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Full Length Research Paper

Effect of nickel toxicity on the alteration of phosphate pool and the suppressing activity of phosphorolytic enzymes in germinating seeds and growing seedlings of rice

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To determine the effects of nickel on the phosphate pool and the activity of phosphorolytic enzymes in rice germinating seeds and growing seedlings, rice *CVS.* Malviya-36 and Pant-12 were germinated for 120 h, submitted to 200 and 400 µM $NiSO_4$ and the level of phosphate pool and the activities of key phosphorolytic enzymes acid phosphatase, alkaline phosphatase and inorganic pyrophosphatase were observed in germinating seeds and growing seedlings. The result showed that in germinating rice seeds, 400 µM $NiSO_4$ treatment caused a decline in phosphate pool at 24 h of germination but during the later period of 72 to 120 h, an increase in phosphate level was observed in both endosperms and embryoaxes, while in seedlings raised for 10 to 20 days, a decline in total phosphate pool was noticed. With a nickel treatment level of 400 µM in situ, about 19 to 38% decline in acid phosphatase activity was observed in endosperms and embryoaxes at 96 h of germination and 23 to 52% decline in activity in roots and shoots in 15 day grown seedlings. Similarly the seeds germinating in presence of 400 µM Ni^{2+} showed about 17 to 41% decline in alkaline phosphatase activity in endosperms and about 51 to 59% in embryoaxes at 72 h of germination while about 21 to 60% inhibition of alkaline phosphatase activity was noticed in roots and shoots of 15 day grown seedlings under 400 µM Ni^{2+} treatment. The activity of inorganic pyrophosphatase was also inhibited substantially in germinating rice seeds and growing rice seedlings in presence of 200 µM or 400 µM Ni^{2+}. The results suggested that alteration in the level of phosphate pool and inhibition in the activities of phosphorolytic enzymes might contribute to reduced metabolic activities, delayed germination of rice seeds and decreased vigour of seedlings in Ni^{2+} polluted environment.

Key words: Germination, nickel, phosphate, phosphatase, rice, seedling.

INTRODUCTION

The physiological and biochemical processes underlying germination and early seedling growth are important for establishment of plant in its environment (Soeda et al., 2005). Phosphorus is an essential macronutrient for all living organisms, it plays vital physiological role in energy transduction and metabolic regulations and serves as key component of many biomolecules (Vincent et al., 1992, Duff et al., 1994). Various stressful conditions of the environment including high metal contents in the growth medium, adversely affect phosphate metabolism in plants (Shah and Dubey, 1997, 1998; Ehsanpour and Amini, 2003; Parida and Das, 2004; Mihoub et al., 2005). Disruptions of metabolic processes are associated with modulation in the activities of different enzymes. Among the enzymes of phosphate metabolism, optimum activities of phosphatases are essential in the cells for regulation of many physiological processes including maintenance of soluble phosphate level required for normal growth (Yan et al., 2001; Vance et al., 2003).

The phosphatases, traditionally classified as acid phosphatases, alkaline phosphatases and inorganic

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pyrophosphatases (Barret-Lennard et al., 1982). Acid phosphatases are ubiquitously present in germinating seed parts and also in different cellular compartments suggesting the involvement of enzymes in various metabolic and bioenergetic events (Bozzo et al., 2004). Acid phosphatases represent a group of enzymes that usually display broad substrate specificity, whereas alkaline phosphatases generally display low substrate specificity (Turner and Plaxton, 2001). Inorganic pyrophosphatases are metalloenzymes that require Mg^{2+} for their stability and catalyze hydrolysis of pyrophosphate to two phosphate molecules and provide energy to various biosynthetic reactions (Geigenberger et al., 1998).

Heavy metals exert direct lesion effects on germination of seeds and affect various physiological and biochemical processes in growing plants (Dubey and Pessarakli 2002; Ahsan et al., 2007; Kuriakose and Prasad, 2008). Nickel is an essential micronutrient and has beneficial effects in the process of seed germination of many crops (Mishra and Kar, 1974; Rout et al., 2000). However, its high concentration is toxic to the plants and causes adverse effects on metabolic and physiological processes like respiration, photosynthesis, metabolism of carbohydrates, proteins etc. (Espen et al., 1997; Parida et al., 2003; Maheshwari and Dubey, 2007). Due to increase in anthropogenic activities, soil pollution by heavy metals including Ni^{2+} has progressively increased (Rubio et al., 1994). On the average, total Ni^{2+} content of soil varies from 2 to 750 mg kg^{-1} with maximum content in serpentine soils (Seregin and Kozhevnikova, 2006). Ni^{2+} is readily taken up by rice roots (Rubio et al., 1994) and therefore, Ni^{2+} toxicity is indeed a problem for rice crop in many areas of the world which have high soil Ni^{2+} contents (Rubio et al., 1994; Wang, 2000; Seregin and Kozhevnikova, 2006). In order to get more insight into the influence of excess nickel on the process of germination and early seedling growth in rice, the present investigation was to examine the metabolic pool of phosphate and behaviour of acid phosphatase, alkaline phosphatase and inorganic pyrophosphatase in endosperms and embryoaxes of germinating rice seeds as well as in roots and shoots of rice plants growing under increasing concentration of nickel in the medium.

