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THE EFFECT OF WATER STRESS ON NITRATE REDUCTASE ACTIVITY AND NITROGEN AND PHOSPHORUS CONTENTS IN *CUMINUM CYMINUM* L.

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Abstract

Cumin (*Cuminum cyminum* L.) is a plant with great medicinal importance cultivated in many regions such as Iran, India, Indonesia, Afghanistan, Pakistan, Lebanon, Syria and Turkey. In this research, nitrogen and phosphorus concentrations and nitrate reductase enzyme activity were studied in cumin under flooding stress. Cumin plants were cultivated in pots containing garden soil (in 1 cm depth, 15-20°C, 14 h light and 10 h darkness). Germination took place after 2 weeks. Flooding stress was applied 6 weeks after germination on a number of pots according to their field capacity (FC) (2, 3, and 4 fold) for 1 week; a number of pots were also considered as controls with field capacity. Plants were then harvested and chemical analysis of the factors under study was done using roots and shoots of the plants exposed to flooding conditions and the control plants. The experiment had a completely randomized design in which four levels of water in the soil (2FC, 3FC, 4FC) were compared. Analysis of variance was carried out using SPSS software and means were compared by Duncan's test at [≤0.05 significance level. The results showed that in comparison with control plants, nitrogen and phosphorus concentrations were significantly lower in both shoots and roots of flooded plants. This decrease was more pronounced in treated plants exposed to 4×field capacity conditions. Nitrogen concentration in roots and shoots of treated plants showed a significant decrease in comparison with control plants and this was more noticeable in treated plants exposed to 4×field capacity conditions. Moreover, concentration of nitrite produced from nitrate reduction catalyzed by nitrate reductase enzyme in roots and shoots of treated plants had a significant increase in comparison with control plants. Treated plants exposed to 4×field capacity conditions showed the most increase. Also the study showed that cumin seeds could survive in flooding environment for 14 days. Thus, flooding stress in cumin seeds decreased nitrogen and phosphorus concentrations in roots and shoots. This stress increased the activity of nitrate reductase enzyme in both roots and shoots.

Introduction

Cumin (*Cuminum cyminum* L.), an important commercial seed spice belonging to the Umbellifereae family, is valued for its aroma, medicinal and therapeutic properties. Historically, Iran has been the principal supplier of cumin, but currently the major sources are India, Sri Lanka, Syria, Pakistan, and Turkey. The area under cultivation of cumin in Iran is about 50 000 hectares with an average annual production of 50000 tonnes. In Iran, this crop is mainly cultivated in Eastern Khorasan, Sabzevar, Birjand, Gonabad, Sorkhe, Garmsar and Kerman (Riazi, 1997).

Cumin seeds contain 3-4% volatile oil and about 15% fixed oil. Cumin powder is an important ingredient in curry mixes and some bakery products. Due to its, it is used volatile oil in perfumery and foods, especially in oriental dishes as a flavour enhancer. In Iranian folk medicine, the fruit of this plant has been used to treat diarrhoea, toothache and epilepsy (Zargari, 1989).

In tropical and subtropical regions, severe crop losses are caused by prolonged seasonal rainfall. Excess water produces anoxic soil conditions within a few hours (Gambrell & Patrick, 1978). A major issue of concern for plant survival in many regions of the world is flooding. Under flooding stress, the soil characteristic changes and during soil inundation various alterations in the availability of different nutrients rapidly take place. Key physiological processes may be more sensitive to a particular stress than others and therefore limit overall growth and productivity in a stressful environment. From a single measurement after establishing a flood treatment, Minchin & Summerfield (1976) concluded that flooding decreased N_2 fixation relatively more than vegetative growth in cowpea (*Vigna unguiculata* [L.] Walp), and they suggested that decreased N_2 fixation was responsible for decreased vegetative growth. From their data it was not possible to discern if changes in N_2 fixation preceded the decrease in vegetative growth which would be necessary in establishing a cause and effect relationship and in determining possible acclimation to flooding stress. Other researches showed that the total nitrogen content in plant tissue has been decrease under flooding stress in various fruit species, such as citrus (Labanauskas *et al.*, 1972), apple (Olien, 1989), avocado (Slowick *et al.*, 1979), and blueberry (Herath & Eaton, 1968). In the wax-apple tree, the total nitrogen in the leaves was found to be significantly lower after 35 days of flooding treatment compared to the un-flooded control while the total amount of carbohydrates increased, resulting in a significant increase in the C/N ratio (total carbohydrate/total nitrogen) (Hsu *et al.*, 1999). Under water logging conditions, the activity of several metabolic pathways is reduced or altered (Kozlowski, 1997 and references therein). Shifts occur in carbohydrate, protein, organic acid, lipid metabolism and mineral contents. Exposures to water logging (min for 2 hrs) can have striking effects on the composition and quantity of proteins and can increase or decrease the activities of the vital enzymes involved (Subbaiah & Sachs, 2003). The mechanisms by which flood-tolerant plants survive water logging are complex and involve interactions of morphological, anatomical and physiological adaptations (Hook, 1984). Metabolic adaptation to water logging is associated with several metabolic adjustments which lead to the modulation of different enzymes viz., glucose-phosphate isomerase,

