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BRIEF COMMUNICATION

Influence of ethephon and 2,5-norbornadiene on antioxidative enzymes and proline content in salt-stressed spinach leaves

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Abstract

The effects of ethephon, an ethylene generating compound, and 2,5-norbornadiene (NBD), an inhibitor of ethylene action, on peroxidase (POD; EC 1.11.1.7), catalase (CAT; EC 1.11.1.6), polyphenol oxidase (PPO; EC 1.14.18.1) activities and proline content in salt-stressed spinach leaves were investigated. POD and PPO activities were increased by NaCl + ethephon + NBD combination and reduced by NBD. Also, ethephon increased the CAT activity while ethephon + NBD reduced CAT activity. NaCl + ethephon increased proline content. The antagonistic effect of ethephon and NBD was seen on POD and PPO activity and proline accumulation, but was not on CAT activity.

Additional key words: catalase, ethephon, NaCl, 2,5-norbornadiene, peroxidase, polyphenol oxidase, *Spinacia oleracea*.

One of the major abiotic stresses effecting plant productivity is water stress resulting from drought or salinity. Plants respond to salinity stress through morphological, physiological and metabolic modifications occurring in all plant organs. NaCl salinity is known to decrease seed germination, shoot and root length, hydrolytic enzyme activity during germination and also affect other metabolic processes (Dash and Panda 2001). Oxygen radicals are generated during plant metabolism, and they need to be scavenged for maintenance of normal growth. Accumulation of oxygen radicals is increased by environmental stresses. Peroxidase (POD), polyphenol oxidase (PPO) and catalase (CAT) enzymes are assumed to deal with the detoxification of reactive oxygen species in plants (Nandwall *et al.* 2000).

Ethylene is a plant hormone, has capable of altering the development of higher plants in a variety of ways. Ethylene has been implicated as a factor that controls the timing of seed germination, the rate and dimensions of etiolated seedling growth and leaf expansion, the initiation and progressing of abscission and fruit ripening, and the expression of a number of stress-related responses in plants (Anthony and Schaller 1996). Treatment with 2,5-norbornadiene (NBD) suppressed germination as well as the stimulation of ethylene (Sutcliffe and Whitehead 1995). Application of NBD from the start of imbibitions strongly inhibited pea germination and the inhibition was mostly counteracted by ethephon (Petruzelli *et al.* 1995). When pea seedlings were grown in the presence of increasing concentrations of NBD, the length increased until the concentration of approximately 1 cm^3 dm^{-3} was reached. When the concentration was further increased, the epicotyl length decreased (Edward and Shang-Fa 1984). The rise in ACC oxidase activity was strongly suppressed by the treatment with NBD, concurrently NBD strongly retarded the progress in senescence (Kasai *et al.* 1996). Continuous NBD treatment prevented in-rolling symptom of petals and maintained fresh flowers for a long time during the continuous NBD treatment time. Continuous NBD treatment from climacteric flowers delayed senescence without wilting until 10^{th} day when NBD was treated every day (Kim *et al.* 1995). It was found that NBD inhibits ethylene-induced phosphorylation (Berry *et al.* 1996). Treatment of the tissue with NBD resulted in substantial reduction in lipoxygenase, ascorbate

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Abbreviations: ACC - 1-aminocyclopropane-1-carboxylic acid; CAT - catalase; NBD - 2,5-norbornadiene; POD - peroxidase; PPO - polyphenol oxidase.
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peroxidase and guaiacol peroxidase activity (Mi-Young *et al.* 1996).

The present investigation was undertaken to study effects of ethephon and NBD on some antioxidative enzyme (POD, PPO and CAT activities) and proline content in salt-stressed spinach leaves.

Seeds of spinach (*Spinacia oleracea* L. cv. Gladiator) were grown in plastic pots and supplied with standard Hoagland's solution every 2 d. They were maintained in growth chambers under control condition (12 h light/22 °C and a 12 h dark/18 °C) for 30 d with irradiance of 400 μmol m^{-2} s^{-2}. Then, the experiment was carried out in eight groups. First group was control, second group was sprayed until runoff with 10 mM ethephon containing 0.05 % Triton X-100, third group was applied with 3 mM NBD, fourth group was applied with ethephon + NBD, fifth group was applied with 150 mM NaCl, sixth group was applied NaCl + ethephon, seventh group was applied with NaCl + NBD and eighth group was applied with NaCl + ethephon + NBD. The leaves of all groups were cut after 5 h. Spinach leaves were frozen in liquid nitrogen and stored at -80 °C.

