrove and tobacco plants

The internal transcribed spacer (ITS) regions were sequenced from the extracted DNA of the endophytic fungal cultures isolated from mangrove leaves and roots. A BLAST search of sequences obtained from endophytic fungi belonging to different isolates taken from mangrove leaves and roots [GenBank accession numbers: JQ269820 (*A. flavus* from *Avicennia*

officinalis leaf); JQ269821 (*A. flavus* from *Kandelia candel* leaf); JQ269822 (*A. flavus* from *Rhizophora mucronata* leaf) ; JQ269823 (*A. flavus* from *Excoecaria agallocha* leaf); JQ269824 (*A. flavus* from *Avicennia officinalis* root) ; JQ269825 (*A. flavus* from *K. candel* root) ; JQ269826 (*A. flavus* from *E. agallocha* root); JQ269827 (*A. flavus* from *R. mucronata* root); JQ269828 (*A. flavus* from tobacco plant leaves) demonstrated that they were most similar (100%) to that of the ITS ribosomal RNA gene sequences of *A. flavus*. Thus, the identities of different isolates of *A. flavus* were confirmed by molecular analysis.

Total antioxidant content

We observed a wide range of phenol concentrations in mangrove plant infusions and extracts of fungal endophytes (Table 1). The values varied from 0.2 to 1.4 mg ml⁻¹ of total phenol as measured by the Folin–Ciocalteu method. Total flavonoid values (Table 1) also varied (from 0.1 to 5.6 mg ml⁻¹) among the four mangroves and among their respective root and leaf endophytes. Total phenolic and flavonoid contents were minimal in the case of *Avicennia officinalis* leaf and maximum for *Kandelia candel* root. The extract of *Aspergillus flavus*, isolated as an endophyte from *Excoecaria agallocha* root, had the lowest phenolic/flavonoid content, while *A. flavus* isolated from *E. agallocha* leaves had the highest level of total phenols/flavonoids (Table 1).

DPPH scavenging assay

The free radical-scavenging activities of four mangrove plants and their endophytic fungi along with the reference standard BHA were determined by the DPPH assay. Decrease in absorbance of the DPPH radical caused by the antioxidant was attributable to radical scavenging by hydrogen donation. The reaction was visible as a color change from purple to yellow. Extracts obtained from leaves of *Kandelia candel*, *Excoecaria agallocha*, *Rhizophora mucronata*, and roots of *K. candel* had the highest DPPH radical scavenging activity and lower EC₅₀ values than the standard BHA. EC₅₀ is the concentration at which the 50% scavenging activity is obtained. A low EC₅₀ indicates a high antioxidant activity at a reduced concentration. The other leaves and roots and their respective endophytes of mangroves had moderate concentration-dependent inhibition of the DPPH radical compared to that the standard BHA (Table 2a-2e).

Hydrogen peroxide scavenging assay

Mangrove plant extracts had very poor H₂O₂ scavenging activity compared to the standard sodium pyruvate (Table 2a-2e). At extract concentrations of 200 µg ml⁻¹, all mangrove plants had lower activity than sodium pyruvate. The EC₅₀ values (Table 2a-2e) of the plant extracts also were found higher than that of the standard. Leaf endophytic fungi of *Avicennia officinalis* and *Excoecaria agallocha* and root endophyte of *A. officinalis* had lower EC₅₀ values and high percentage inhibition (at 200 µg ml⁻¹) than standard sodium pyruvate (Table 2a-2e).

Hydroxyl radical scavenging assay

Leaves and roots of *Avicennia officinalis*, *Kandelia candel* and *Rhizophora mucronata* had lower EC₅₀ values and higher percentage inhibition (at 200 µg ml⁻¹) than standard, mannitol. Among the endophytes tested, *Excoecaria agallocha*, *K. candel* root endophytes, and *E. agallocha* leaf endophyte showed less EC₅₀ values and also high percentage of inhibition (at 200 µg/ml) than mannitol (Table 2a-2e).

Metal chelating assay

Metal chelating capacity was significant since the extracts of all the samples reduced the concentration of the catalyzing transition metal in lipid peroxidation. In this assay, both extract and EDTA interfered with the formation of the Fe²⁺-ferrozine complex, suggesting that extract has chelating activity and captures the ferrous ion before ferrozine does. The absorbance of the Fe²⁺-ferrozine complex decreased dose-dependently; otherwise activity increased with increasing concentration from 50 to 200 µgml⁻¹. The leaf extract of *Excoecaria agallocha* had maximum activity among test samples but all extract activities were lower than that of EDTA the activity (Table 2a-2e).

β-carotene–linoleate model system antioxidant assay

Sample extracts of mangrove plants and endophytic fungi hindered β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system. Leaf and root extracts of all four mangroves had lower EC₅₀ values and higher or equivalent percentage inhibition in comparison with the BHT standard (Table 2a-2e). The extracts of root endophytes of *Kandelia candel*,

Excoecaria agallocha and *Rhizophora mucronata* had lower EC₅₀ values and almost equivalent percentage inhibition in comparison with the BHT standard (Table 2a-2e).