MATERIALS AND METHODS

Plant material

Seeds of two rice (*Oryza sativa* L.) *cvs.*, Malviya-36 and Pant-12 were used in the experiments. The seeds were obtained from the Institute of Agricultural Sciences, Banaras Hindu University, India. Seeds were surface sterilized with 0.1% sodium hypochlorite solution for 10 min and then rinsed with double distilled water. After soaking for 24 h in water, seeds were spread in petriplates lined with thin bed of sterilized moist cotton using uniform quantities of deionized distilled water which served as control or $NiSO_4.7H_2O$ solutions of 200 M (11.74 ppm) and 400 M (23.48 ppm) concentrations which served as treatment solutions. Seeds were germinated for 5 days at 28 ± 1°C with 80% relative humidity in humidity cum BOD incubator (Narang Scientific Works, New Delhi), maintaining a regular cycle of 12 h light (80 to 90 mol m^{-2} s^{-1} irradiance) followed by dark period. Seedlings were raised in sand cultures in plastic pots. Sand in each pot was saturated with either Hoagland nutrient solution (Hoagland and Arnon, 1950), which served as control, or with nutrient solutions containing 200 M and 400 M $NiSO_4.7H_2O$, which served as treatment solutions. Five day germinated seeds were transplanted in the pots and pots were kept for growth of seedlings in green house for 20 days with similar specification of temperature, humidity and light as for germinating seeds. Every alternate day, the pots received control or respective treatment solutions to saturate the sand. The germinated seeds were taken out at 24 h intervals up to 120 h and the endosperms and embryoaxes were separated. Seedlings were uprooted at 5 days periodic intervals up to 20 days and roots and shoots were separated. All estimations were performed in triplicate in endosperms and embryoaxes of germinating rice seeds as well as in roots and shoots of growing seedlings.

Extraction and estimation of phosphate

Total phosphate was extracted from endosperms and embryoaxes of germinating rice seeds as well as from roots and shoots of growing rice seedlings. About 100 mg oven dried (70°C for 72 h) samples were acid digested and phosphate was estimated according to the method of Fiske and Subbarow (1925). To 1 ml of sample extract, 1 ml of 5 M H_2SO_4 and 1ml of 2.5 percent ammonium molybdate solution were added. To this mixture, 0.1 ml of reducing reagent was prepared by mixing 0.2 g of 1,2,4-aminonapthosulfonic acid (ANSA) with 1.2 g of sodium metabisulphite and 1.2 g of sodium sulfite. From this properly powdered hypochlorite solution for 10 min and then rinsed with double distilled water. After soaking for 24 h in water, seeds were spread in petriplates lined with thin bed of sterilized moist cotton using uniform quantities of deionized distilled water which served as control or $NiSO_4.7H_2O$ solutions of 200 M (11.74 ppm) and 400 M (23.48 ppm) concentrations which served as treatment solutions. Seeds were germinated for 5 days at 28 ± 1°C with 80% relative humidity in humidity cum BOD incubator (Narang Scientific Works, New Delhi), maintaining a regular cycle of 12 h light (80 to 90 mol m^{-2} s^{-1} irradiance) followed by dark period. Seedlings were raised in sand cultures in plastic pots. Sand in each pot was saturated with either Hoagland nutrient solution (Hoagland and Arnon, 1950), which served as control, or with nutrient solutions containing 200 and 400 M $NiSO_4.7H_2O$, which served as treat-ment solutions. Five day germinated seeds were transplanted in the pots and pots were kept for growth of seedlings in green house for 20 days with similar specification of temperature, humidity and light as for germinating seeds. Every alternate day, the pots received control or respective treatment solutions to saturate the sand. The germinated seeds were taken out at 24 h intervals up to 120 h and the endosperms and embryoaxes were separated. Seedlings were uprooted at 5 days periodic intervals up to 20 days and roots and shoots were separated. All estimations were per-formed in triplicate in endosperms and embryoaxes of germinating rice seeds as well as in roots and shoots of growing seedlings.

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reagent was added. The reducing reagent was prepared by mixing 0.2 g of 1,2,4-aminonapthosulfonic acid (ANSA) with 1.2 g of sodium metabisulphite and 1.2 g of sodium sulfite. From this properly powdered the activity of inorganic pyrophosphatase (EC 3.6.1.1) was assayed in endosperms/embryoaxes and roots/shoots according to the method of Tominaga and Takeshi (1974). Fresh samples weighing 200mg were extracted in 5 ml of 0.1 M glycine-NaOH buffer (pH 8.8) using chilled mortar and pestle. Enzyme extract was dialyzed for 12 h in cold against the extraction buffer, with 3 to 4 changes of the buffer. The assay mixture contained 4 µmoles of $Na_4P_2O_7$, 100 µmoles glycine-NaOH buffer (pH 8.8), 10 µmoles of $MgCl_2$ and enzyme in a total volume of 2 ml. After incubation for 20 min at 30°C, the reaction was stopped by the addition of 0.5 ml of 30% TCA. Precipitate, if any formed, was removed by centrifugation and the supernatant was used for the determination of phosphate according to the method of Fiske and Subbarow (1925). One nkat of pyrophosphatase activity is expressed as one nmol of phosphate liberated per second under the experimental conditions and specific activity as enzyme units mg^{-1} protein.

