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Piriformospora indica, an excellent system for heavy metal sequestration and amelioration of oxidative stress and DNA damage in *Cassia angustifolia* Vahl under copper stress

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ABSTRACT

Present investigation reveals copper induced phytotoxicity, oxidative stress and DNA damage in *Cassia angustifolia* Vahl and its amelioration by employing a symbiotic fungus, *Piriformospora indica*. Seeds were germinated on Knop's medium containing five Cu levels (0, 1, 10, 50, 100 and 200 mg L^{-1}), with and without *P. indica*. Colonization with *P. indica* significantly ($P < 0.05$) ameliorated Cu induced oxidative stress. However, maximum amelioration was observed at 50 mg L^{-1} Cu with *P. indica*. Atomic absorption spectroscopy revealed that *P. indica* colonization significantly inhibited Cu accumulation in shoots. Maximum decline in Cu accumulation in shoots was observed at 50 mg L^{-1} (27.27%) with *P. indica* over Cu alone. Besides, *P. indica* colonized seedlings stored 16.86% higher Cu in roots as compared to Cu alone at 200 mg L^{-1}. Similarly, maximum proline accumulation increased up to 19.32% over Cu alone at 50 mg L^{-1} Cu with *P. indica*. Significant elevation in antioxidant enzyme levels of superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase and glutathione reductase was seen with *P. indica*. Contrary to increase in antioxidant level, toxic parameters such as lipid peroxidation and hydrogen peroxide decreased significantly with *P. indica*. Maximum decline in lipid peroxidation (13.76%) and hydrogen peroxide (18.58%) was observed at 50 mg L^{-1} with *P. indica* over Cu alone. *P. indica* significantly reduced DNA damage as well as changed the protein profile in *C. angustifolia* seedlings. Thus, *P. indica* proved to be an excellent system to alleviate Cu induced oxidative stress and might be useful as a phytostabilization tool.

1. Introduction

Copper, an essential micronutrient is required for the normal plant growth as it acts as a cofactor in various enzymes such as superoxide dismutase, amino oxidase, plastocyanin and polyphenol oxidase. However, at higher concentrations, it causes growth inhibition, chlorosis and reduction in biomass due to increased production of reactive oxygen species and interfering with cellular molecules (Yruela, 2005; Burkheadd et al., 2009; Lange et al., 2017). High levels of Cu naturally occur in soils but mining, smelting and waste disposal contribute to increased level in soil. Typically, Cu concentrations in soil generally range from 2 to 250 ppm and plant tissues contain from 20 to 30 μg g^{-1} DW (Mittler et al., 2004; Khatun et al., 2008). Copper is a redox–active metal which is directly involved in production of reactive oxygen species (ROS) which results in oxidative stress consequently leading to lipid peroxidation, DNA and protein damage (Panda et al., 2016). Plants inherently have certain mechanisms to combat oxidative damage such as enzymatic antioxidants (SOD, CAT, POX and GR) and non-enzymatic antioxidants such as glutathione, thiols and carotenoids to counter damaging effects of ROS (Inzé and Van Montagu, 1995; Thounaojam et al., 2014; Munne-Bosch and Pinto-Marijuan, 2016). There are certain techniques by which amount of heavy metals can be removed or restricted to prevent harmful effect in plants and animals. These include physical, chemical and biological techniques. Phytoremediation technique is an effective strategy to remediate heavy metal contaminated land as it is simple and highly cost efficient as compared to other techniques (Sarwar et al., 2017). Symbiotic association of plants with arbuscular mycorrhizal fungi also play an important role in plant defence system as it can effectively restrict and alleviate heavy metals in the soil (Emamverdian et al., 2015; Latef et al., 2016). Various

Abbreviations: GSH, Glutathione; GSSG, Glutathione Disulfide; NBT, Nitro Blue Tetrazolium; NADP, Nicotinamide Adenosine Dinucleotide Phosphate; SOD, Superoxide dismutase; CAT, Catalase; APX, Ascorbate peroxidase; GPX, Guaiacol peroxidase; GR, Glutathione reductase; H_2O_2, Hydrogen peroxide; MDA, Malondialdehyde; SDS, Sodium dodecyl sulphate; PAGE, Polyacrylamide Gel Electrophoresis

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genes involved in heavy metal tolerance such as ZIP transporters, metallothioneins and glutathione-s-transferases were up regulated in mycorrhiza colonized plants. Additionally, heavy metal responsive genes up regulated in response to heavy metal exposure in colonized plants might be localized to fungal structures such as arbuscules (Hildebrandt et al., 2007). Therefore, these plant–fungi associations might be effectively utilized in enhancing heavy metal phytostabilization potential of plants.

Piriformospora indica is a symbiotic mycorrhizal fungus as it has a beneficial role in plant growth and yield under normal and stressed condition (Varma et al., 1999). It enhances antioxidant defence system of plants which is crucial in stress tolerance. It also induces local and systemic disease resistance by modulating the level of antioxidants to combat oxidative stress (Vadassery et al., 2009). It has also shown a significant capacity to immobilise heavy metals in roots which can be very promising in phytoremediation (Shahabivand et al., 2017). Very few studies (Shahabivand et al., 2012; Sartipnia et al., 2013; Hui et al., 2015; Gill et al., 2016) have been reported in literature regarding heavy metal stress in plants where *P. indica* successfully alleviated phytotoxicity. Therefore, practical application of *P. indica* in clean up of heavy metal contaminated lands can prove to be a major tool in functioning of sustainable environment.

