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SUPPRESSION IN MITOCHONDRIAL ELECTRON TRANSPORT IS THE PRIME CAUSE BEHIND STRESS INDUCED PROLINE ACCUMULATION

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Exposure of six day old rice (*Oryza sativa*) seedlings to salt or cadmium stress lead to an increase in the level of proline with a simultaneous decline in the mitochondrial electron transport activity. Mitochondrial electron transport inhibitors - rotenone, antimycin A or potassium cyanide also stimulated proline accumulation in rice seedlings with a concurrent decline in the mitochondrial electron transport activity. Four to five fold enhancement in proline level was noted in seedlings after 48 h exposure to electron transport inhibitors. A significant rise in the level of NADH was also noted in seedlings exposed to salt stress, cadmium stress or any of the electron transport inhibitors. These results show for the first time that the suppression in the mitochondrial electron transport activity lead to proline accumulation. Our results also suggest that the increase in the ratio of NADH to NAD^+ due to the suppression in mitochondrial electron transport might be the prime reason behind proline accumulation in plants exposed to environmental stresses.

Subsequent to the discovery of drought induced proline accumulation in *Lolium perenne* (1), it was realized that the accumulation of this imino acid is one of the most frequent metabolic responses exhibited by plants exposed to any kind of environmental stress (2,3). Inspite of being the subject of intensive research during the last four decades, the actual reason behind proline accumulation remains controversial (4,5). Proline accumulation has been speculated to be a compensatory mechanism for better plant survival during stress conditions as it was suggested to play an important role in - osmoregulation (2,5), acting as carbon and nitrogen source (6), protection of enzyme denaturation (7), regulation of cytosolic acidity (8) and/or scavenger of free radicals (4,9).

The suppression in the mitochondrial electron transport activity and the increase in NADH to NAD^+ ratio have been reported independently in plants exposed to various stresses (10,11,12). This made us to propose in an earlier communication that proline synthesis from glutamate might be an adaptive mechanism to reduce the accumulation of

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NADH during stress conditions (3). In the present communication, this hypothesis has been confirmed using mitochondrial electron transport inhibitors - rotenone, antimycin A and potassium cyanide, which also induced proline accumulation in the rice seedlings. Our results, provide a probable answer to a long-standing puzzle in plant stress biology i.e. the reason behind proline accumulation in plants exposed to extremely different kinds of stresses.

MATERIAL AND METHODS

Seeds of rice (*Oryza sativa*) cv. Ratna, were procured from Indian Agricultural Research Institute, New Delhi (India). Seeds were surface sterilised with 0.1% mercuric chloride and placed on filter paper (Whatman no. 1) bridges in test tubes containing 20 ml of B_5 (13) liquid medium under sterile conditions. These were then incubated at 25±2°C in dark for getting etiolated seedlings. After incubating for six days, the seedlings were transfered to B_5 medium and B_5 medium supplemented with NaCl (200 mM, to induce salt stress), $CdCl_2$ (5 mM, to induce cadmium stress), KCN (5 mM), antimycin A (10 μg/ml) or rotenone (50 mM) for various time intervals.

For proline estimation the shoots were extracted in 3% sulfosalycylic acid and the homogenate was centrifuged at 10,000 rpm for 15 min and the supernatant was used for proline quantification. Proline content was determined by the method of Bates et al. (14). Concentration of proline in the sample was computed from a standard curve of L-proline.

The mitochondria were isolated from shoots of etiolated seedlings by the method of Kolloffel (15). The protein content in the mitochondrial preparations was estimated according to Bradford (16). The mitochondrial electron transport activity was measured in the isolated mitochondrial preparations by estimating the uptake of O_2 due to the oxidation of NADH by using Clark-type O_2 electrode as described earlier (10).

The amount of NADH in the shoot extracts was determined according to Peine et al. (17).

RESULTS AND DISCUSSION

Six day old etiolated rice seedlings showed 3.6 and 3.2 fold increase in the level of proline upon exposure to $CdCl_2$ (5 mM) and NaCl (200 mM) stress for 48 h, respectively (Table 1). Various environmental stresses including heavy metal stress and salt stress have been reported to induce proline accumulation in a variety of plants (2,3). Another frequently reported metabolic change, common in plants exposed to most of the environmental stresses is the suppression in the mitochondrial electron transport (10,11). Even in the present investigations a considerable decline in the mitochondrial electron transport activities was noted in the rice seedlings exposed to NaCl or $CdCl_2$ stress for 48 h (Table 1). The mitochondria isolated from the seedlings exposed to these stresses showed approximately 30 and 35 per cent lower capacity to oxidise NADH compared to those from control seedlings.

TABLE 1. Proline levels and electron transport activity of mitochondria in shoots of rice seedlings exposed to B_5 medium (control), B_5 medium supplemented with NaCl (200 mM) or $CdCl_2$ (5 mM) for 48 h. Data represent mean of values from three independent experiments ±SD.

Treatment	Proline (μg/g fresh wt.)	Electron transport activity (n mol O_2/min/mg protein)
Control	48±05	63.5±7.2
NaCl	158±20	45.0±3.9
$CdCl_2$	175±18	41.2±4.2

The concurrent suppression in the mitochondrial electron transport activity and accumulation of proline in rice seedlings exposed to either of these extremely different kinds of environmental stresses, viz. salt (NaCl) or heavy metal ($CdCl_2$) stress, made us to speculate that there is a possible link between these metabolic events.