In all enzyme preparations, protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin (BSA, Sigma) as standard.

Statistical analysis

All the experiments were performed in triplicate. Values in the figures indicate Mean ± s.d. based on three independent determinations. Differences among control and treatments were analyzed by one factorial ANOVA followed by Tukey's test using software SPSS.10. Asterisks (* and **) were used to identify the level of significance of the difference between control and nickel treatments on the corresponding days as $p \leq 0.05$ (*) and $p \leq 0.01$(**).

RESULTS

Effect of Ni^{2+} on level of phosphate

Phosphate content was observed in endosperms and embryoaxes of germinating seeds of both rice *cvs.* (Malviya-36 and Pant-12), during 0 to 120 h period (Figure 1). Ni^{2+} treatment resulted in marked alteration in the level of Pi in both endosperms and embryoaxes. Compared to controls, phosphate level declined at 24 h of germination while increased level of Pi was observed in endosperms and embryoaxes of Ni^{2+}-treated seeds during 72 to 120 h of germination. A higher Ni^{2+} treatment level of 400 µM resulted in an apparent 30 to 32% increase in the level of Pi in endosperms and about 53 to 78% increase in Pi level in embryoaxes at 120 h of germination.

The level of phosphate pool increased in roots and shoots of control grown seedlings during 5 to 15 days of growth and thereafter, it remained either at similar level (Malviya-36, root) or declined by 20^{th} day (Figure 2). In seedlings growing in presence of 200 µM or 400 M Ni^{2+}, a marked decline in phosphate content was observed in both roots and shoots of the two rice cultivars. Seedlings grown in presence of a higher Ni^{2+} concentration of 400 µM showed about 23 to 52% decline in phosphate content in roots and about 20 to 50% decline in shoots at 15 day of growth.

Effect of Ni^{2+} on acid phosphatase activity

A genotype specific difference in the level of acid phosphatase activity was observed during germination of seeds of the two rice cultivars, with *cv.* Pant-12 showing higher enzyme activity than *cv.* Malviya-36 in both endosperms and embryoaxes under both controls as well as Ni^{2+} treatments (Figure 3a). A significant decline in acid phosphatase activity was observed in endosperms and embryoaxes of germinating rice seeds, in roots and shoots of growing seedlings with increasing concentration of nickel treatment. A 400 µM Ni^{2+} led to about 19 to 38% decline in acid phosphatase activity in endosperms and about 23 to 36% decline in enzyme activity in embryoaxes at 96 h of germination of seeds compared to the enzyme activity values in controls. The activity of enzyme declined gradually during 5 to15 days of growth period and it increased thereafter by 20^{th} day in control as well as Ni^{2+} treated rice seedlings (Figure 3b). A 400 µMNi^{2+} treatments led to about 37 to 52% decline in acid phosphatase activity in roots and about 23 to 47% decline in activity in shoots.

Effect of Ni^{2+} on alkaline phosphatase activity

In endosperms of both rice cultivars, the activity of alkaline phosphatase increased after 24 h of germination and it decreased gradually thereafter, whereas in the embryoaxes, maximum enzyme activity was noted during 48 to 72 h of germination (Figure 4a). With increase in the level of Ni^{2+} treatment, a concomitant decline in alkaline phosphatase activity was observed in both endosperms and embryoaxes of germinating seeds. A higher nickel treatment level of 400 µM resulted in about 17 to 41% decline in alkaline phosphatase activity in endosperms and about 52 to 59% decline in activity in embryoaxes at 72 h of germination.

Figure 4b shows the effect of increasing level of Ni^{2+} treatment on alkaline phosphatase activity in roots and shoots of both rice *cvs.* Malviya-36 and Pant-12. An organ specific alteration in the level of alkaline phosphatase activity was observed in roots and shoots of seedlings during a 5 to 20 day growth period. In roots of the seedlings, in control as well as Ni^{2+} treatments, alkaline phosphatase activity increased during early days of growth, it was maximum at 10 days and decreased thereafter, whereas in shoots, a regular increase in enzyme activity was observed during 5 to15 days of growth and either it further increased by 20^{th} day (Malviya-36, shoot) or decreased a little (Pant-12, shoot). In both roots and shoots of the two rice cultivars, with increase in the level of Ni^{2+} treatment, a decline in alkaline phosphatase activity was observed. Shoots always maintained higher alkaline phosphatase activity

