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Influence of insecticide phorate on chemical composition and enzyme profile of root exudates and root extracts of *Brassica juncea*

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Abstract

'Rhizosphere effect', a phenomenon of enhanced degradation of pollutants in rhizosphere is frequently reported, however the underlying mechanism is poorly understood. It is known that root exudates play a significant role in this phenomenon. Thus, the effect of phorate present in the growth medium on the composition of root exudates and extracts of *Brassica juncea* was studied in the preset investigation. Results indicated significant increase in proteins (88.4 and 26.2%), total glutathione (13.2 and 58.3%), and ascorbic acid (13.4 and 100%) content of root exudates and root extracts, respectively of the plants when grown in presence of phorate. An increase in riboflavin concentration was observed in case of root extracts only. On the contrary, no significant changes were observed in total carbohydrate, free thiol groups, amino acids, sugars, antioxidative enzymes, phenolic acids and flavonoids present in root exudates and extracts of plants in presence of phorate. Results of the present investigation provide some limited but preliminary evidence on the fact that exudation behaviour of roots was altered on sensing the presence of contaminants (phorate) in the rhizosphere, which can be one of the reasons for rhizospher effect.

Key words

Brassica juncea, Contaminants, Phorate, Root extracts, Root exudates

Introduction

Apart from nutrient uptake, plant roots also affect the rhizosphere micro-environment by releasing a wide range of organic and inorganic compounds into the rhizosphere. Compounds like amino acids, sugars, organic acids, sterols, enzymes *etc.,* are also secreted from roots, which presumably stimulate the catabolic capabilities of microflora, thus resulting in enhanced degradation of the pollutants. Soil chemical changes related to the presence of these compounds and products of their microbial turnover are important factors affecting microbial populations, availability of nutrients, solubility of toxic elements in rhizosphere, and thereby, the ability of plants to cope with adverse soil-chemical conditions.

Root exudates are responsible for wide array of interactions going in the rhizosphere like plant-microbe and plant-plant. It has been reported that plant root exudates accelerates the rate of contaminant removal from soil or water (Xie *et al*., 2012; Liu *et al*., 2015). Components of root exudates have been reported to increase the mobility of contaminants in soil thereby facilitating the phytoremediation process (Le Fevre *et al.* 2013; Balseiro Romero *et al.* 2013). But, till now little attention has been given towards identification of the active component involved and the exact mechanism by which they mediate their effect. Therefore, in the present study an initial attempt was to investigate the effect of presence of phorate, on organophosphate insecticide on the composition of root exudates of *B. juncea*. Pesticides are widely used in

agriculture and insect vector control programs, as around 4 million tons of pesticides are applied to crops annually round the world (Miller, 2004). Organophosphorus compounds account for around 38% of total pesticides used globally (Post, 1998) and phorate {O, O-diethyl S-[(ethylthio) methyl] phosphorodithioate} is one of the high toxicity organophosphate compound extensively used to control insects (Abhilash and Singh, 2009) on various field crops.

A significant fraction of applied insecticides gets dissipated from application site and cause adverse effect on biotic and abiotic components of the ecosystem. When present in soil, how they will influence the composition of root exudates and thus the rhizospheric micro-environment is important but not well studied area of study. Therefore, in the present investigation attempts were made to study the chemical composition and enzyme profile of root exudates of *B. juncea* in absence and presence of phorate.

Materials and Methods

Collection of root exudates : Root exudates from *B. juncea* (Indian mustard) were collected by sand culture method according to Gaidamak (1971) and root extract was obtained by extracting roots in calcium chloride according to Steingrobe *et al*. (2002).

In sand culture method, *B. juncea seeds* were surface sterilized and germinated in glass assemblies containing acid washed and sterilized sand, and placed in growth chamber at 25 ºC temperature and photoperiod of 12 hr. Plants were allowed to grow for 20days and watered daily with half strength Hoagland medium upto 40% of its water holding capacity. To study the difference in exudation behavior of plants in response to phorate, one set was given phorate treatment (sand was spiked with phorate at 5 mg kg^{-1} concentration) while other was left untreated. After development of roots (after 20 days), the amount of water added to pots was increased (60-70% of the water holding capacity of the sand) so that water along with root washings overflows and gets collected in the flask connected to the glass assemblies. In this manner, root exudates were collected for next ten days.