Frozen leaves of 0.5 g were ground in liquid nitrogen. The powder was homogenised at 4 °C in the extraction solutions [0.1 M potassium phosphate buffer, pH 6.5, contained 0.5 M sodium chloride and 2 % (m/v) polyvinyl pyrrolidone for POD activity, 50 mM potassium phosphate buffer at pH 7.5 containing 5 mM dithiothreitol for CAT activity and 0.05 M sodium phosphate buffer at pH 6.5 containing 5 mM ascorbic acid and 1 % (m/v) polyvinyl pyrrolidone for PPO activity]. The suspension was centrifuged (12 000 *g* for POD, 27 000 *g* for CAT and 20 000 *g* for PPO activity) for 15 min at 4 °C. The supernatants were filtered through *Whatman No. 4*. Crude extract (0.02, 0.01, and 0.05 cm^3 for POD, CAT and PPO, respectively) was added to 3 cm^3 of substrate mixture containing 15 mM guaiacol and 7.5 mM of 30 % H_2O_2 in 0.1 M potassium phosphate buffer (pH 6) for POD, 20 mM of 30 % H_2O_2 and 52 mM potassium phosphate buffer (pH 7.5) for CAT, and 25 mM catechol in 0.20 M sodium phosphate buffer (pH 6.5) for PPO activity. One unit of POD enzyme activity represents the amount of enzyme catalysing the oxidation of 1 μmol guaiacol per 1 min (measured at 470 nm) (Angelini *et al.* 1990). One unit of CAT activity was defined as the amount of enzyme catalysing the decomposition of 1 μmol H_2O_2 per min (at 240 nm) calculated from the coefficient of absorbance for H_2O_2 at 240 nm of 0.036 cm^2 μmol^{-1} (Lück 1965). One unit of PPO activity was defined as the amount of enzyme that can used an increase in absorbance (420 nm) of 0.001 per min (Flurkey 1986).

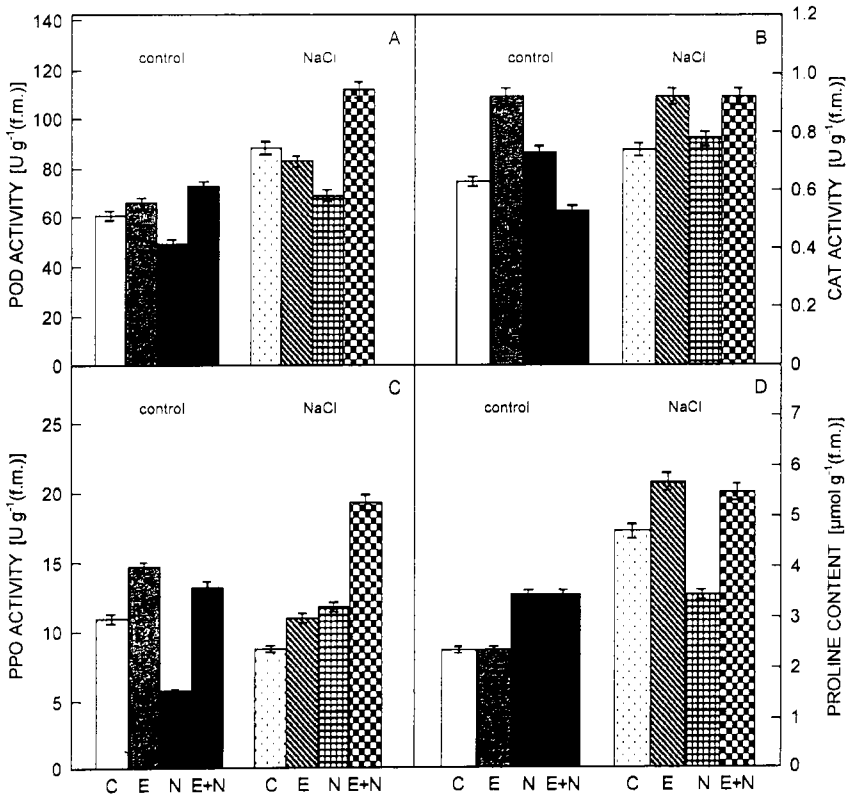

Fig. 1. Effects of ethephon (E), NBD (N) and ethephon + NBD (E+N) on POD (*A*), CAT (*B*), PPO (*C*) activities and proline content (*D*) in unstressed (control) and NaCl-stressed spinach leaves. Means ± SE, $n = 3$. C - untreated leaves.