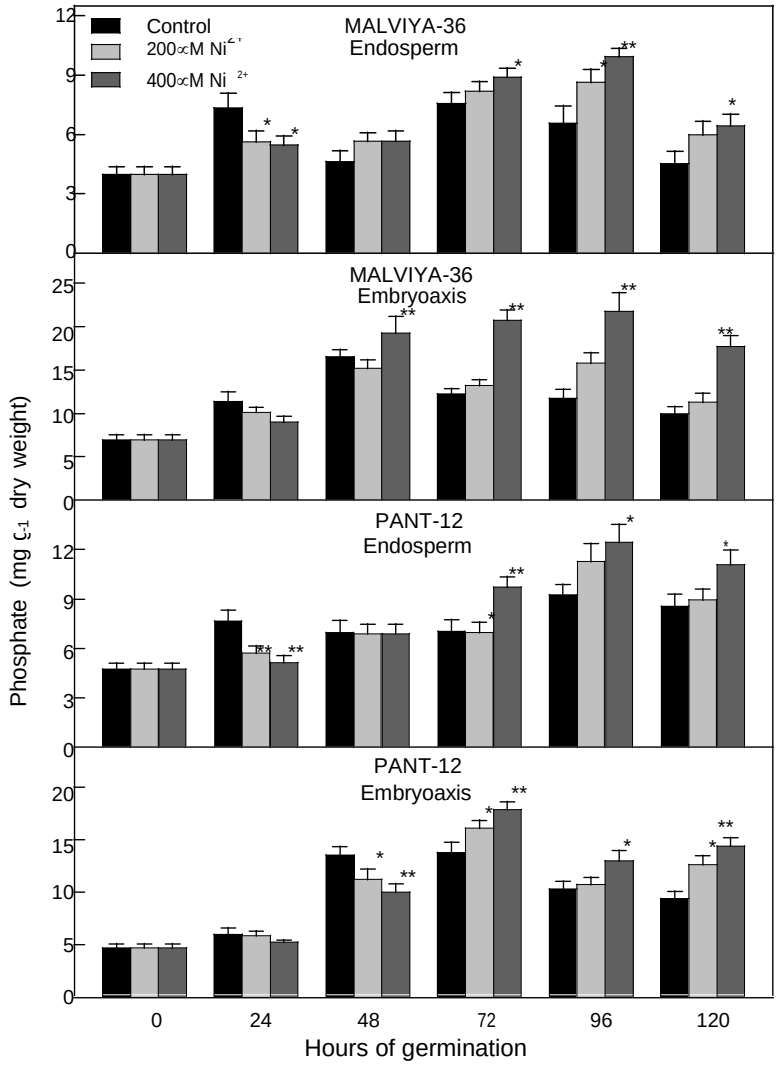

Figure 1. Level of phosphate in endosperms and embryoaxes of seeds of rice *cvs.* Malviya-36 and Pant-12 at increasing hours of germination under 0 (control), 200 and 400 µM Ni^{2+} in the medium. Values are Mean ± s.d. based on three independent determinations and bars indicate standard deviations. * and ** represent significant differences compared to controls at $p \leq 0.05$ and $p \leq 0.01$ respectively according to Tukey's test.

compared to roots under both controls as well as Ni^{2+} treatments. A Ni^{2+} treatment level of 400 µM resulted in about 32 to 60% decline in alkaline phosphatase activity in roots and about 21 to 35% decline in the activity in shoots in 15 day grown seedlings.

Effect of Ni^{2+} on inorganic pyrophosphatase activity

Pyrophosphatase increased in endosperms and embryoaxes of both the rice cultivars reaching maximum at 72 h and it declined thereafter (Figure 5a). Increasing concentration of nickel in the germination medium resulted in a significant decline in the activity of inorganic pyrophosphatase. A higher level of 400 µM Ni^{2+} treatment resulted in about 33 to 57%t decline in enzyme activity in endosperms and about 32 to 36% decline in activity in embryoaxes at 72 h of germination.

In control grown seedlings of both the rice cultivars, activity of pyrophosphatase increased during 5 to 10 days of growth, it was maximum at day 10 and declined thereafter (Figure 5b). With increase in the level of Ni^{2+} treatment, a concomitant decline in inorganic pyro-phosphatase activity was observed. Seedlings grown in presence of 400 µM Ni^{2+} for 20 days showed about 50 to 75% decline in inorganic pyrophosphatase activity in roots and about 18 to 42% decline in enzyme activity in shoots compared to the activity in control grown seedlings.