For root extracts, plants were grown in acid washed and sterilized sand in growth chamber at same conditions as stated above and harvested after 30 days. Here also, two treatments were kept, one without phorate (control) and another with 5 mg kg^{-1} phorate (experimental). The experiment was run in triplicates. After 30 days, plants were uprooted, washed in running tap water for 5 min and then by autoclaved distilled water. Plants, fifty in number were placed in 100ml, 0.05M calcium chloride ($CaCl_2$) solution in 250 ml conical flask at room temperature. Roots were allowed to exude for one hour in solution. Plants were then placed in fresh $CaCl_2$ solution for another one hour.

Root exudates and extract were sterilized by passing through 0.2µm membrane filter, concentrated 100 folds by lyophilization, purified by dialysis and stored at -4 ºC until further analysis.

Analysis of root exudates and root extracts : Root exudates and extract were analyzed for total carbohydrate content using Anthrone's method (Dreywood, 1946), proteins by Bradford's test (Bradford, 1976), and total free thiol groups by Ellman's test (Kuwata *et al.* 1982). Free amino acids, sugars and organic acids were determined by thin layer chromatography (TLC) using silica gel plates. Butanol: glacial acetic acid: water (4:1:1) was mobile phase for amino acids and 2% ninhydrin in acetone was used as developer. For sugars, chloroform: methanol: 0.25% potassium chloride (KCl) (5: 4: 1) was solvent and orcinol (1%) in 5% H_2SO_4 was the indicator solution used. Analytical grade rhamnose, xylose, maltose, sucrose, raffinose, glucose, mannose, fructose, ribose, lactose, galactose were used as standards. Organic acids were detected on silica gel 60-F245 (Merck) plates using analytical grade acetic acid, butyric acid, salicyclic acid, dihydroxy benzoic acid, lactic acid, 2-ketoglutaric acid, malonic acid, oxalic acid, tartaric acid, citric acid, sulphanic acid, maleic acid as standards. The solvent system used as mobile phase for monocarboxylic acid was composed of propanol: ammonium hydroxide (7: 3) and that for dicarboxylic acids was propanol: ammonium hydroxide: water (6: 2: 2). Bromocresol green was used as developing agent.

Activities of various antioxidative enzymes like ascorbate peroxidase, catalase, glutathione S transferase and glutathione (GSH) and hydrolytic enzymes like phosphomonoesterase, phosphodiesterase and phosphotriesterase were also determined. Acid phosphatase activity (EC 3.1.3.2) was estimated at acidic pH (5.0) using para-nitrophenyl phosphate as substrate, according to Ozkanca and Flint (1996). Phosphodiesterase I (EC 3.1.4.1) and organophosphate hydrolase (EC 3.1.8.1) activities were assayed at pH 7 using bis-para-nitrophenyl phosphate and phorate, respectively as substrates (Singh *et al.,* 2004). One unit of enzyme activity in all three cases was equivalent to mmol l^{-1} of paranitrophenol released mg^{-1} protein min^{-1}. Total glutathione content was determined according to Akerboom and Sies (1981). Glutathione S transferase (EC 2.5.1.18) activity towards chloro-2,4-dinitrobenzene (CDNB) was determined according to Ewards and Owen (1986), and enzyme activity was expressed as nmol l^{-1} of glutathione–CDNB conjugate produced mg^{-1} protein min^{-1}. Catalase (EC 1.11.1.6) activity was estimated according to

Brannan *et al.* (1981). One catalase unit is the amount of enzyme responsible for the decomposition of 1 μmol l^{-1} of H_2O_2. L-Ascorbate peroxidase (EC1.11.1.11) activity was measured according to Beyer and Fridovich (1987). One unit of enzyme catalyzes oxidation of 1 μmol l^{-1} of ascorbate mg^{-1} protein min^{-1}.