Reducing power scavenging assay

A significant linear correlation ($r^2 = p < 0.05$) was established between total phenolics and reducing ability of extracts of mangrove plant parts and their endophytes (Fig. 1).

Infection of tobacco plant with endophytic fungus

The analysis of the leaves of endophyte-infected tobacco plants after 25 days indicated that they were successful. Each leaf was infected. We identified the isolated fungus using morphological and molecular characters. The sequenced ITS region of the re-infected endophytic fungus that of *Aspergillus flavus* (GenBank accession number: JQ269828). We determined the effect of *A. flavus* infection on the growth of tobacco plants by monitoring plantlet growth for 30 days after infection/inoculation. Total biomass of infected plantlets showed maximal in comparison with the control (Fig. 2a, b).

Discussion

A common consequence of most abiotic and biotic stresses is increased production of ROS. These ROS may lead to the unspecific oxidation of proteins and membrane lipids or may cause DNA injury. The control of oxidant levels is achieved by antioxidative systems. These defense systems are composed of metabolites such as ascorbate, glutathione, tocopherol, etc. (Tian et al. 2008). Mutualistic associations of endophytes also enhance host plant defense responses against various biotic and abiotic stresses (Redman et al. 2002; Rodriguez et al. 2004; Rubini et al. 2005; Swarthout et al. 2009; Tian et al. 2008). Thus, we tested total antioxidant capacity (TAC) of four different mangrove plants and their predominant endophytic fungus using a suite of *in vitro* assay systems, such as the β -carotene-linoleate model system, iron chelating capacity, reducing power, and hydroxyl radical/hydrogen peroxide/DPPH radical scavenging.

DPPH is usually used as a substrate to evaluate activity of antioxidants. In the present study, DPPH assays of mangrove plant extracts demonstrated maximum production of antioxidants and antioxidant activity in *Kandelia candel*, *Excoecaria agallocha*, *Rhizophora mucronata* leaves (Table 1; Table 2a-2e) where the % occurrence of endophytes was highest (Table 3). We made similar

observations on *K. candel* roots. Low percentages of occurrence of endophytes in *Avicennia officinalis* leaves and other mangrove leaves and roots were correlated with poor total antioxidant and antioxidant activity (Table 1; Table 2a-2e). Increased endophytic fungal biomasses observed in other studies have been associated with increased host tolerance of a range of stresses (Baltruschat et al. 2008; Swarthout et al. 2009; Tanaka et al. 2006; Tian et al. 2008). Hydroxyl radicals are the major ROS causing lipid peroxidation and enormous biological damage (Hernández et al. 1995; Yang et al. 2004). Extracts of mangroves and fungi added to the reaction mixture removed hydroxyl radicals from the sugar and prevented the reaction. EC₅₀ values (Table 2a-2e) indicated that extracts of leaves and roots of *A. officinalis*, *K. candel*, *R. mucronata*, and the endophytic fungi from *E. agallocha* roots and leaves are better hydroxyl radical scavengers than the standard mannitol. Chelating agents, that form r-bonds with metals are effective as secondary antioxidants because they reduce redox potential, thereby stabilizing the oxidized form of the metal ion (Gordon 1990). Thus, we used a metal chelating assay. Ferrozine quantitatively forms complexes with Fe²⁺. We observed metal chelating ability with increases in the concentrations of all the extracts of mangrove plant and endophytes (Table 2a-2e). Reducing properties are generally associated with the presence of reductones (Pin-Der-Duh 1998), which exert antioxidant action by breaking the free radical chain through donation of a hydrogen atom (Gordon, 1990). The high correlation values of mangrove leaf endophytes than that of the mangrove leaves infers that the endophytic fungal association may favor the plant under adverse conditions (Fig. 1). Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups, and also by forming hydroxyl radicals that cause many of its toxic effects (Miller et al. 1993). The better performance in hydrogen peroxide assays of fungal endophyte extracts than the mangrove plants extracts again indicates that endophytic fungi may promote ROS resistance in mangrove plants. The sample extracts of mangrove plants and endophytic fungi hindered the extent of β-carotene bleaching by neutralizing linoleate-free radicals and other free radicals formed in the system (Jayaprakasha et al. 2001). Thus, our DPPH, hydroxyl, hydrogen peroxide and β-carotene–linoleate model system antioxidant assays show that when the host fails to produce antioxidants adequately, the associated endophytic fungi in roots and leaves have enhanced antioxidant potential that may benefit the host (Table 2a-2e). We also found that total phenol contents of endophytic *Aspergillus flavus* were significantly higher than in the free living fungus (data not shown). Thus production of novel antioxidant metabolites by endophytic *A. flavus* (Zheng et al. 2008) and expression of antioxidant genes (Jitesh et al. 2006) are enhanced under stress conditions. Our results are congruent with earlier observations on

endophyte-facilitated salt tolerance in agricultural plants like barley (Baltruschat et al. 2008), tomato (Mittova et al. 2004) and wheat (Marshall et al. 1999).

