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BRIEF COMMUNICATION

NaCl-inhibited chlorophyll synthesis and associated changes in ethylene evolution and antioxidative enzyme activities in wheat

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Abstract

Effect of NaCl was studied on chlorophyll (Chl) synthesis and its intermediates (protoporphyrin IX, Mg-protoporphyrin IX, and protochlorophyllide), dry mass, ethylene evolution, and activities of superoxide dismutase (SOD) and peroxidase (APX) in wheat (*Triticum aestivum* L.) seedlings at 24, 48, and 72 h after germination. A conspicuous decrease in Chl synthesis, associated with increase in ethylene evolution and SOD and APX activities, was noted as NaCl concentration was increased from 0 to 100 mM.

Additional key words: dry mass, peroxidase, superoxide dismutase, *Triticum aestivum*

In arid and semi-arid regions, salinity is a common problem as it results in reduced plant growth and productivity. It also affects chlorophyll (Chl) synthesis, dry mass accumulation, and plant hormone concentration (Grant 1992, Mishra *et al.* 1997, Saha and Gupta 1999, Mishra *et al.* 2000, Barathi *et al.* 2001, Chakraborty *et al.* 2002). Plants develop different strategies for the protection against salinity stress. Production of antioxidative enzymes is one part of the mechanism that plants require for the protection against stress. The objective of the reported research was to evaluate the effect of salinity stress on tetrapyrrole intermediates in Chl biosynthetic pathway, protoporphyrin IX (Proto IX), Mg-protoporphyrin IX (MPE), and protochlorophyllide (Pchlide) and associated changes in ethylene evolution and activities of superoxide dismutase (SOD) and peroxidase (APX) in wheat seedlings.

Pre-soaked wheat (*Triticum aestivum* L. cv. HD 2204) seeds were germinated in Petri dishes in the dark and after germination, 30 cm^3 each of 0, 25, 50, 75, and 100 mM NaCl was added with four replications, and the dishes were transferred to light [photon flux density (PPFD) 360 μmol m^{-2} s^{-1}, 16-h photoperiod, temperature 25 °C]. To maintain NaCl concentrations, the solution in each Petri dish was decanted and freshly prepared NaCl solution, added every morning and evening. Chlorophyll (Chl) and its biosynthetic intermediates (Proto IX, MPE, and Pchlide), dry mass, ethylene evolution, and SOD and APX activities were determined at 24, 48, and 72 h after germination. At every sampling time, four leaves were harvested, fresh mass was recorded, and the material was used for Chl determination, or the material was dried at 80 °C for 48 h and percent dry mass was calculated.

For chlorophyll estimation, fresh leaves were homogenised in 80 % acetone and centrifuged at 6 000 *g* at 4 °C for 10 min. Absorbance was recorded on *UV 160A* spectrophotometer (*Shimadzu*, Tokyo, Japan) at 645 and 663 nm (Arnon 1949). For determination of intermediates of Chl biosynthesis equal amount of hexane was added to the supernatant. The top hexane layer was discarded and the bottom layer was again extracted with one-third volume of hexane. The bottom hexane extracted acetone residue (HEAR) solvent mixture was taken for estimation of Proto IX, MPE and Pchlide, using a *8000 C*

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Abbreviations: APX - peroxidase; BSA - bovine album serum; Chl - chlorophyll; EDTA - ethylenediaminetetraacetic acid; MPE - Mg-protoporphyrin IX; NBT - nitroblue tetrazolium; Pchlide - protochlorophyllide; PPFD - photon flux density; Proto IX - protoporphyrin IX; SOD - superoxide dismutase.

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photon-counting spectrofluorometer (*SLM Aminco*, New York, USA), as described by Hukmani and Tripathi (1992). The HEAR samples were excited at 400, 420, and 440 nm and emission spectra were recorded at 622, 632, 638, and 675 nm. Excitation and emission slit widths were 4 nm.