Riboflavin was detected using spectrophoto-fluorometer at 460-nm activation wavelength and 530-nm emission wavelength and ascorbic acid was determined by titrimetric method according to official methods of analysis of the association of official analytical chemist (Helrich, 1990).

Phenolic acids in root exudates were detected by high performance liquid chromatography (Waters Delta Prep, preparative chromatography system with PDA detector, Waters) with photodiode array detector. All the samples were diluted appropriately in HPLC grade methanol and filtered through a 0.2 μm membrane filter. The compounds detected were identified by the comparison with their retention times with those of pure standards. The HPLC system consisted of Waters pumping system, PDA detector and software. Chromatographic separations were performed on a C18, column (250 × 4.0 mm ID, 5 μm) and gradient elution program with two solvents (Tian *et al.* 2009). Solvent A consisted of 10% methanol and 2% acetic acid in water, and solvent B included of 90% methanol and 2% acetic acid in water. For elution program, the following proportions of solvent B were used: 0-25 min, 0-15% B; 25-45 min, 15-50% B; 45-53min, 50-0% B at a flow rate of 1.0 ml min^{-1}. Analytical grade gallic acid, vanillin, ellagic acid, caffeic acid, gentisic acid, syringic acid, *p*-coumaric acid, ferulic acid, benzoic acid, salicyclic acid, chlorogenic acid, phytic acid were used as standards.

Attempts were made to identify flavonoids present in root exudates by MALDI-TOF analysis according to Wang and Sporns (2000). Samples (2 μl) were coated with 8 μl CHCA (α-hydroxy 2-chloro cinnamic acid) matrix and then subjected to laser ionization. Flavonoids were largely identified on the basis of mass spectra in absence of any reference standards.

All the experiments were carried out in triplicates and results are presented as Average ±SD. Data for chemical and enzyme analysis was subjected to student's t test to determine statistical significance. Correlation between different parameters was estimated by calculating Pearson's correlation coefficient (R).

Results and Discussion

The quantity of compounds present in root exudates and extracts greatly varies with factors like plant species, its age and method of collection (Wadhwa and Narula, 2012). No significant change ($P>0.05$) in carbohydrate content of root exudates, as well as, root extracts was observed between control and experimental samples (Table 1). Carbohydrate content varied significantly in root exudates and extracts, higher value was obtained in all root exudates as compared to root extracts. The differences in results of samples collected by two methods indicate that the quantity and components of the same differed with the method for its collection. The chemical composition of root exudates is not only dependent on plant age, species, type of soil, climatic and geographical conditions and environmental stress, but also on the method of its collection and analysis (Shaw and Burns, 2004). Carbohydrate content of root exudates is mainly contributed by high molecular weight mucilaginous substances secreted by root tissues; however, low molecular weight compounds like sugars also contribute to it to some extent. Mucilaginous substances are generally excreted as waste materials by plants without any known specific function.

In the present investigation, protein content of both root exudates and extract was found to increase by 88.4% and 26.2% respectively ($P<0.05$), in response to phorate (Table 1,4). Direct involvement of proteins in phytoremediation of inorganic contaminants including heavy metals and radionuclides have been demonstrated by many workers (Gleba *et al*., 1999) but their role in phytodegradation of organic contaminants is seldom reported. In a study by Suresh *et al*. (2005), involvement of few root enzymes of *B. juncea* in biodegradation of DDT was postulated. They observed that hairy root cultures of plant were capable of degrading DDT by approximately 75%, in ten days, *in-vitro*. Also, the involvement of extracellular proteins secreted by roots of *Brassica napus* plants in development and stress resistance in plants have been speculated (Basu *et al*., 2006). Similarly, increase in secretion of proteins from roots of both plants in response to phorate, as observed in this case, suggests that they have some important role in degradation/ transformation of target compound (phorate). However, the nature of proteins exuded needs to be determined. No significant difference ($P>0.05$) was observed in free thiol content of exudate samples collected by both the methods. Root exudates were not found to contain any of the amino acids, but root extracts were found to contain few of them. *B. juncea* roots extract contained threonine and 2-amino butyric acid, and no change in composition of amino acid was observed in presence of phorate (Table 1). Proteinogenic amino acids released into the rhizosphere have been suggested to have some role in direct acquisition of nutrients by plants. Though, in *B. juncea*, no change in the composition of amino acids in root extract could be observed; another peculiar observation which needs to be addressed here was the appearance of 2-amino butyric acid, a non-protein amino acid in the samples. Detection of 2-amino butyric acid and