C. angustifolia is drought tolerant fast growing shrub which is frequently used in wasteland development (Sharma et al., 1999). Metal accumulating plants are generally slow growing which greatly limits their full utilization in phytoremediation (Pilon-Smits, 2005). Therefore, the ability of *C. angustifolia* to grow in wastelands and enhanced heavy metal accumulation can provide a detoxification strategy to combat heavy metal pollution. We have previously reported that it can accumulate significant amount of heavy metals in its roots and has a great prospect in phytoremediation (Nanda and Agrawal, 2016). The present study was carried out to further explore the potential of mycorrhizal association of *C. angustifolia* with *P. indica* regarding heavy metal distribution in various plant parts and its beneficial effects. So far, there is no report of any study regarding alleviation of heavy metal stress in *C. angustifolia*, an important source of human drug. Therefore, following objectives were set to determine the potential of *P. indica* association with *C. angustifolia*: a) effect of this association on various physiological parameters, b) its effect on oxidative stress and defense system and c) its effect of heavy metal accumulation in various plant parts and its scope in phytoremediation.

2. Materials and methods

2.1. Plant material and experimental conditions

Seeds of *C. angustifolia* Vahl were surface sterilized and germinated on Knop's medium (Knop, 1865) according to the detailed protocol described in Nanda and Agrawal (2016). $CuSO_4$ was used for heavy metal treatment in the present study. Aqueous solution of $CuSO_4$ (Merck, Germany) was added to adjust Cu concentration in the medium. Treatment was given with five Cu levels (0, 1, 10, 50, 100 and 200 mg L^{-1}), with and without *P. indica*. Range of Cu concentrations were selected on the basis of inhibition of growth of seedlings after exposure.

2.1.1. Co-cultivation of C. angustifolia with P. indica

The stock cultures of *Piriformospora indica* were obtained from Prof. Ajit Varma, AIMT (Amity University), Noida, India. Stock cultures of *P. indica* were sub–cultured in liquid modified Kaefer medium (pH 6.5) (Hill and Kafer, 2001). For co-cultivation of *C. angustifolia* seeds with *P. indica*, a 5 mm^2 pit was prepared on the solid culture medium with help of a needle and same size of matured mycelium was placed in the pit (Sharma and Agrawal, 2013). Surface sterilized seeds were carefully placed in the mycelium filled pit for proper infection.

2.1.2. Analysis of root colonization

Root colonization analysis was performed according to the modified protocol of Dickson and Smith (1998) and Phillips and Hayman (1970). Root colonization in *C. angustifolia* co–cultivated with *P. indica* was analysed after 28 d of inoculation. Roots of *C. angustifolia* were washed thoroughly with ddH_2O to remove attached medium and mycelium. Roots were then cut into 1.0 cm pieces, boiled for 10 min in 10% KOH solution and incubated in 1 M HCl for 10 min. Roots were washed with autoclaved ddH_2O and stained with cotton blue overnight. Stained root segments were observed under light microscope at 40× magnification.

2.2. Chlorophyll estimation

Chlorophyll estimation was carried out according to Arnon (1949). Leaf samples (0.1 g) were homogenized in 5 mL chilled 80% acetone in dark conditions. Absorbance of supernatant was recorded at 645 and 663 nm after centrifugation at 5000 × *g* for 10 min at 4 °C.

2.3. Metal accumulation

Treated seedlings were washed thoroughly with autoclaved ddH_2O to remove residues of medium followed by 0.1 M HNO_3 to remove metals adsorbed on root surface. Control and treated plant material was digested in HNO_3/$HCLO_4$ (3:1, v/v). After complete digestion, 10 mL of 0.1 N HNO_3 was added and analysed in atomic absorption spectrophotometer (AAS) (Shimadzu, Japan). Limit of detection (LOD) was determined by analyzing the known concentration of samples at which it was reliably detected. Limit of quantification (LOQ) was determined by analyzing the known concentration of samples at which it was reliably quantified.

2.4. Antioxidant enzyme assays

2.4.1. Superoxide dismutase (SOD) assay

SOD assay was performed according to a modified NBT method of Beyer and Fridovich (1987). Each reaction mixture contained phosphate buffer (pH 7.5), riboflavin (2 µM), methionine (13 mM), NBT (7.5 µM) and 50 µL protein extract. Detailed protocol has been described previously in Nanda and Agrawal (2016).

2.4.2. Catalase (CAT) assay

Determination of CAT activity in the material was assayed according to Aebi (1974). Catalase activity was measured by monitoring the decrease in H_2O_2 concentration at 240 nm. Complete protocol has been described in Nanda and Agrawal (2016).

2.4.3. Ascorbate peroxidase (APX) assay

APX activity was determined according to the modified protocol of Nakano and Asada (1981). APX was assayed using ascorbic acid and H_2O_2 as a substrate. Kinetic changes were measured at 290 nm for 500 s at 25 °C. Protocol for APX estimation has been described in detail in Nanda and Agrawal (2016).

2.4.4. Guaiacol peroxidase (GPX) assay

Determination of GPX activity was performed according to the protocol of Thimmaiah (1999). GPX was measured using guaiacol as a substrate. Kinetic changes were recorded at 470 nm for 300 s at 25 °C. Chemical constituents and method has been previously illustrated in Nanda and Agrawal (2016).

2.4.5. Glutathione reductase (GR) assay

GR activity was determined according to the protocol of Schaedle and Bassham (1977). GR was assayed by monitoring a decrease in absorbance at 340 nm due to NADPH oxidation. Complete protocol has been given in detail in Nanda and Agrawal (2016).