Proline content in 0.5 g of fresh leaf was estimated spectrophotometrically following the ninhydrin method described by Bates *et al.* (1973) by using proline standard. All the experiments were done in triplicates and the data represent means ± SD.

Total POD activity was increased by ethephon, ethephon + NBD and salt, but NBD application decreased the activity significantly (Fig. 1*A*). When the values of the chemicals + NaCl were compared to value with only NaCl, ethephon + NBD combination increased POD activity, and ethephon and NBD decreased POD activity. An increase in POD activity is a common response to oxidative and abiotic stresses. Enhanced productions of oxygen free radicals are responsible for peroxidation of membrane lipids and the degree of peroxidative damage of cells was controlled by the potency of antioxidative peroxidase enzyme system. It is known that expression of POD in plant cells is controlled by the ethylene (Retig and Rudich 1972). It was reported that ethylene increases POD activity in sweet potato, cucumber, pea, tomato, and tobacco (Abeles *et al.* 1988, Cassab *et al.* 1988). Also, increased total POD activities in response to salinity were reported (Siegel 1993, Sancho *et al.* 1996). Ethephon enhanced POD activity in the cucumber since ethephon released ethylene (Retig and Rudich 1972). In this study, the same results for ethephon and NaCl were observed on POD activity. In addition, it was shown that NBD reduced POD activity, while NaCl + ethephon + NBD combination increased POD activity.

CAT activity was increased by NaCl, ethephon and NBD but decreased by ethephon + NBD (Fig. 1*B*). When the values of the chemicals + NaCl were compared to value with only NaCl, CAT activity was increased by all applications. NaCl treatment increased CAT activity also in cucumber plant (Lechno *et al.* 1997). In mangrove plants, CAT activity immediately increased after transfer from water to high salinity (Takemura *et al.* 2000). The corresponding result was found in our study. For the first time, effects of ethephon, NBD and ethephon + NBD were investigated and it was found that CAT activity was increased by them except for ethephon + NBD. Especially, ethephon may play an important role on CAT regulation although antagonistic effect of ethephon and NBD is not seen on CAT activity.

PPO activity was increased by ethephon and ethephon + NBD while NBD and NaCl decreased the activity (Fig. 1*C*). When the values of the chemicals + NaCl were compared to value with only NaCl, PPO activities were increased by ethephon, NBD and ethephon + NBD. Similarly, Ke and Saltveith (1988) found also that ethylene may cause the accumulation of the flavonoids, catechin and epicatechin, which are readily oxidised to brown substances by PPO in iceberg lettuce. Generally, longer exposure to ethylene resulted in higher browning intensity, slightly higher soluble phenolic content and PPO activity in lettuce (Couture *et al.* 1993). In another study, it was found that ethylene treatment increased PPO activity in rice (Peng and Yamauchi 1993). On the other hand, PPO activities of bean seeds decreased as salt content increased (Kocaçalışkan and Kabar 1990). In the present study, the results for ethephon and NaCl support previous studies. For the first time, it was found that NBD decreased PPO activity in controls, while ethephon, NBD and ethephon + NBD increased PPO activity under salinity.

NBD, ethephon + NBD and NaCl increased proline content but ethephon did not change it (Fig. 1*D*). When the values of chemicals + NaCl were compared to value with only NaCl, all applications increased proline content except for NBD. Proline accumulation in plant is also a striking consequence of water, salt, temperature and ultraviolet stresses (Demir 2000). Proline is an important parameter of the stress tolerance capacity of plants (Dash and Panda 2001). Proline has been assigned as a storage compound or a protective agent for cytoplasmic enzymes and cellular structure (Pandey and Ganaphaty 1985). Proline accumulation was also considered as a consequence of stress-induced damage to cells (Hanson and Hitz 1982). In the present study, NaCl + ethephon combination caused the highest increase in proline content. Antagonistic effect of ethephon and NBD was seen on proline content.

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