Table 1 : Characterization of root exudates and root extracts of *B. juncea* in presence and absence of phorate

Sample	Carbohydrate content (mg l^{-1})	Proteins (mg ml^{-1})	Free thiol groups (µM)	Amino acids	Sugars	Organic acids
Exudate C	1482.6±21	0.0086±0.005	0.098±0.03	——	Xylose	Tartaric acid
Exudate E	1491.5± 33	0.0162±0.004	0.102±0.04	——	Xylose	Tartaric acid
Extract C	250.2±12	0.175±0.004	0.047±0.08	Threonine, 2 amino butyric acid	——	Tartaric acid,
Extract E	270.5±33	0.2209±0.01	0.048±0.02	Threonine, 2 amino butyric acid	——	Tartaric acid, 2-ketoglutaric acid

Note**:** C= without phorate; E= with phorate

Table 2 : Enzyme activities detected in root exudates and root extracts of *B. juncea*

Sample	Acid phosphatase activity (U)	Phosphodiesterase activity (U)	OPH activity (U)	GSH content (nM ml^{-1})	GST (U)	APX (U)	CAT (U)
Exudate C	33.11±4.5	21.21±5.3	143.6±45.3	1.970±0.38	250.7±35	0.0175±0.03	5.58±1.54
Exudate E	34.97±3.5	22.691±3.9	138.5±52.4	2.230±0.21	168.57±78	0.0188±0.07	4.70±1.23
Extract C	29.38±7.2	14.14±4.4	136.0±23.8	1.204±0.34	——	——	——
Extract E	24.90±2.4	14.51±3.2	128.2±8.3	1.787±0.4	——	——	——

Note: U=Units; OPH=organophosphorus hydrolase; GSH=glutathione; GST=glutathione s transferase; APX=ascorbate peroxidase; CAT=catalase Exudate = root exudates; Extract= Root extract; C= control (in absence of phorate); E= Experimental (in presence of phorate)

several other non-protein amino acids in root exudates of few plant species have been reported (Phillips *et al*., 2004). Presence of non-protein amino acids in plants have been ascribed plentiful of functions like they are generally known to act as either inhibitor of predators, pathogens or competing plants, thereby helping the plant to survive under colossal competitive environment (Bell, 2003).

Xylose was only sugar detected in root exudates of *B. juncea* in control, as well as, in experimental samples. Results of the present investigation are in accordance with previous studies where xylose, fructose, glucose and maltose were detected in root exudates of *Brassica juncea* **(***Carvalhais et al*., 2011). Sugars act as stimulators of bacterial and fungal growth, however, their exudation has also been related with nutrition stress and has been suggested as a mechanism to enhance mycorrhizal association (Li *et al*., 2013).

Root exudates of *B. juncea* showed presence of tartaric acid, both in control and experimental samples. However, root extracts were found to contain tartaric acid and 2-keto glutaric acid in control and tartaric acid in experimental samples. Organic acids like malic acid, citric acid, tartaric acid and oxalic acid have been detected in the root exudates of *B. juncea* plants by earlier workers (Carvalhais *et al*., 2011). It has been suggested that these organic acids are responsible for the observed negative-rhizosphere effect in *Barssica* plants. Exudation of organic acids, especially citrate, is a mechanism to increase the availability of phosphorus. Release of organic acids causes decline in soil pH, thus favouring solubilization of unavailability phosphorus, consequently turning it available to plants. Secretion of organic acids in response to metal nutrient deficiency like iron (Fe) is also well documented (Kozdroj and Elsas, 2000). Presence of heavy metals (mostly toxic) in rhizosphere and low availability of phosphorus in soil has been reported to cause enhanced secretion of acids from root exudates (Pearse *et al*., 2006).

