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Potential of vetiver (*Vetiveria zizanoides* L. Nash) for phytoremediation of phenol

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Abstract

Aseptically grown *Vetiveria zizanoides* were evaluated for their potential for phytoremediation of phenol from Murashige and Skoog's liquid medium. Phenol was found to be completely removed from incubation medium at the end of 4 days by *V. zizanoides* plantlets, when medium was supplemented with 50 and 100 mg L^{-1} phenol, while with 200, 500, and 1000 mg L^{-1} of phenol, 89%, 76% and 70%, respectively, were removed. Phenol removal was found to be associated with inherent production of peroxidase and hydrogen peroxide. Coupled with H_2O_2 formation, the levels of antioxidant enzymes like superoxide dismutase and peroxidase showed an enhancement when plants were exposed to phenol, whereas catalase levels initially showed a decline due to the utilization of H_2O_2 by peroxidase for phenol oxidation. However, when peroxidase levels declined, there was an enhancement in catalase levels to minimize the presence of H_2O_2 in the medium. Having confirmed that the removal of phenol was by *V. zizanoides* plantlets, in the next phase, micropropagated plantlets and well-developed plants grown in hydroponics were used under *in vivo* conditions to study the effect of phenol (200 mg L^{-1}) on plant growth and reuse. Although plant growth was reduced in presence of phenol, the results of the reuse study indicated the possibility of plants getting adapted to phenol without any decline in potential for phenol remediation.

Keywords: Catalase; Hydrogen peroxide; Peroxidases; Phenol; Superoxide dismutase; *Vetiveria zizanoides*

1. Introduction

Phytoremediation, the use of green plants to extract, sequester and detoxify organic or inorganic pollutants as a cheap and eco-friendly alternate technology has evoked a lot of interest in the last few years (Cunningham et al., 1995; Macek et al., 2000; Newman and Reynolds, 2004; Eapen and D'Souza, 2005a, b). Phytoremediation has been used for remediation of many recalcitrant pollutants such as polychlorinated biphenyls (Smith et al., 2007), trichloroethylene (Newman et al., 1997) and 2,4,6-trinitrotoluene (Sung et al., 2003). Phenols are major pollutants (Wentz, 1989) in aqueous effluents from coal conversion processes, petroleum refineries and in waste waters from production of fungicides, herbicides and insecticides and are a major risk to human health (Sharma et al., 1997; Entezari and Petrier, 2004). Thus, it is desirable to remediate phenol from industrial effluents. Current methods for removal of phenols include solvent extraction, adsorption on activated carbon, chemical oxidation and microbial degradation (Klibanov et al., 1983). Although conventional methods are effective, they also pose certain limitations (Klibanov et al., 1983) such as high cost. In our laboratory, we have carried out extensive work on phenol degradation using acclimatized activated sludge (Joshi and D'Souza, 1999; Israni et al., 2002; Melo et al., 2005).

Among the different bioremediation techniques available, phenol remediation through enzyme-catalyzed polymerization has been recognized as an alternate detoxification method (Bollag et al., 1988; Tatsumi et al., 1996). Involvement of peroxidase in oxidative polymerization of phenol in the presence of hydrogen peroxide (H_2O_2) has been demonstrated (Maloney et al., 1986; Yu et al., 1994; Roper et al., 1996). Besides, one can also use materials which are a source of peroxidase for phenol remediation. Hence, plant pieces (Dec and Bollag, 1994) and plant hairy roots of

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Daucus carota (de Araujo et al., 2002), *Brassica napus* (Agostini et al., 2003) and *Brassica juncea* (Singh et al., 2006) producing peroxidase have been used and shown to remove phenol or its derivatives from solutions.

Unlike in most studies wherein H_2O_2 was added as an external source for phenol remediation, our earlier work on *B. juncea* hairy roots showed that they could remove phenol from solution without the need for external addition of H_2O_2 (Singh et al., 2006). Thus, it was demonstrated that there was inherent production of H_2O_2 besides peroxidase, when the roots were exposed to phenol. The generation of reactive oxygen species (ROS) such as H_2O_2 is a common event associated with normal plant biochemical processes. Plants also generate these ROS when exposed to a number of different stresses. Thus, increased accumulation of H_2O_2 alerts the plant cell of environmental stresses (Maksymiec and Krupa, 2006). ROS causes oxidative damage through actions such as lipid peroxidation with membrane destruction, protein inactivation or DNA mutation (Ye et al., 2006). To mitigate the oxidative damage, plants have developed a complex defense antioxidant system including low molecular weight antioxidants as well as enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) (Winston, 1990).