Successful transfer of *A. flavus* to tobacco plants suggests the fungus may be effectively used in other agriculturally relevant plants. Total biomass of tobacco plantlets increased with *A. flavus* infection (Fig. 2a and 2b); a similar effect occurs upon infection of tobacco and *Arabidopsis* with the endophyte *Piriformospora indica* Verma, Varma, Kost, Rexer & Franken, (Sherameti et al. 2005). The stimulation of host growth by endophytic fungi is attributable to a fungal phytohormone effect (Nassar et al. 2005). Thus, endophytic fungi with broad host ranges can confer effective tolerance of ROS under abiotic stress conditions, such as high salinity (Rodriguez and Redman 2008). Transfer of effective endophytes of mangroves to agricultural plants will probably not only facilitate stress tolerance but also controls yield loss.

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References

- Baltruschat, H., J. Fodor, B.D. Harrach, E. Niemczyk, B. Barna, G. Gullner, A. Janeczko, K. Kogel, P. Schäfer, I. Schwarczinger, A. Zuccarol and A. Skoczowski. 2008. Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytol.* 180: 501-510.
- Bandarnayake, W.M., 2002. Bioactivities, bioactive compounds and chemical constituents of mangrove plants. *Wetlands Ecol. Manage.* 10: 421-452.
- Carmichael, J.W., W.B. Kendrick, I.L. Connors and L. Sigler. 1980. *Genera of Hyphomycetes*. The University of Alberta Press, Edmonton. p. 386.
- Clay, K and J. Holah. 1999. Fungal endophyte symbiosis and plant diversity in successional fields. *Science* 285: 1742-1744.
- Dinis, T.C.P., V.M.C. Madeira and L.M. Almeida. 1994. Action of phenolic derivatives (acetoaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxy radical scavengers. *Arch. Biochem. Biophys.* 315: 161-169.
- Ellis, M.B. 1971. *Dematiaceous Hyphomycetes*. CAB International, Wallingford. pp. 608.
- Elizabeth, K and M.N.A. Rao. 1990. Oxygen radical scavenging activity of curcumin. *Int. J. Pharm.* 58: 237-240.

- Gordon, M.H. 1990. The mechanism of antioxidant action in vitro. In: (B.J.F. Hudson, ed.), Food antioxidants. Elsevier Applied Science, London. pp. 1–18.
- Hernández, J.A., E. Olmos, F.J. Corpas, F. Sevilla and L.A. Del Río. 1995. Salt-induced oxidative stress in chloroplasts of pea-plants Plant Science 105: 151–167.
- Jayaprakasha, G.K., R.P. Singh and K.K. Sakariah. 2001. Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models in vitro. Food Chem. 73: 285–290.
- Jitesh, M., S.R. Prasanth, K.R. Sivaprakash and A. Parida. 2006. Monitoring expression profiles of antioxidant genes to salinity, iron, oxidative light and hyperosmotic stresses in the highly salt tolerant gray mangrove, *Avicennia marina* (Forsk.) Vierh. by mRNA analysis. Plant Cell Rep. 25: 865-876.
- Kumaresan, V., T.S. Suryanarayanan and J.A. Johnson. 2002. Ecology of mangrove endophytes. Fungal Divers. Res. Ser. 7: 145 – 166.
- Long, L.H., P.J. Evans and B. Halliwell. 1999. Hydrogen peroxide in human urine: implications for antioxidant defense and redox regulation. Biochem Biophys. Res. Comm. 262: 605 609.
- Marshall, D., B. Tunali and L.R. Nelson. 1999. Occurrence of fungal endophytes in Species of wild Triticum. Crop Science 39: 1507-1512.
- Miller, M.J., H. Sadowska-Krowicka, S. Chotinaruemol, J.L. Kakkis and D.A. Clark. 1993. Amelioration of chronic ileitis by nitric oxide synthase inhibition. J. Pharm. Exp. Therap. 264:11-16.
- Mittova, V., M. Guy, M. Tal and M. Volokita. 2004. Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*, J. Exp. Bot. 55: 1105–1113.
- Murashige, T and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
- Nassar AH, K.A. El-Tarabily, and K. Sivasithamparam. 2005. Promotion of plant growth by an auxin-producing isolate of the yeast *Williopsis saturnus* endophytic in maize (*Zea mays* L.) roots. Biol. Fertil. Soils 42: 97-108.
- Oyaizu, M., 1986. Studies on product of browning reaction prepared from glucose amine. Jap. J. Nutr. 44: 307–315.
- Pin-Der-Duh, X., 1998. Antioxidant activity of burdock (*Arctium lappa* Linné): its scavenging effect on free-radical and active oxygen. J. Amer. Oil Chem. Soc. 75: 455–461.
- Ravindran, C., T. Naveenan and G.R. Varatharjan. 2010. Optimization of alkaline cellulase production by marine derived fungus *Chaetomium* sp., using agricultural and industrial wastes as substrates. Bot. Mar. 53: 275-282.
- Ravindran, C and T. Naveenan. 2011. Adaptation of marine derived fungus *Chaetomium globosum* (NIOCC 36) to alkaline stress using antioxidant properties. Process Biochem. 46: 847-857.
- Redman, R.S., K.B. Sheehan, R.G. Stout, R.J. Rodriguez and J.M. Henson. 2002. Thermotolerance generated by plant/fungal symbiosis. Science 298: 1581.