glyceraldehyde-3-phosphate dehydrogenase, nitrate reductase and phosphatases (Sachs *et al*., 1996). The intracellular phosphatases, present in cytosol, plastids and vacuoles, are responsible for the Pi-hydrolysis from organic compounds during seed germination, favoring internal Pi mobilization and translocation from senescent tissues (Lee, 1988; Duff *et al*., 1994). There is prevailing hypothesis about the role of phosphatases in plants and its relation to plant nutritional status i.e., plants adapted to Pi stress would present high leaf or root P-ase activity as a sign of hydrolyzing and remobilizing Pi, by root secretion and/or leaf synthesis, making Pi more available to plant, from soil or other plants parts (Lee, 1988; Barret-lenard *et al*., 1993). Abiotic stresses like salt, osmotic and water stress, have been reported to increase acid or alkaline phosphatase activity by maintaining a certain level of inorganic phosphate in the plant cells (Olmos & Hellin, 1997). However, the variation that occurs in phosphatase activities during very early water logging conditions is poorly understood and information on physiological events involved in this process is scarce. Nitrate reductase (NR, E.C. 1.6.6.1), the first enzyme in the nitrate assimilation pathway, is a limiting factor of plant growth and development (Solomonson & Barber, 1990) and is influenced by a variety of environmental factors (Crawford, 1995). Under flooding stress, the composition and quantity of proteins and amino acids, and the activities of related enzymes are important. In particular, nitrate reductase and glutamine synthetase, the two key enzymes in nitrate reduction and ammonia assimilation influencing the total nitrogen balance, are affected by flooding (Buwalda *et al*., 1988; Garcia-Novo & Crawford, 1973; Reggiani *et al*., 1988). Nitrate reductase is the key enzyme in nitrate reduction. Garcia-Novo & Crawford (1973) and Lambers (1976) reported that the activity of nitrate reductase in roots of flood-tolerant plants increased rapidly during flooding, as did the amino acid synthesis capability. However, wax-apple trees tend to respond differently to flooding. The activity of nitrate reductase in roots of flooded tree was not found to increase with flooding, as it did in other plants; instead, it decreased significantly (Hsu *et al*., 1999). This might be attributable to reduced nitrate uptake by the roots. The synthesis of nitrate reductase is substrate regulated (Beevers & Hageman, 1969; Hewitt, 1975). In the present research, nitrogen and phosphorus concentrations and nitrate reductase enzyme activity were studied in cumin under flooding stress.

Materials and Methods

Plant materials and growing conditions: Cumin seeds procured from the local market were allowed to germinate at 25 ± 1°C in the pots filled with cumin farm soil. Seedlings were raised in an experimental greenhouse under non-flooding conditions and were exposed to daylight with photosynthetic active radiance of 1220-1236 ($\mu M\ m^{-2}\ s^{-1}$). Six-week-old healthy seedlings of uniform size were selected for flooding treatments. The treatments were based on field capacity (FC) of the cultured soil (2, 3 and 4 FC) and cultures were maintained in an experimental greenhouse. After 14 days of treatment, plants were harvested and different biochemical assays were carried out.