Several enzymes have been reported to be important constituents of root exudates in many plants; therefore, activities of certain hydrolytic and antioxidative enzymes were detected in root exudates and extracts (Table 2). No significant change in acid phosphatase activity in samples was observed in root exudates and extracts of *B. juncea* (Table 2,4) Acid phosphatase activity in root exudates is regulated by the availability of plant assimilable forms of phosphorus in soil (Sarapatka *et al*., 2004). In scarcity of available phosphorus, plants are known to secrete high amount of enzyme which facilitates the conversion of unavailable phosphorus to available ones; the latter is then easily taken up by plants.

Root exudates and extracts showed both phosphodiesterase and organophosphate hydrolase activities, however, no significant variation was observed between the activities of enzymes in control and experimental samples (Table 2,4). Total glutathione content in root exudates and extracts was found to increase by 13.2% and 58.3%, respectively, ($P<0.05$) in plants exposed to phorate (Table 2, 4). High level of GSH in roots have been

Table 3 : Riboflavin, ascorbic acid, phenolic acids and flavonoids in *B. juncea* root exudates

Sample	Riboflavin ($\mu g\ ml^{-1}$)	Ascorbic acid ($mg\ ml^{-1}$)	Phenolic acids	Flavonoids
Exudate C	3.37±0.54	0.744±0.34	caffeic acid, coumaric acid, gentisic acid, vanillic acid	kaempferol, kaempferol rutinoside, myricetin 3-rutinoside, isohamnetin rutinoside, quercetin 3-rutinoside
Exudate E	3.59±0.86	0.844±0.68	caffeic acid, coumaric acid, gentisic acid, vanillic acid	kaempferol, kaempferol rutinoside, myricetin 3-rutinoside, isohamnetin rutinoside, quercetin 3-rutinoside
Extract C	0.58±0.06	0.124±0.04	_____	isohamnetin rutinoside, quercetin 3-rutinoside and rutin
Extract E	1.36±0.23	0.248±0.06	_____	isohamnetin rutinoside, quercetin 3-rutinoside and rutin

Exudate= Root exudate; Extract = Root extract; C= Control (in absence of phorate); E= Experimental (in presence of phorate)

Table 4 : Percent change in the chemical composition and enzyme profile of root exudates and root extracts of *B. juncea*

Component	% change in root exudates on phorate exposure	% change in root extracts on phorate exposure
Carbohydrate content (mgL^{-1})	+6.0 (NS)	+8.1 (NS)
Protein content ($mg\ ml^{-1}$)	+88.4 (P<0.05)	+26.2 (P<0.05)
Free thio groups (µM)	+4.1 (NS)	+2.1 (NS)
Riboflavin ($\mu g\ ml^{-1}$)	+6.5 (NS)	+134.4 (P<0.05)
Ascorbic acid ($mg\ ml^{-1}$)	+13.4 (P<0.05)	+100 (P<0.05)
Acid phosphatase activity (U)	+5.5 (NS)	-15.3 (NS)
Phosphodiesterase activity (U)	+7.0 (NS)	+2.6 (NS)
Organophosphate hydrolase (OPH) activity (U)	-3.6 (NS)	-5.7 (NS)
Glutathione content (nM/ml)	13.2 (P<0.05)	+58.3 (P<0.05)
Glutathione S Transferase activity (U)	-32.8 (P<0.05)	_____
Ascorbate Peroxidase activity (U)	+7.4 (P<0.05)	_____
Catalase (U)	+15.8 (P<0.05)	_____-

Note: +ve sign indicates increase and -ve sign indicates decrease; NS= Non significant

reported in response to the presence of heavy metals in soil as it acts as precursors for phytochelatins synthesis (Jozefczak *et al.,* 2012) Taking into account, the protective role of GSH in detoxification/phytodegradation of organic contaminants, it may be hypothesized that it is secreted in high concentrations from plant roots in response to the presence of phorate in rhizosphere.