For ethylene measurement, leaf material was trimmed to small pieces, weighed, and placed in 30 cm^3 tubes, which were stoppered with rubber secure cap and placed in light for 2 h under the same conditions as used for plant growth. Ethylene content in the gas phase of the tubes was determined from 1 cm^3 samples removed from the tubes and injected into a *GLC 5700* (*Nucon*, New Delhi, India) gas chromatograph fitted with a flame ionisation detector and 1.8 m × 4 mm glass column packed with 80 - 100 mesh *Porapack-N*. The oven temperature was 100 °C and flow rates of nitrogen, hydrogen and oxygen were 30, 30 and 300 cm^3 min^{-1}, respectively. Ethylene identification was based on the retention time compared with a pure ethylene standard.

Superoxide dismutase (EC 1.15.1.1) activity was determined according to the methods of Giannopolitis and Ries (1977) and Beyer and Fridovich (1987). Leaf samples were homogenised in a pre-chilled mortar and pestle for 2 min with 1.5 g of quartz sand and 10 cm^3 of homogenising solution containing 50 mM HEPES buffer and 0.1 mM Na_2EDTA (pH 7.6). The homogenate was centrifuged at 15 000 *g* for 15 min, and then filtered through *Whatman 42* filter paper to produce the crude extract, which was used for SOD assay. A 5 cm^3 reaction mixture containing 50 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3 (pH 10.0), 13 mM methionine, 0.025 % (m/v) *Triton X-100*, 63 μmol nitroblue tetrazolium (NBT), 1.3 μmol riboflavin, and an enzyme extract was illuminated for 15 min (360 μmol m^{-2} s^{-1}) and a control set of reaction mixtures was not illuminated to correct for background absorbance. A unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of NBT as monitored at 560 nm.

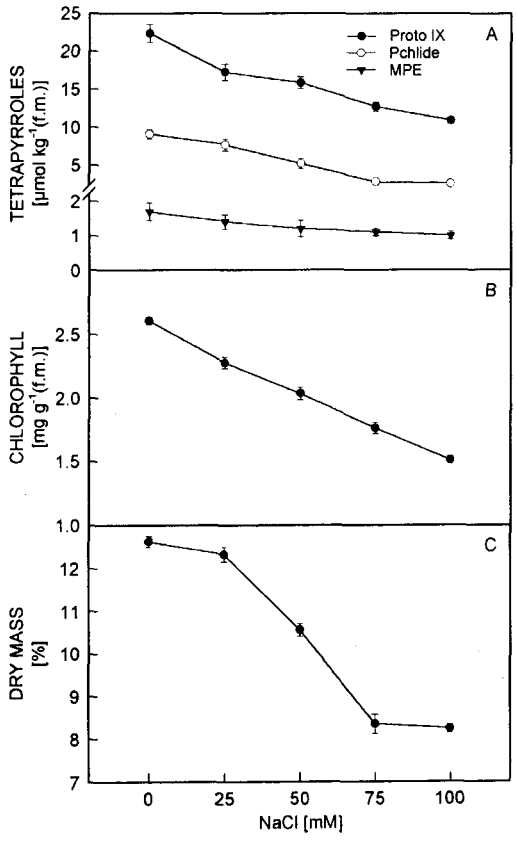

Fig. 1. Effect of NaCl concentrations (treatments - 72 h) on contents of tetrapyrroles Proto IX, Pchlide, and MPE (*A*), chlorophyll (*B*), and dry mass (percentage of fresh mass) (*C*) in wheat. Means ± SD, $n = 4$.