Plants, due to their ability to grow using sunlight and nutrients and due to their robust biomass are preferred as bioremediation agents for xenobiotic pollutants. Vetiver (*Vetiveria zizanoides* L. Nash) is a high biomass, fast growing grass species known for its massive root system and is recognized as a suitable plant for solving many of the environmental problems (Truong, 2000). The plant is known to be tolerant to toxic metals (Pang et al., 2003; Chen et al., 2004; Boonyapookana et al., 2005) and is used for rehabilitation of mine wastes. There are reports on the use of this plant for phytoremediation of soils contaminated with heavy metals (Chen et al., 2004), polycyclic aromatic hydrocarbons (Paquin et al., 2002), petroleum (Brandt et al., 2006) and 2,4,6-trinitrotoluene (Markis et al., 2007a, b). To the best of our knowledge, there are no reports on the use of *V. zizanoides* for the remediation of phenol and its influence on antioxidant enzymes.

Use of *in vitro* plants as a model system for studying degradation of organic pollutants has the advantage that any contamination with microbes, which may lead to erroneous results can be avoided. Besides, *in vitro* developed plantlets due to their small size have the additional advantage of requirement for small volumes of solutions for experiments. In the present study, results on remediation of phenol from solutions using *in vitro* grown plantlets of vetiver under aseptic condition as well as *in vivo* plants cultivated under hydroponics conditions, in the absence of external supplementation of H_2O_2 are presented. The influence of phenol on growth of vetiver plants and on H_2O_2 and antioxidant enzymes such as peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) are also presented.

2. Materials and methods

2.1. Plant material

Vetiver grass (*V. zizanoides* L. Nash) was used for the experiments. Shoots multiplied by axillary bud development on MS medium (Murashige and Skoog, 1962) supplemented with 2 mg L^{-1} benzyladenine (BA) and 0.1 mg L^{-1} indole acetic acid (IAA) were transferred to liquid MS medium in test tubes supplemented with 1 mg L^{-1} α-naphthalene acetic acid (NAA) without a raft for rooting (Eapen, 2007). One-month old *in vitro* plantlets [(2.0 ± 0.13 g fresh weight (fw)] having well-developed roots growing on half strength MS liquid medium in test tubes were used for the experiments under aseptic conditions. In another set of experiment, plantlets developed under *in vitro* conditions were kept in test tubes under non-sterile condition in Hoagland's (Hoagland and Arnon, 1938) liquid medium and used for studies. In the final stage, field grown plants cultivated in hydroponics in Hoagland's medium were used in bench scale studies.

2.2. Phenol removal using in vitro grown vetiver plantlets

Autoclaved half strength MS liquid medium with filter-sterilized phenol was added to test tubes each having a single vetiver plantlet (2.0 ± 0.13 g fw), so that roots were in contact with the medium. In all studies, phenol at a concentration of 200 mg L^{-1} was used, except in one experiment where different concentrations of phenol were tested. To study the effect of different concentrations, phenol in the range of 50–1000 mg L^{-1} (viz. 50, 100, 200, 500, and 1000 mg L^{-1}) was incorporated into half strength MS medium and phenol in the medium estimated from day 1 to 12. Plantlets were routinely contacted with 10 mL of the above medium under sterile condition and incubated at 25 °C under white fluorescent light (12.2 μM photon ms^{-2} s^{-1}) at a photoperiod of 12 h. Samples, 0.5 mL each, were drawn out at different time periods and used for estimation of phenol. Samples drawn before the incubation of plantlets in the medium supplemented with phenol, were used to determine the initial phenol concentration. Medium without plantlets served as control in order to ensure that the disappearance of phenol was not because of evaporation, whereas medium with heat inactivated plantlets served as control to ensure that the disappearance of phenol was not because of physical adsorption in each set of experiment.

2.3. Mode of phenol removal and effect on antioxidant enzymes

To understand the mode of phenol removal and its effect on antioxidant enzymes, vetiver plantlets were incubated in 10 mL of half strength MS medium supplemented with 200 mg L^{-1} phenol under aseptic conditions and the roots of plantlets assayed for enzyme activity as described below.

For determination of enzyme activity, roots of vetiver plantlets (200 mg) incubated in medium containing 200 mg L^{-1} of phenol at different time periods of 1, 2, 3, and 4 days were homogenized in liquid nitrogen and extracted in 2 mL of 0.1 M Na-phosphate buffer pH 7. The resulting cell homogenate was centrifuged at 7000 × *g* for 15 min at 4 °C and the cell free extract assayed for enzyme activity. All the steps in the preparation of enzyme extract were carried out at 0–4 °C.

Peroxidase activity was determined at 25 °C with a spectrophotometer (JASCO V-530, Japan) following the formation of tetraguaiacol as described by Singh et al. (2006). One unit of peroxidase activity (*U*) represents the amount of enzyme catalyzing oxidation of 1 μmol of guaiacol in 1 min at 25 °C. Activity was measured at the end of 1, 2, 3, and 4 days after addition of phenol.

Superoxide dismutase activity was measured by the method of Nishikimi et al. (1972) using the root extract. One unit of SOD was defined as the enzyme amount causing 50% inhibition reduction of nitroblue tetrazolium (NBT) to farmazan. A system devoid of enzyme served as control.