- Rodriguez, R and R. Redman. 2008. More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *J. Exp. Bot.* 59: 1109–1114.
- Rodriguez, R.J., R.S. Redman and J.M. Henson. 2004. The role of fungal symbioses in the adaptation of plants to high stress environments. *Mitig. Adapt. Strat. Global Change* 9: 261–272.
- Rubini, M.R., R.T. Silva-Ribeiro, A.M.V. Pomella, C.S. Maki, W.L. Araújo, D.R. dos Santos and J.L. Azevedo. 2005. Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellis pernicioso*, causal agent of Witches' Broom Disease. *Int. J. Biol. Sci.* 1: 24-33.
- Sherameti, I, B. Shahollari, Y. Venus, L. Altschmied, A. Varma and R. Oelmuller. 2005. The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. *J. Biol. Chem.* 280: 26241-26247.
- Strobel, G., E. Forda, J. Worapong, K.J. Harper, A.M. Arif, D.M. Grant, P.C.W. Fung and R.M.W. Chau. 2002. Ispoestacin, an isobenzofuranone from *Pestalotiopsis microspora*, possessing antifungal and antioxidant activities. *Phytochem* 60: 179–183.
- Swarthout, D., E. Harper, S. Judd, D. Gonthier, R. Shyne, T. Stowe and T. Bultman. 2009. Measures of leaf-level water-use efficiency in drought stressed endophyte infected and non-infected tall fescue grasses. *Environ. Exp. Bot.* 66: 88–93.
- Tan, R.X and W.X. Zou. 2001. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep.* 18: 448–459.
- Tanaka, A., M.J. Christensen, D. Takemoto, P. Park and B. Scott. 2006. Reactive Oxygen Species play a role in regulating a fungus–perennial ryegrass mutualistic interaction. *Plant Cell* 18: 1052–1066.
- Tian, P., Z. Nan, C. Li and G. Spangenberg. 2008. Effect of the endophyte *Neotyphodium lolii* on susceptibility and host physiological response of perennial ryegrass to fungal pathogens. *Eur. J. Plant Pathol.* 122: 593–602.
- White, T.J., Bruns, T.D., Lee S.B., Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: (M A Innis, D H Gelfand, J J Sninsky and T J White, eds) *PCR Protocols: A guide to methods and applications*. Academic Press, London. pp. 315– 322.
- Yang, Y.L., J.K. Guo, F. Zhang, L.Q. Zhaob and L.X. Zhang. 2004. NaCl induced changes of the H⁺-ATPase in root plasma membrane of two wheat cultivars. *Plant Science* 166: 913–918.
- Yildirim, A., A. Mavi and A.A. Kara. 2001. Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. *J. Agric. Food Chem.* 49: 4083-4089.
- Zheng, P.L., L.W. Gao, J.Q. Zhou, Y.H. Sima and J.W. Wang. 2008. Antioxidant activity of aqueous extract of a *Tolypocladium* sp. fungus isolated from wild *Cordyceps sinensis*. *Afric. J. Biotech.* 7: 3004-3010.
- Zhishen, J., T. Mengcheng and W. Jianming. 1999. The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* 64: 555-559.

Figure Legend

Figure 1: Relationships between total phenolic content and reducing power of **Mangrove leaves** (*Avicennia officinalis* $r^2 = 0.8089$; *Kandelia candel* $r^2 = 0.8557$; *Excoecaria agallocha* $r^2 = 0.9117$; *Rhizophora mucronata* $r^2 = 0.8704$); p value = 0.00224; **Mangrove root** (*A. officinalis* $r^2 = 0.9969$; *K. candel* $r^2 = 0.9990$; *E. agallocha* $r^2 = 0.9950$; *R. mucronata* $r^2 = 0.9950$); p value = 0.02; **Leaf endophyte** (*A. officinalis* $r^2 = 0.9668$; *K. candel* $r^2 = 0.9509$; *E. agallocha* $r^2 = 0.9971$; *R. mucronata* $r^2 = 0.9742$); p value = 0.004; **Root endophyte** (*A. officinalis* $r^2 = 0.9882$; *K. candel* $r^2 = 0.9774$; *E. agallocha* $r^2 = 0.9461$; *R. mucronata* $r^2 = 0.9815$); p value = 0.004.