Secretion of few antioxidative enzymes like super oxide dismutase (SOD) and peroxidases (TPX) have been reported (Basu *et al*., 2006) in *Brassica napus* in response to various stress conditions. In the present investigation, enzyme activities like glutathione S transferase, ascorbate peroxidase and catalase could be detected only in the samples collected by root exudates and none of the enzyme activities were observed in root extracts. Yet, no significant (P>0.05) change between the activities of any of the enzymes, in root exudates and extracts was observed following exposure to phorate except glutathione S transferase, which was found to decline in experimental samples (Table 2, 4)

Riboflavin content of root extracts of *B. juncea* was 134.4% high (P<0.05) in experimental samples as compared to controls (Table 3,4). Release of riboflavin from plant roots is reported to be induced by Fe deficiency (Welkie, 2000). Release of riboflavin by plant roots also have important role in plant-microbe interactions. Some riboflavin in root zone is degraded to lumichrome, which stimulates root respiration (Phillips *et al*., 1999) and may, therefore, contribute to exogenous CO_2 required for growth by this organism (Matiru and Dakora, 2004). In this way riboflavin in root exudates play significant role in plant-microbial interactions. Ascorbic acid concentration in the experimental exudate and extract samples was found to be higher by 13.4 and 100.0%, respectively as compared to control. Exudation of ascorbic acid from plant roots have been demonstrated by several workers (Tu *et al*., 2004).

Phenolic acids in root exudates have been associated with number of functions. Commonly, they act as nutrient source for microflora of soil. Many of them have been reported as chemoattractant signals to microbes, microbial growth promoters, nod gene inducers and inhibitors in rhizobia. Many phenolic compounds are important in plant's defense against pathogens and act as phytoalexins and have allelophathic functions (Wu *et al.,* 2010; Bhattacharya *et al.,* 2010). Thus, phenolic acids are important chemicals in plant-microbe interaction. Secretion of phenolic compounds from

plant roots have also been frequently reported under iron and phosphorus deficient conditions (Jin *et al*., 2007). Caffeic acid, coumaric acid, gentisic acid and vanillic acid were phenolic compounds detected in root exudates of *Brassica juncea* (Table 3). A number of phenolic acids have been detected in *Brassica* species but their exudation by plant roots has seldom been reported. Irtelli and Navari-Izzo (2006) detected phenolic acids like gallic acid, protocatechuic acid, *p*-hydroxy benzoic acid, vanillic acid, chlorgenic acid in *Brassica juncea* plants and they reported increase in phenolic acid content in plants growing in presence of cadmium. Gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid were identified from potherb mustard (*Brassica juncea*, Coss.) (Fang *et al*., 2008). Also, sinigrin, gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, and sinapic acid are some common phenolic acids present in *Brassica juncea*. Caffeic acid and its derivative, p-hydroxy benzoic acid, vanillic acid, gentisic acid, totocatechuic acid and syringic acid have been reported in Broccoli (*Brassica oleracea*) (Vallejo *et al., 2003).*

Root exudates were found to contain kaempferol, kaempferol rutinoside, myricetin 3-rutinoside, isohamnetin rutinoside, quercetin 3-rutinoside and rutin, whereas root extracts showed the presence of isohamnetin rutinoside, quercetin 3-rutinoside and rutin (Table 3). Kaempferol and kaempferol rutinoside and their glucosides have been reported to be present in *Brassica juncea* plants (Kim *et al*., 2002; Jung *et al*., 2009). Flavonoids in root exudates mainly function as signaling compounds in symbiotic and pathogenic plant-microbe interactions (Hassan and Mathesius, 2012). None of flavanoid components have been reported in root exudate/extract of *B. juncea*; thus, this is for the first time that some of the flavonoid components of root exudates and extracts have been identified.

Hence, on the basis of our observations it may conclude that composition of root exudates and root extracts of *B. juncea* contain wide array of chemical compounds which have different physiological functions in nature. On the basis of experimental evidence of current investigation, it may be concluded that the chemical composition and enzymatic activities in root exudates changes in presence of contaminants in growth medium. This may be a mechanism of plant defense against harmful pollutants.

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