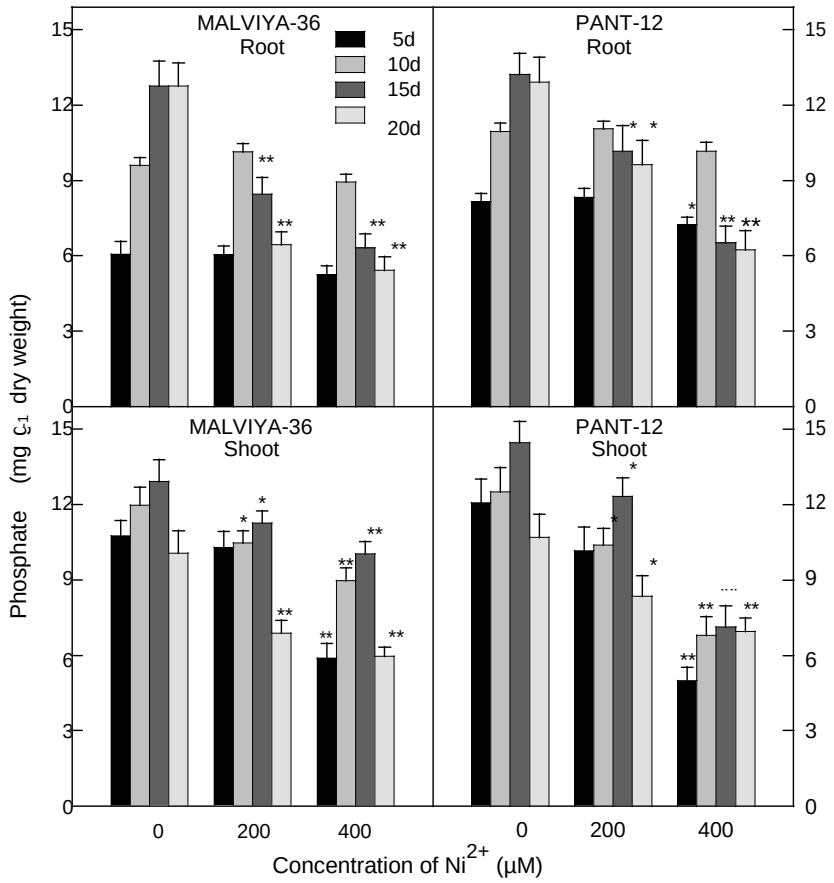

Figure 2. Level of phosphate in roots and shoots of the seedling of rice *cvs*. Malviya-36 and Pant-12 at different days of growth under 0 (control), 200 µM and 400 µMNi^{2+} in the medium. Values are mean ± s.d. based on three independent determinations and bars indicate standard deviations. * and ** represent significant differences compared to controls at $p \leq 0.05$ and $p \leq 0.01$ respectively according to Tukey's test.

Discussion

Results of the present study indicate an alteration in phosphate pool and decline in the activities of phosphohydrolytic enzymes (acid phosphatase, alkaline phosphatase and inorganic pyrophosphatase) in endosperms and embryoaxes of germinating rice seeds and in roots and shoots of growing seedlings in presence of 200 or 400 µM Ni $^{2+}$ in the medium. Our earlier studies indicated that Ni^{2+} concentrations of 200 µM was moderately toxic and 400 µM highly toxic for the growth of these two rice cultivars and led to inhibition in the activities of hydrolytic enzymes ribonuclease and protease in the seedlings (Maheshwari and Dubey, 2007).

Phosphorus is one of the most significant determinants of plant growth (Vance et al., 2003) and it is preferentially assimilated in the form of orthophosphate anion (Duff et al., 1994). Besides being structural component of many biomolecules, free inorganic phosphate plays vital role in energy transfer reactions including gene transcription, enzyme regulation, etc. (Mimura, 1995). Abiotic stressful conditions of the environment like salinity, water stress, and excessive level of heavy metals in the soil lead to altered status of Pi within the plant tissues (Dubey and Sharma, 1989; Shah and Dubey 1998). It has been suggested that an optimum level of Pi is to be maintained in plant tissues when subjected to stressful conditions in order to maintain subcellular osmoticum and for proper functioning of energy transfer reactions (Treeby and Van Steveninck, 1988). In our studies, a marked alteration in the level of inorganic phosphate was noted in both endosperms and embryoaxes due to Ni^{2+} treatment, and especially during 72 to 120 h of germination, an elevated level of Pi was noted in Ni^{2+} treated seeds, while a decline in the level of Pi was observed in roots as well as shoots of nickel-grown rice seedlings compared to the level in controls. The decline in Pi content might be either due to decline in uptake of phosphate or due to inhibition in the activity of phosphatases resulting in decreased