Figure 2a and 2b: Inoculation of the mangrove endophytic fungus *Aspergillus flavus* into tobacco plant. (a1): control plant without endophytic fungus infection. (a2): infected plant. (b): Biomasses of tobacco plantlets with and without fungal infection. Values are mean \pm SD (n=3).

Table 1 Total phenolic and flavonoid contents of leaves and roots of mangrove plants and their endophytic fungus (*Aspergillus flavus*). Values are mean±SD (n=3).

Mangrove plants	Total phenols (mg ml ⁻¹)	Total flavonoids (mg ml ⁻¹)
<i>Avicennia officinalis</i>		
Leaf	0.4±0.008	1.0±0.34
<i>A. flavus</i> (Leaf endophyte)	0.5±0.02	0.7±0.11
Root	0.9±0.03	2.5±0.03
<i>A. flavus</i> (Root endophyte)	0.2±0.008	0.1±0.04
<i>Kandelia candel</i>		
Leaf	0.8±0.02	1.7±0.39
<i>A. flavus</i> (Leaf endophyte)	0.3±0.01	0.6±0.01
Root	1.4±0.013	5.6±0.16
<i>A. flavus</i> (Root endophyte)	0.3±0.003	0.1±0.01
<i>Excoecaria agallocha</i>		
Leaf	0.9±0.009	1.9±0.15
<i>A. flavus</i> (Leaf endophyte)	0.4±0.02	0.8±0.06
Root	0.6±0.007	2.4±0.20
<i>A. flavus</i> (Root endophyte)	0.3±0.007	0.1±0.01
<i>Rhizophora mucronata</i>		
Leaf	1.0±0.02	1.8±0.19
<i>A. flavus</i> (Leaf endophyte)	0.3±0.01	0.6±0.15
Root	1.0±0.02	3.6±0.31
<i>A. flavus</i> (Root endophyte)	0.3±0.003	0.1±0.008

Table 2A Scavenging and iron chelation activities of mangrove leaf extracts (with EC₅₀ values). Values are mean ±SD (n=3).

Sample concentration (µg ml ⁻¹)	DPPH scavenging (%)	EC ₅₀ (µg ml ⁻¹)	H ₂ O ₂ scavenging (%)	EC ₅₀ (µg ml ⁻¹)	OH- scavenging (%)	EC ₅₀ (µg ml ⁻¹)	β-Carotene antioxidant activity (%)	EC ₅₀ (µg ml ⁻¹)	Iron chelation (%)	EC ₅₀ (µg ml ⁻¹)
<i>Avicennia officinalis</i>										
50	18±3	239±15	-	565±9	2±3	275±11	76 ±5	56±2	8±2	351±9
100	23 ±2		8±0		23 ±4		80±1		11 ±3	
150	33±2		9±0		29±1		84±2		18±0	
200	40±4		19±1		32±1		85±1		31±0	
<i>Kandelia candel</i>										
50	34±0	105±2	-	1269±20	-	338±9	76±1	57±1	2±0	372±20
100	53±1		-		18±3		79±1		4±0	
150	71±1		5±1		23±1		86 ±0		20±0	
200	80±0		8±1		26±1		87 ±0		25±0	
<i>Excoecaria agallocha</i>										
50	59±2	67±2	2±0	550±14	9±2	473 ±16	87±1	40±2	15±0	271 ±21
100	80±0		6±2		15±1		91±0		26±4	
150	90±0		13±1		17±1		92±1		29±1	
200	91 ±0		18±1		21 ± 1		95±1		35±7	
<i>Rhizophora mucronata</i>										
50	45±1	80±1	-	1081±7	-	342±25	83 ±2	47 ±5	9±4	732±6
100	72±0		-		-		84±1		9±1	
150	87±0		3±0		18±4		95±2		10±1	
200	87±1		10±0		32±4		97±2		16±1	

Table 2B Mangrove root extract scavenging activity (with EC₅₀ values). Values are means ±SD (n=3).

Sample concentration (µg ml ⁻¹)	DPPH scavenging (%)	EC ₅₀ (µg ml ⁻¹)	H ₂ O ₂ scavenging (%)	EC ₅₀ (µg ml ⁻¹)	OH-scavenging (%)	EC ₅₀ (µg ml ⁻¹)	β-Carotene antioxidant activity (%)	EC ₅₀ (µg ml ⁻¹)	Iron chelation (%)	EC ₅₀ (µg ml ⁻¹)
<i>Avicennia officinalis</i>										
50	20±0	169±3	2±2	515±17	8±3	260±12	78±2	54±3	16±1	437±7
100	33±1		9±2		9±2		82±3		19±1	
150	46±0		13±1		29±1		85±1		21±0	
200	55±2		19±1		35±2		86±9		23±0	
<i>Kandelia candel</i>										
50	24 ±4	45±4	1±1	430±13	33±2	216±10	82±1	46±1	22±1	281±5
100	61 ±2		12±3		36±1		89±7		25±0	
150	75±1		17±1		37±2		90±7		30±1	
200	83±1		21 ± 1		41 ± 1		94±2		34±1	
<i>Excoecaria agallocha</i>										
50	16±2	193±5	5±2	446±18	-	497±30	78±1	53±2	17±1	463±5
100	29 ±2		12±2		-		84±3		19±0	
150	40±0		17±2		5±3		86±3		20±0	
200	50±1		21±1		25±3		90±1		21±0	
<i>Rhizophora mucronata</i>										
50	2±1	140±2	3±1	863±14	27±4	120±9	79±2	55±2	16±0	467±7
100	38±1		8±1		48±4		82±0		18±1	
150	60±1		11 ± 1		60±2		90±2		19±1	
200	70±1		14±0		75±2		99±1		22±0	