Nitrate reductase (NR) assay: NR was prepared and assayed based on the method described by Sagi *et al*., (1997). Shoot and root samples from control and treated plants were frozen in liquid nitrogen immediately after harvesting. Crude extracts were obtained by maceration with acid-washed sand in an ice-cold extraction medium containing 25 mM Tris-HCl (pH 8.5), 3 mM dithiothreitol, 1 mM ethylenediaminetetraacetate, 10 µM flavine adenine dinucleotide sodium salt, 1 µM sodium molybdate, 2% (w/v) casein, 10 µM leupeptin, 5 mM reduced glutathione and 3% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 30,000 *g* for 15 min in a refrigerated centrifuge (Beckman, L7 Ultracentrifuge, USA) at 4°C. The resulting supernatant was used for assays of NR and NO_3^-. The activity of NR (E.C. 1.6.6.1) was assayed in a modified reaction mixture containing 15 mM K-phosphate buffer (pH 7.5), 25 mM Tris-HCl buffer (pH 7.5), 12.5 mM KNO_3 and 0.4 mM nicotenamide adenine dinucleotide (reduced) by nitrite accumulation, which was analyzed by sulphanilamide and *N*-(1-napthyl) ethylenediamine dihydrochloride addition and subsequent measurement of absorption at 540 nm.

Determination of total nitrogen: Total nitrogen contents in leaves of control and flooding stressed plants were determined following the colorimetric procedure described by Baethgen & Alley (1989). This essentially involved two steps: (i) digestion of the sample with concentrated H_2SO_4 and H_2O_2 (30%) to convert the N compounds in the sample to NH_4^+ form; and (ii) determination of NH_4^+ in the digest. For estimation of total nitrogen, 1 ml of aliquot from the plant digest was placed in a 25ml volumetric flask. To this, 5.5 ml of working buffer solution (0.1 M Na_2HPO_4, 5% Na–K tartarate, 5.4% NaOH) was added and stirred thoroughly. Then 4 ml of Na-salicylate and Na-nitroprusside solution (15–0.03%) was added and the solution was mixed again. Following this, 1 ml of Na-hypochlorite solution was added and the solution was again mixed thoroughly. The reagents were added in the sequence stated in order to avoid the formation of solid residues. The solution was allowed to stand for 45 min at 25°C to ensure complete color development. The absorbance was then read in a spectrophotometer (Jasco V 530, Japan) at 650 nm. The nitrogen content was determined from a standard curve prepared using (NH_4) SO4 solution.

Extraction and assay of total phosphorous (P): For total soluble Pi determination, only fresh tissue samples were used. They were homogenized with 5ml of 10% (v/v) HClO4 at 4°C. After centrifugation at 5000g at 4°C, the supernatant was collected for Pi analysis. Pi content of the resultant soluble fraction was measured by the formation of a blue molybdenum complex according to Tsvetkova & Georgiev (2003). Appropriate aliquots were briefly mixed with 5ml 0.1 M acetate buffer pH 4.0, 0.5 ml 1% (w/v) ammonium molybdate in 0.05 N H_2SO_4, 0.5 ml 1% (w/v) Na-ascorbate. To avoid the delay in the conversion of the blue colour of molybdate- phosphoric complex, 1 mM $CuSO_4.5H_2O$ was added into the ascorbate solution. The blue colour of the complex was obtained after 10 min and the absorption was determined using spectrophotometer at 620 nm.

Statistical analysis: Statistical analysis of the results was carried out according to Duncan's multiple range tests. Data were subjected to a one way ANOVA following the method of Rohlf & Sokal (1995).

Results

By increasing the flooding stress, content of nitrogen in shoot and root of treated plants significantly decreased in comparison with control plants but there were no significant differences between nitrogen contents in different parts of treated plants (Tables 1 and 2). Total phosphorus content in different parts of treated plants decreased by an increase in the flooding stress and the differences between control and treated plants were significant ($\alpha<0.05$). The results are shown at Tables 1 and 2. The nitrate reductase (NR) activity in shoots and roots of *Cuminum cyminum* plants was also affected by flooding stress. A significant difference was observed between the control and treated plants. The NR activity in the root of treated plants was more than in shoots (Tables 1 and 2).

Table 1. The effect of flooding stress on contents of phosphorus, nitrogen and nitrate reductase activity of root in Cuminum cyminum plants.