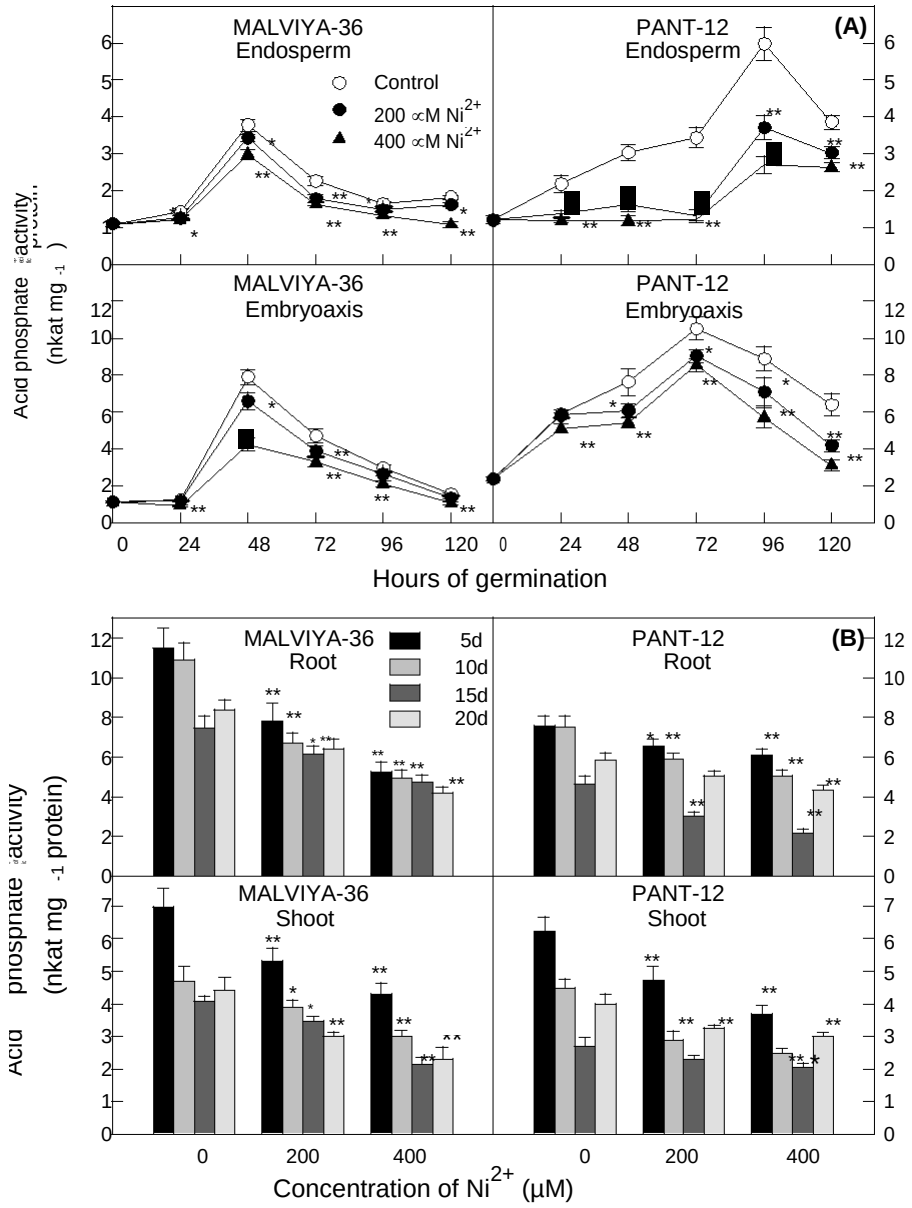

Figure 3. Specific activity of acid phosphatase in; (A) endosperms and embryoaxes seeds of rice *cvs.* Malviya-36 and Pant-12 at increasing hours of germination as well as in; (B) roots and shoots of seedlings at different days of growth under 0 (control), 200 µM and 400 µMNi^{2+} in the medium. Values are mean based on three independent determinations and bars indicate standard deviations. Asterisks * and ** indicate values that differ significantly from controls at $p \leq 0.05$ and $p \leq 0.01$ respectively.

hydrolysis of phosphate monoesters under stressful conditions. This suggests that Ni^{2+} toxicity impairs the mobilization as well as utilization of phosphate in germinating rice seeds and this might contribute to reduce growth of rice seedlings in high Ni^{2+} containing medium.

Acid phosphatase is the key enzyme involved in acquisition, transport and recycling of phosphorus (Yan et al., 2001). The activity of enzyme is abundant in germinating seeds and ripening fruits (Bozzo et al., 2002). Ungerminated seeds have low level of acid phosphatase activity and the activity gets enhanced during germination and early seedling growth (Biwas and Cundiff, 1991; Thomas, 1993). Expression of acid phosphatase is modulated by a variety of developmental and unfavorable environmental conditions. An increase in acid phosphatase activity has been found in plant parts during phosphate deficiency (Duff et al., 1994), water deficit (Ehsanpour and Amini, 2003) and salinity stress (Parida and Das, 2004). In our studies, an inhibition in the activity of acid phosphatase was observed throughout the