Table 2C Mangrove leaf endophyte extract scavenging activity (with EC₅₀ values). Values are mean ±SD (n=3).

Sample concentration (µg ml ⁻¹)	DPPH scavenging (%)	EC ₅₀ (µg ml ⁻¹)	H ₂ O ₂ scavenging (%)	EC ₅₀ (µg ml ⁻¹)	OH-scavenging (%)	EC ₅₀ (µg ml ⁻¹)	β-Carotene antioxidant activity (%)	EC ₅₀ (µg ml ⁻¹)	Iron chelation (%)	EC ₅₀ (µg ml ⁻¹)
<i>Avicennia officinalis</i>										
50	32±1	196±4	21±1	361±21	-	906±23	64±5	71±6	14±4	401 ±17
100	37±0		23 ±4		-		73±1		17±0	
150	40±1		25±2		2±1		82±2		21 ±2	
200	46±1		27±1		14±2		85±1		24±1	
<i>Kandelia candel</i>										
50	24±1	227±6	10±1	492±10	-	353±24	66±1	68±5	12±0	590±5
100	28±1		15±1		10±4		75 ±3		15±0	
150	36±1		17±1		20±4		81±3		16±0	
200	41 ± 1		20±1		27±2		88±2		17±0	
<i>Excoecaria agallocha</i>										
50	32±1	210±2	10±1	395±14	-	267 ±6	73±1	61±4	12±1	652±12
100	37±0		18±2		20±1		78±3		14±0	
150	39±0		21 ±2		22±1		83 ±2		14±0	
200	42±0		24±1		39±1		87±0		16±1	
<i>Rhizophora mucronata</i>										
50	3±1	267±5	-	690±16	-	379±11	67±1	79±2.6	7±2	881 ±22
100	25±1		2±0		-		69±1		8±1	
150	31±0		12±1		17±2		71±1		8±1	
200	33 ±0		13±0		28±1		74±0		13±1	

Table 2D Mangrove root endophyte extract scavenging activity (with EC₅₀ values). Values are means ±SD (n=3).

Sample concentration (µg ml ⁻¹)	DPPH scavenging (%)	EC ₅₀ (µg ml ⁻¹)	H ₂ O ₂ scavenging (%)	EC ₅₀ (µg ml ⁻¹)	OH- scavenging (%)	EC ₅₀ (µg ml ⁻¹)	β-Carotene antioxidant activity (%)	EC ₅₀ (µg ml ⁻¹)	Iron chelation (%)	EC ₅₀ (µg ml ⁻¹)
<i>Avicennia officinalis</i>										
50	19±1	323 ±5	13±1	378±16	-	502±14	63±3	78±5	3±0	979±33
100	22±0		19±2		-		72±2		6±2	
150	25±1		23 ±2		13±2		73±0		9±1	
200	31±0		25±2		20±1		77±1		10±0	
<i>Kandelia candel</i>										
50	3±0	317±6	3±1	466±10	-	269±5	74±0	60±5	2±0	721±30
100	15±1		11±0		21 ± 1		80±3		5±1	
150	21±1		18±2		29±1		83±1		8±2	
200	32±2		20±1		34±1		85±1		14±3	
<i>Excoecaria agallocha</i>										
50	17±1	410±5	6±2	457±13	30±2	193±7	79±1	53±2	2±1	804±31
100	18±0		10±1		37±0		82±1		5±0	
150	19±1		17±1		40±1		86±1		7±1	
200	26±0		22±1		46±1		89±1		13±4	
<i>Rhizophora mucronata</i>										
50	2±0	337±3	2±1	557±17	-	905±14	75±2	61±4	3±0	1253±9
100	4±1		7±1		-		77±1		4±0	
150	20±0		12±2		3±1		81±0		5±0	
200	31±0		18±3		13±2		83±2		9±1	

Table 2E Artificial standard scavenging activity (with EC₅₀ values). Values are mean ±SD (n=3).