To confirm this speculation, the rice seedlings were exposed to various mitochondrial electron transport inhibitors viz. potassium cyanide (KCN), antimycin A and rotenone. As evident from figure 1, the proline content rose sharply in seedlings exposed to any of these electron transport inhibitors. The extent of enhancement in the level of proline increased with time in seedlings subjected to any of these treatments (Fig. 1). The electron transport inhibitor induced proline accumulation was significantly higher than that observed under NaCl or $CdCl_2$ stress. The electron transport activity of mitochondria isolated from the seedlings exposed for 48 h to rotenone, antimycin A or KCN was about 57, 52 and 66 per cent lower than those from controls.

The level of NADH was higher in the seedlings exposed to any electron transport inhibitor (Fig. 2). The NADH level was also higher in seedlings exposed to NaCl or $CdCl_2$ stress compared to controls. As shown in figure 2, the NADH content in the seedlings exposed to NaCl and $CdCl_2$ stress was 2.0 and 2.3 fold higher respectively, than that of control seedlings. Such an increase in NADH can be attributed to the observed decline in the mitochondrial electron transport activity.

The stress induced enhancement in the level of NADH would result in a decline in the level of NAD^+. Lawlor and Khanna-Chopra (12) have also observed an increase in the NADH/NAD^+ ratio in plants subjected to water stress. Such an increase in NADH/NAD^+ ratio would result in suppression of some important metabolic reactions such as conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglyceric acid mediated by the key enzyme of glycolysis, glyceraldehyde-3-phosphate dehydrogenase (18). In order to let such essential reactions to continue, the living systems have evolved a number of stratagies to readily maintain the ratio of NADH/NAD^+. The activation of some of the reactions such as conversion of pyruvate to ethanol or lactic acid (19), oxaloacetate to malate (20) and glyoxylate to glycolate (21) can readily supply NAD^+. However, the accumulation of organic acids such as lactate, malate and glycolate causes a disturbance in several other metabolic reactions by lowering the cellular pH (8,21,22).

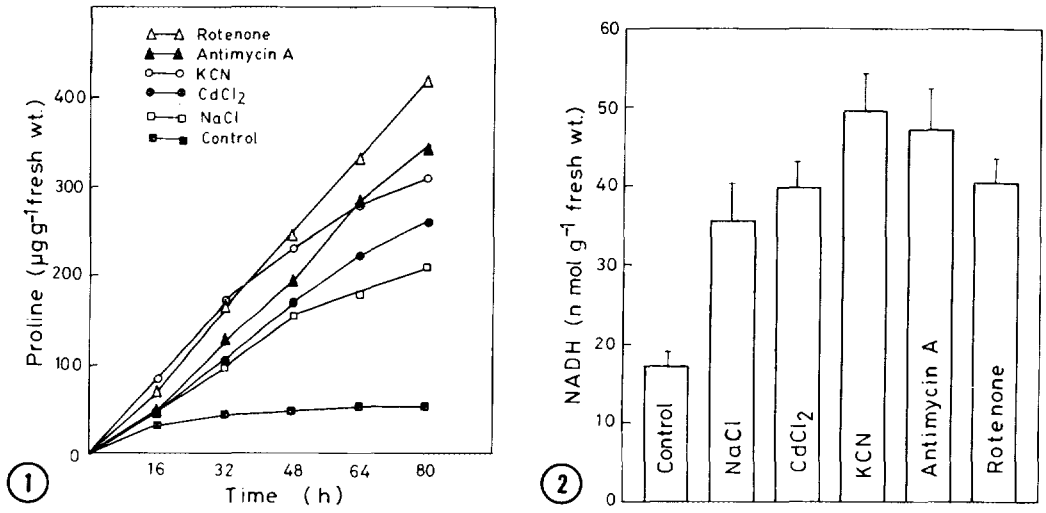

FIG. 1. Change in the level of proline with time upon exposing the six day old rice seedlings to B_5 medium (control), B_5 medium supplemented with NaCl (200 mM), $CdCl_2$ (5 mM), rotenone (50 mM), antimycin A (10 µg/ml) or KCN (5 mM). Data represent mean of values from three independent experiments.

FIG. 2. NADH levels in shoots of rice seedlings exposed to B_5 medium (control), B_5 medium supplemented with NaCl (200 mM), $CdCl_2$ (5 mM), rotenone (50 mM), antimycin A (10 µg/ml) or KCN (5 mM) for 48 h. Vertical lines represent standard deviation of the mean of values from three independent experiments.

As proposed by us in our earlier communication, proline accumulation is due to its fresh synthesis from glutamate in order to maintain the ratio of NADH/NAD^+ (3). It is well known that the synthesis of each molecule of proline from glutamate would oxidise two molecules of NADH (instead of one in the above mentioned reactions). In addition, in contrary to the above reactions which do not involve any alteration in the number of carboxylic groups associated with the products and their respective substrates, proline is a monocarboxylic acid while glutamate is a dicarboxylic acid. Unlike the organic acids produced in other reactions (associated with the oxidation of NADH), proline is a compatible solute (7) whose accumulation do not alter the cellular pH. Infact, it has been proposed that proline accumulation could play a role of redox buffer by storage of excess reductants in a non toxic form (23).

Therefore, our results for the first time show that the suppression in the mitochondrial electron transport is the prime cause behind proline accumulation and the excessive synthesis of proline during various environmental stresses may be an appropriate adaptive mechanism evolved by the plants to reduce excess of NADH resulting due to inhibition of mitochondrial electron transport activity.

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