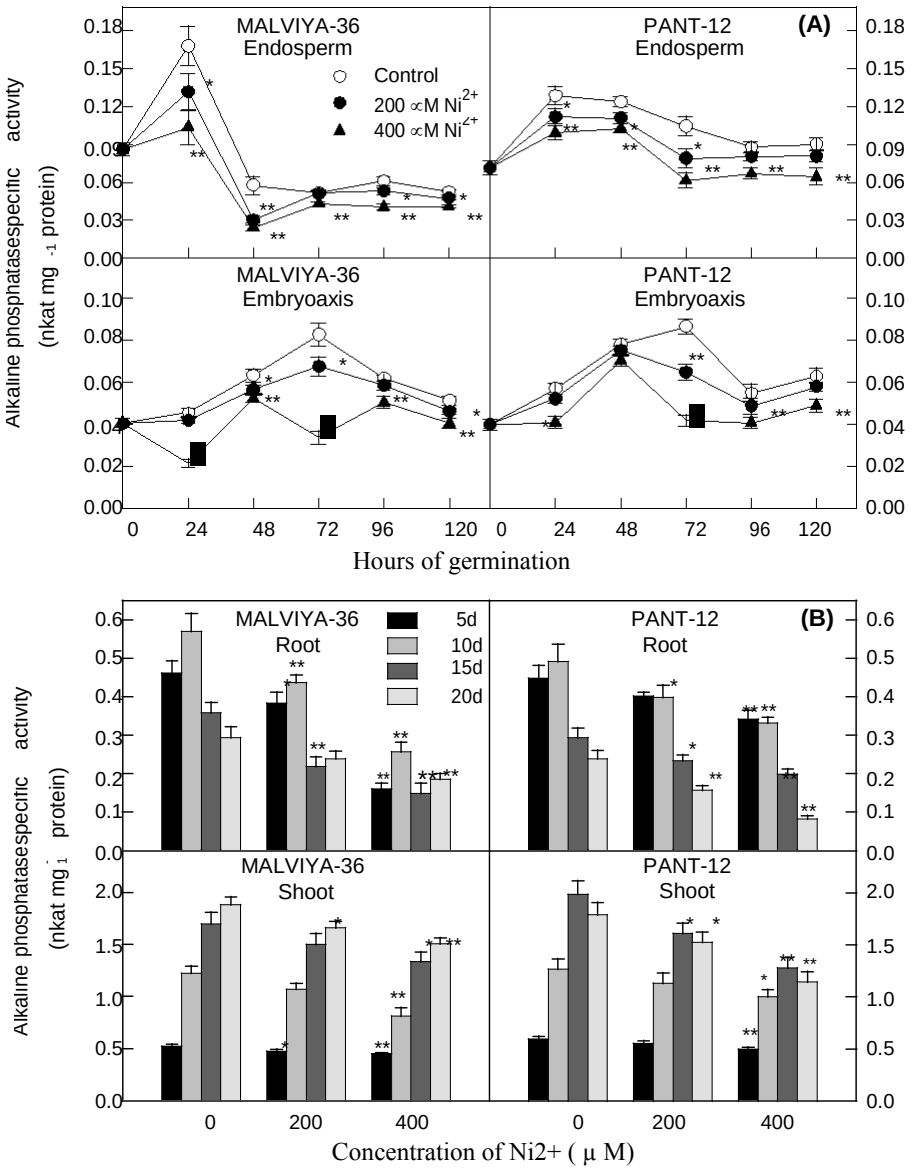

Figure 4. Specific activity of alkaline phosphatase in; (A) endosperms and embryoaxes of seeds of rice *cvs.* Malviya-36 and Pant-12 at increasing hours of germination as well as in; (B) roots and shoots of seedlings at different days of growth under 0 (control), 200 µM and 400 µMNi^{2+} in the medium. Values are mean based on three independent determinations and bars indicate standard deviations. Asterisks * and ** indicate values that differ significantly from controls at $p \leq 0.05$ and $p \leq 0.01$ respectively.

growth period under study due to Ni^{2+} treatment. Similarly, many workers have reported an inhibition in acid phosphatase activity under increasing levels of Cd^{2+}, Cu^{2+}, and Zn^{2+} in germinating seeds (Shah and Dubey, 1997; Granjeiro et al., 1999; Mihoub et al., 2005) and growing seedlings (Shah and Dubey, 1998).

Different behaviour of acid phosphatase activity has been observed in two Alyssum species differing in Ni^{2+} tolerance when grown under increasing levels of Ni^{2+}(Gabbrielli et al., 1989). Nickel tolerant plants showed higher level of acid phosphatase activity when grown in presence of Ni^{2+}, whereas under similar level of Ni^{2+} treatment, in sensitive plants, a decline in acid phosphatase activity was observed (Gabbrielli et al., 1989). Similarly, when barley plants differing in Al^{3+} tolerance were raised under increasing levels of Al^{3+}, a decline in acid phosphatase activity was observed in sensitive plants whereas, Al^{3+} tolerant plants showed increased enzyme activity under similar conditions (Huttová et al., 2002). Our results indicate sensitivity of the two rice cultivars