Sample concentration (µg ml ⁻¹)	DPPH ^a scavenging (%)	EC ₅₀ (µg ml ⁻¹)	H ₂ O ₂ ^b scavenging (%)	EC ₅₀ (µg ml ⁻¹)	Off scavenging (%)	EC ₅₀ (µg ml ⁻¹)	β-Carotene ^d antioxidant activity (%)	EC ₅₀ (µg ml ⁻¹)	Iron chelation ⁶ (%)	EC ₅₀ (µg ml ⁻¹)
Artificial Standard										
50	26±0	123±1	-	424±11	13±1	346±8	67±0	60±1	90±0	36±1
100	54±0		80±2		20±0		83±1		95±0	
150	63±0		13±1		22±1		87±0		98±1	
200	66±1		24±1		29±1		94±1		99±0	

Standards used for various assays: ^aDPPH scavenging assay - BHA; ^bH₂O₂ scavenging assay - Sodium pyruvate; ^cOH scavenging - Mannitol; ^dAntioxidant activity using β-carotene linoleate emulsion system - BHT; ^eMetal chelating assay - EDTA.

Table 3 Percentage frequency of occurrence of endophytes in mangrove leaves and roots (*Aspergillus*).

Mangrove plants	Leaf/root	<i>Aspergillus flavus</i>	% of <i>A. flavus</i>	Unidentified fungi	% Unidentified fungi
<i>Avicennia officinalis</i> - Leaf	74	4	5.4	1	1.3
<i>Kandelia candel</i> - Leaf	75	13	17.5	4	5.3
<i>Excoecaria agallocha</i> - Leaf	76	9	11.8	5	6.6
<i>Rhizophora mucronata</i> - Leaf	81	26	32.0	-	0
<i>Avicennia officinalis</i> - Root	64	7	10.9	3	4.7
<i>Kandelia candel</i> - Root	52	7	13.4	4	7.7
<i>Excoecaria agallocha</i> - Root	43	1	2.3	20	46.5
<i>Rhizophora mucronata</i> - Root	73	6	8.2	5	6.8