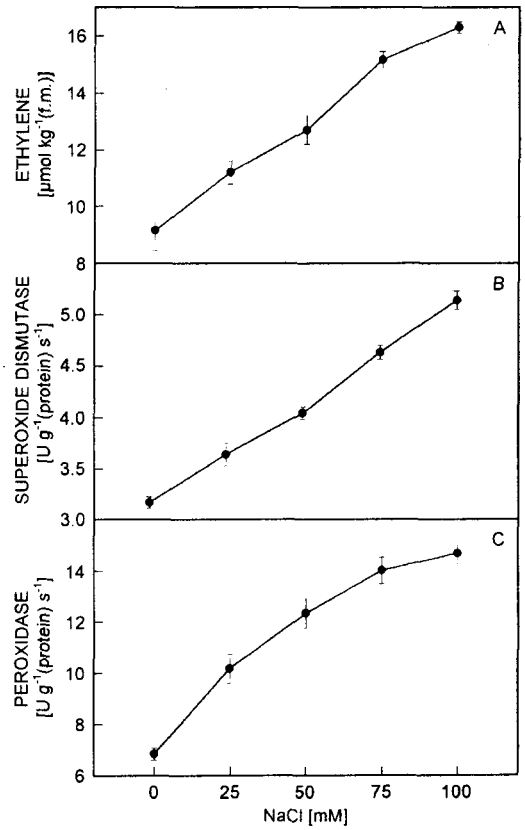

Fig. 2. Effect of NaCl concentrations (treatments - 72 h) on ethylene evolution (*A*), superoxide dismutase (*B*), and peroxidase activity (*C*) in wheat. Means ± SD, $n = 4$.

Peroxidase (E.C.1.11.1.7) was extracted and determined according to Quesada *et al.* (1992). Fresh leaf material was homogenised in 0.05 M acetate buffer (pH 4.0). The homogenate was filtered through layers of cheesecloth and the filtrate was incubated overnight at 4 °C with polyvinylpyrrolidone, and then centrifuged at 15 000 *g* for 40 min. The supernatant was used for determination of soluble peroxidase. Following

incubation of the extracts with 0.26 mM *o*-dianisidine, 8.8 mM H_2O_2 in 20 mM phosphate buffer (pH 6.0 at 25 °C), the absorbance at 460 nm was recorded. A unit of enzyme activity was defined as the one-increment increase in absorbance at 460 nm per min. The protein content in leaf extracts was determined using bovine serum album (BSA) as standard (Bradford 1976).

The observations recorded at 24, 48 and 72 h showed similar pattern of response to NaCl concentrations. To avoid repetition, values are given only for 72 h when the effect was maximal. The contents of Proto IX, MPE, Pchlide, Chl, and dry mass were noted to decrease with increasing NaCl concentration (Fig. 1*A-C*). It is likely that salinity stress reduced Chl formation (and consequently dry mass) through its adverse effect on Proto IX. Earlier Sudhakar *et al.* (1990), Brugnoli and Malco (1991), Sanchez *et al.* (1997), and Sultana and Itoch (2000) observed decrease in growth and dry mass accumulation due to salinity stress. However, no report on the effect of NaCl stress on intermediates of Chl biosynthesis is available although the effect of other stresses, such as chill and heat, has been reported (Tewari and Tripathi 1998).

Another reason for decrease in contents of Chl and tetrapyrroles noted in the present study, could be the observed increase in ethylene evolution due to NaCl stress (Fig. 2*A*). Trebitsch *et al.* (1993) have also reported such a decrease due to increase in ethylene evolution in citrus. Grant (1992), working on maize and Saha and Gupta (1999), on sunflower, have reported an increase in ethylene evolution with increasing NaCl concentration.

NaCl stress increased SOD and APX activities, although they were not saturated even with the highest salt concentration (Fig. 2*B,C*). Antioxidant enzymes are induced in plant response to different stresses (Bowler *et al.* 1992, Smirnoff 1996, Wojtaszek 1997). NaCl stress caused oxidative stress (Meneguzzo *et al.* 1999, Benanides *et al.* 2000) through involvement of activated oxygen species that appeared to be functional, as even twice the activity of SOD and APX than in the control was not enough for protection of thylakoid membranes against oxidative stress.

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