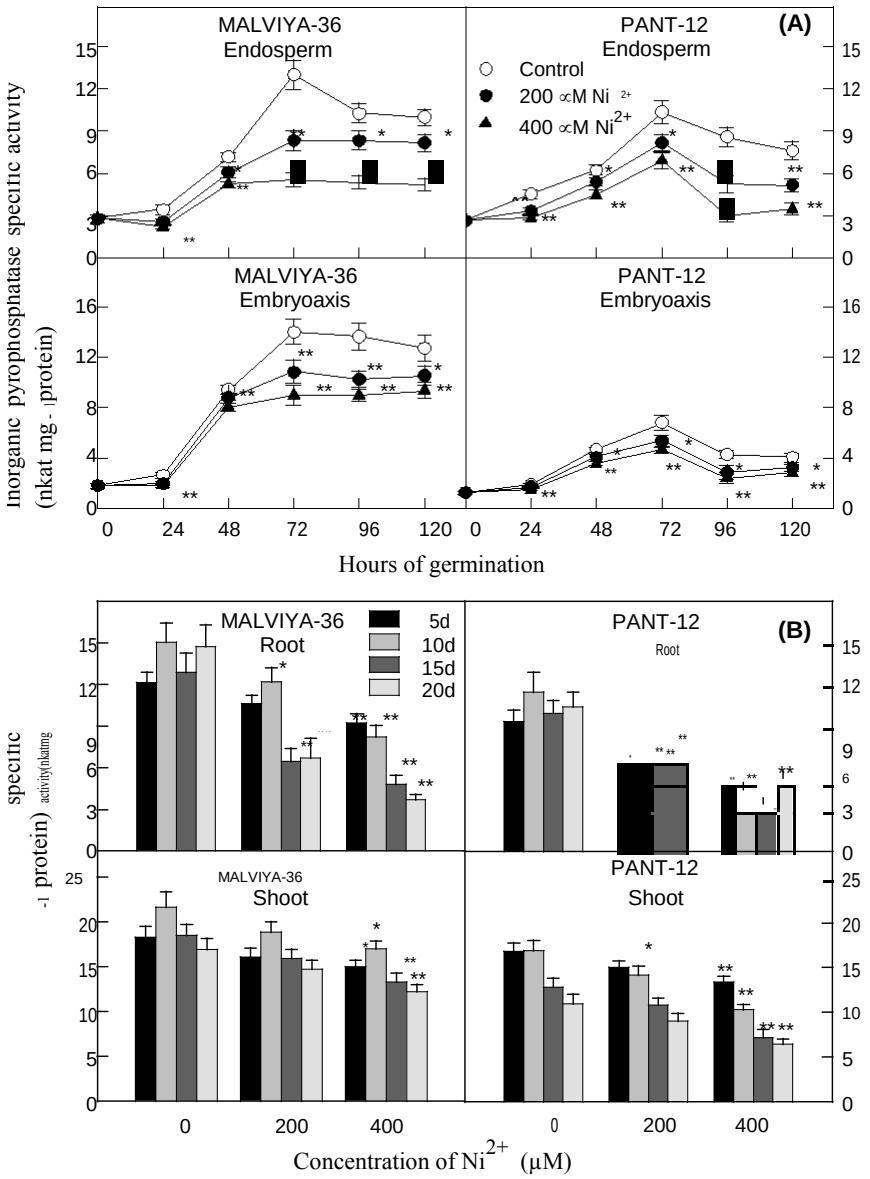

Figure 5. Specific activity of inorganic pyrophosphatase in; (A) endosperms and embryoaxes of seeds of rice *cvs.* Malviya-36 and Pant-12 at increasing hours of germination as well as in; (B) roots and shoots of seedlings at different days of growth under 0 (control), 200 µM and 400 µMNi^{2+} in the medium. Values are mean based on three independent determinations and bars indicate standard deviations. Asterisks * and ** indicate values that differ significantly from controls at $p \leq 0.05$ and $p \leq 0.01$ respectively.

used in the present study towards Ni^{2+} and that 200 µM Ni^{2+} or 400 µM Ni^{2+} cause inhibition in acid phosphatase activity in the seedlings.

Similar to acid phosphatase, the activity of alkaline phosphatase was also inhibited due to Ni^{2+} treatment. Alkaline phosphatase exists as dimeric enzyme containing two Zn^{2+} and one Mg^{2+} in each monomer, required for enzyme activity. Certain studies have indicated that Cd^{2+} and Co^{2+} ions replace Zn^{2+} ions from alkaline phosphatase in nostoc and marine diatoms, causing changes in enzyme conformation which result in inhibition in enzyme activity (Price and Morel, 1990; Husaini and Rai, 1991). Reduction in the activity of alkaline phosphatase has earlier been observed in presence of high concentration of Zn^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and Mo^{2+} (Angosto and Matilla, 1990; Shah and Dubey, 1997; Shah and Dubey, 1998). In the present study, decreased activity of alkaline phosphatase observed due to in situ Ni^{2+} treatment might be as a result of direct inhibition of enzyme activity due to Ni^{2+}.

In our studies, the activity of inorganic pyrophosphatase was inhibited due to in situ Ni^{2+} treatment. Inorganic pyrophosphatase hydrolyzes pyrophosphates, which are formed in various anabolic reactions and render these reactions irreversible (Geigenberger et al., 1998). In this way, pyrophosphatase has prime role in regulating various biosynthetic reactions within the cells. It is suggested that the induction in pyrophosphatase activity is associated with increased stress tolerance (Liang et al., 2005). An increase in H^+ translocating inorganic pyrophosphatase activity was observed under conditions of anoxia, chilling and salinity stress (Carystinos et al., 1995; Liang et al., 2005). However, earlier studies conducted with heavy metals like Cd^{2+}, Co^{2+}, Cu^{2+} showed inhibitory effect of these metals on inorganic pyrophosphatase activity (Maeshima, 1991; Shah and Dubey, 1998). An inhibition in the activity of pyrophosphatase, as observed in our studies in germinating rice seeds and growing rice seedlings under increasing levels of nickel thus suggests that high concentration of nickel ions would limit the hydrolysis of pyrophosphate which would further inhibit the biosynthetic reactions in establishing rice seedlings.

Our results indicate that the presence of 200 and 400 µM Ni^{2+} significantly inhibits the activities of key phosphorolytic enzymes acid phosphatase, alkaline phosphatase and inorganic pyrophosphatase and alters the level of free orthophosphate anion in germinating seeds and growing seedlings. Altered phosphate pool and decline in the activity levels of phosphohydrolases would ultimately contribute to decreased metabolic activity thereby leading to reduced germination and decreased vigour of rice seedlings in excess Ni^{2+} containing medium.

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