Figure 1

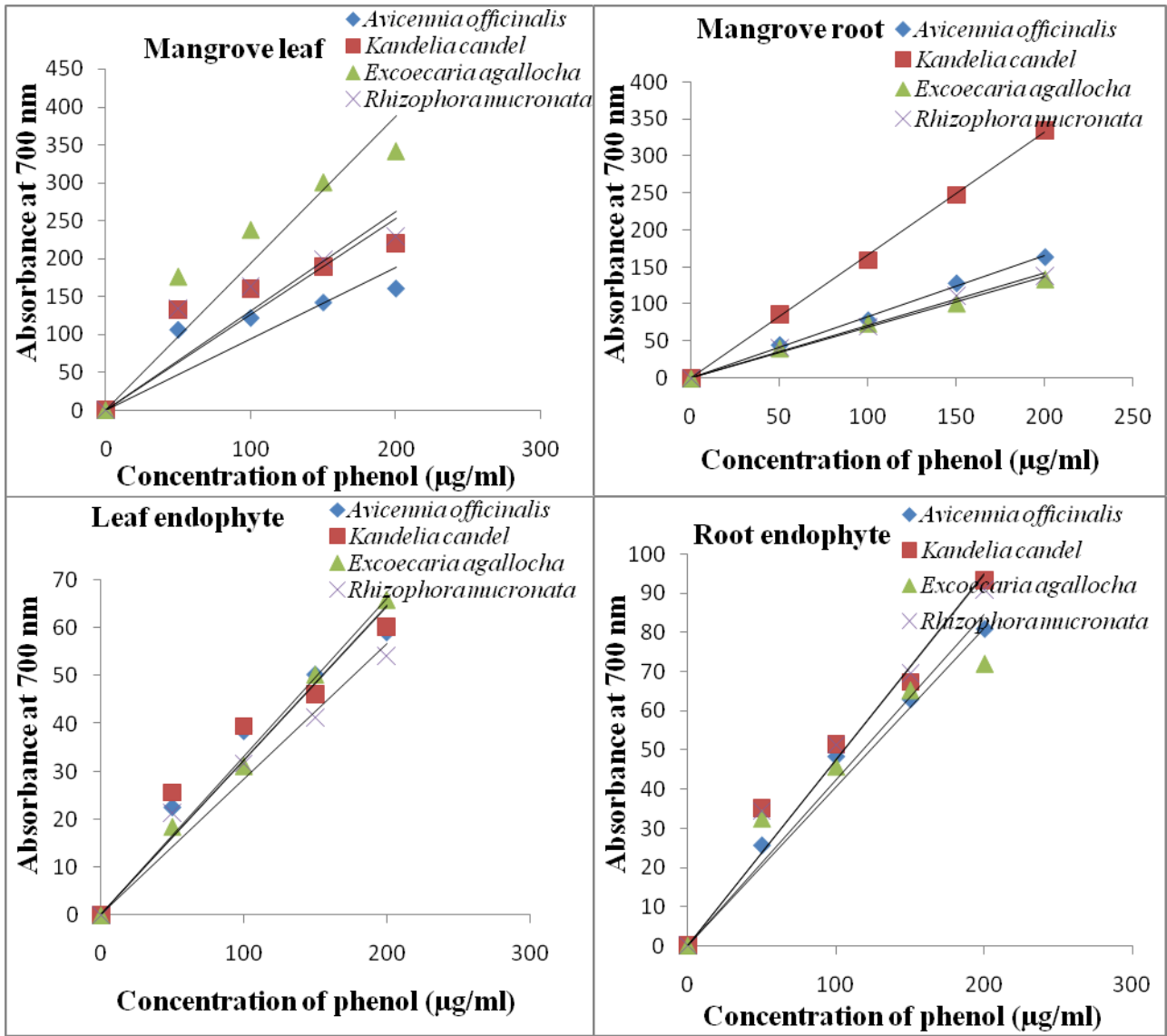


Figure 2a

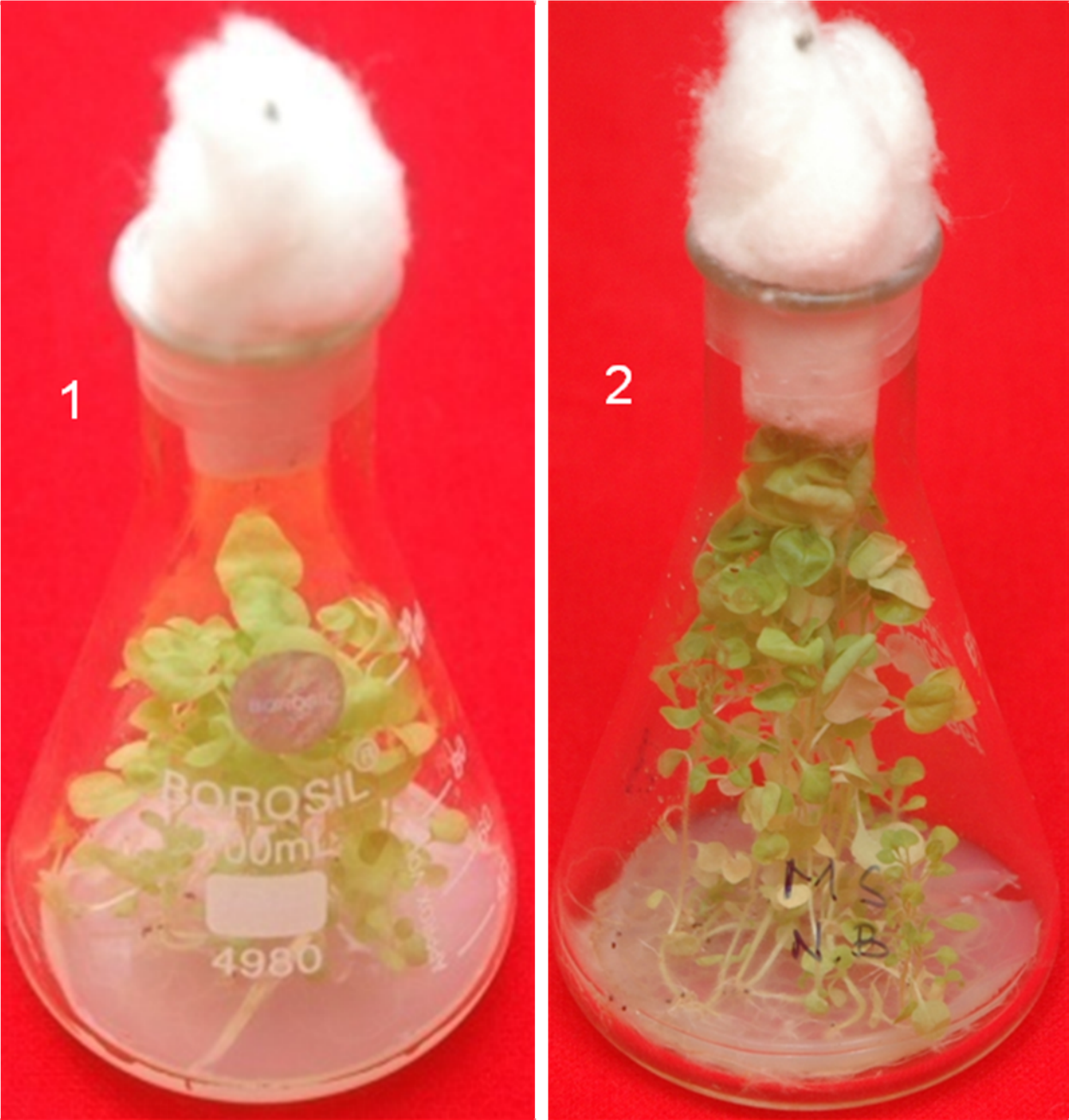


Figure 2b