Nitrate reductase (mg/100g FW)	Nitrogen (g/100g DW)	Phosphorus (mg/100g DW)	Contents Treatments(FC)
305.66 ± 100 [a]	1.45 ± 0.5 [d]	173.66 ± 50 [d*]	FC
626.33 ± 100 [b]	0.873 ± 0.5 [c]	71.00 ± 50 [c]	2FC
631.00 ± 100 [c]	0.615 ± 0.5 [b]	63.66 ± 50 [b]	3FC
648.00 ± 100 [d]	0.593 ± 0.5 [a]	53.00 ± 50 [a]	4FC

*The means were compared by Duncan's Test. The same letters show that differences are not significant at $p\leq0.05$

Table 2. The effect of flooding stress on contents of phosphorus, nitrogen and nitrate reductase activity of shoot in *Cuminum cyminum* plants.

Nitrate reductase (mg/100g FW)	Nitrogen (g/100g DW)	Phosphorus (mg/100g DW)	Contents treatments(FC)
133.33 ± 100 [a]	2.56 ± 0.5 [d]	235.33 ± 50 [d*]	FC
255.66 ± 100 [b]	1.24 ± 0.5 [c]	109.00 ± 50 [c]	2FC
377.33 ± 100 [c]	1.16 ± 0.5 [b]	93.66 ± 50 [b]	3FC
381.33 ± 100 [cd]	1.05 ± 0.5 [a]	86.33 ± 50 [a]	4FC

*The means were compared by Duncan's test. The same letters show that differences are not significant at $p\leq0.05$

Discussion

Flooded cumin plants exhibited lower P concentration in roots than leaves. High rates of P at cumin leaves should be noted that the definition of ''transport'' and ''accumulation'' is on per unit dry weight basis, so after water logging some nutrients such as P transport from root to shoot. These findings are opposite to McKevlin *et al*., (1987), they reported that also, increasing P concentration in roots of flooded plants may interfere with P uptake and immobilize P in roots, thus preventing further transport of P to shoots. In comparison with control plants, Phosphate inorganic (Pi) level of shoots and roots were strongly decreased in different flooding treatments. Depending on the persistence of stresses, plants respond to Pi deficiency with coordinated adaptations on multiple levels comprising well documented morphological, physiological and biochemical changes (Helal, 1990). An integral part of the plant response to Pi deficiency is the induction of both extracellular and intercellular ATP-ases. There are similar reports on the increase in ATP-ase activities in contrast, proportion to the low level of Pi has been demonstrated in numerous species and plant parts under flooding stress, e.g., wheat leaves and roots (Barret-Lennard *et al*., 1982; Mclachlan & Demarco, 1982), maize leaves (Elliot & Lauchli, 1986); sorghum roots (Furlani *et al*., 1984) and common beans roots (Helal, 1990). The expression of higher ATP-ase activities in both tissues (shoots and roots) suggests its global role in enhancing Pi availability and possibility of recycling the organic Pi compounds.

Flooding induced a sharp decrease in plant -N, especially in the roots. However, nitrate reductase activity in leaves of flooded seedlings was always below that of roots. This weak nitrate reductase activity in the leaves of stressed cumin plants could be due to a decrease in nitrate import from root. Nitrate induces activation and induction of nitrate reductase. Thus, the observed decrease of nitrate reductase activity in leaves of flooded cumin plants could be related to a low nitrate translocation from the root (Alaoui-Sosse *et al*., 2005). Nitrate reductase activity can be used as a stress index for plants grown in soils where nitrate is the main form of N available for the plants (Caravaca *et al*., 2003). The increased NR activity found in plants is an identification of plant availability to

promote plant adaptation to flooding stress (Azcon & Tobar, 1998). According to the findings of the present study, increase in NR activity due to flooding stress was considerably higher in roots than shoots. This could be attributed to the effects of flooding stress on roots, which agree with the findings of Azcon & Tobar (1998) who detected higher NR in shoots of stressed mychorrhizal *Allium cepa*. The assimilation sites of N forms may be affected by flooding stress and such aspects are known to affect physiological response by plants (Subramanian & Charest, 1998). The total nitrogen content in plant tissue has been reported to decrease under flooding stress in various fruit species such as citrus (Labanauskas *et al*., 1972) and apple (Olien, 1989). Total nitrogen contents in this study showed a significant decrease with an increase in flooding stress. This agrees with Hsu *et al*., (1999) who reported significant decrease of leaves total nitrogen after 35 days of flooding treatments.

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