BIOLOGIA PLANTARUM 34 (5-6): 415-422, 1992

Effect of iso-osmotic levels of salts and PEG-6000 on enzymes in germinating pea seeds

M. SINGH and B.B. SINGH

Department of Crop Physiology, N.D. University of Agriculture and Technology, Narendra Nagar, Kumarganj, Faizabad-224 229 (U.P), India

Abstract

Effects of iso-osmotic levels of salts (NaCl, $CaCl_2$, Na_2SO_4) and PEG-6000 on the activity of hydrolytic and nitrogen assimilatory enzymes in pea embryo axis and cotyledon were studied. The activity of nitrate reductase and nitrite reductase in embryo axis and cotyledon and the activity of protease and α-amylase in cotyledon decreased with decreasing medium osmotic potential as compared to control at all the stages of seedling growth. The activity of protease and amylase increases with increasing levels of stress in embryo axis. Sodium chloride induced stress had more deleterious effects on the activity of nitrate reductase, nitrite reductase and α-amylase followed by other salts and PEG-6000. On the other hand, $CaCl_2$ induced salt stress was more depressive for protease activity. The maximum increase in the activity of protease and amylase was observed in embryo axis at higher concentration of salts and PEG-6000.

Introduction

The process of seed germination which is initiated with imbibition of water, is accompanied by increased metabolic activity due to increase in enzyme activity. Both catabolic and anabolic processes operate simultaneously in the cotyledons and embryo axis. Harmful effects of water and salt stress on the activity of hydrolytic and nitrogen assimilatory enzymes have been reported in a number of crops (Balasubramaniam *et al.* 1973, Plaut 1974, Mali and Mehta 1977, Gaikwad *et al.* 1987, Tewari and Singh 1991). Sheoran (1980) observed that protease and amylase activity of mungbean decreased in cotyledons whereas it increased in embryo axis, root and leaves with the treatment of NaCl, KCl, Na_2SO_4 and K_2SO_4. However, little attempts have been made so far to isolate osmotic and ionic effects of salt stress with respect to metabolic changes in pea. The present investigation was, therefore, carried

Received 28 February 1990, *accepted* 27 September 1991.
Acknowledgements: The authors are grateful to the Dean, College of Agriculture, N.D. University of Agriculture and Technology, Kumarganj, Faizabad for providing facilities during the course of investigation. One of us (Maharaj Singh) is also grateful to the Indian Council of Agricultural Research, New Delhi for providing a Junior Fellowship.

out to quantify the osmotic and ionic effects of salts using iso-osmotic levels of NaCl, $CaCl_2$, Na_2SO_4 and PEG-6000.

Material and methods

Healthy, uniform seeds of pea (*Pisum sativum* L., cv. Rachna) were placed in sterilized Petri dishes containing discs of filter paper soaked in uniform amount of desired osmotic solution (NaCl, $CaCl_2$, Na_2SO_4 and PEG-6000) of different osmotic potentials (-0.1, -0.2, -0.3, -0.4, -0.5 MPa). The Petri dishes were placed in the dark at 20 ± 1 °C in a B.O.D. incubator during 1989. The experiment was replicated three times under a C.R.D. factorial design. Samples for estimation of enzymes were taken at 72 h, 96 h and 120 h after sowing.

Nitrate reductase was determined according to Jaworski (1971). The fresh samples (embryo axis/cotyledons) were cut into small pieces and suspended in screw cap vials having 4.5 cm^3 medium containing 0.1 M phosphate buffer (pH 7.0), 0.02 M KNO_3, 5 % propanol and added two drops chloramphenicol (0.5 mg cm^{-3}). The vials were capped and kept in dark at 30 °C for overnight. Nitrate released into medium was determined by treating 0.4 cm^{-3} aliquot with 0.3 cm^{-3} each of sulphanilamide and *N*-1-naphthyl ethylene diamine hydrochloride. After 20 min the solution was diluted with distilled water to make the volume upto 5 cm^{-3} and absorbance was measured at 540 nm.

Nitrite reductase was determined according to Ferari and Varner (1971). The fresh sample (embryo axis/cotyledons) were cut into small pieces and suspended in screw cap vials having 4.5 cm^3 medium containing 0.1 M phosphate buffer (pH 7.0), chloramphenicol (0.5 mg cm^{-3}) and sodium nitrate (2 mM). 0.1 cm^3 aliquot was immediately removed to provide a measure of initial nitrite concentration of the reaction mixture in the presence of tissue pieces. After incubation for overnight 0.1 cm^3 aliquot of the medium was again removed for NO_2^- determination. Dimethyl sulphoxide was then added to the medium and the flask were kept on hot plate until the medium started boiling (20-30 s). After cooling, 0.1 cm^3 aliquot was removed for NO^- determination. The difference between this nitrate concentration and the initial concentration gives the amount of nitrate reduced. Residual NO_2^- was determined colorimetrically by adding 0.3 cm^3 each of sulphanilamide and *N*-1-naphthyl ethylene diamine hydrochloride reagent; absorbance was recorded at 540 nm.

α-Amylase: The activity of α-amylase in the cotyledons and embryo axis of pea seedling was assayed according to Bernfield (1955). Enzyme was extracted in phosphate buffer, 0.1 M, pH 6.7. The homogenate was centrifuged under cold condition at 4000 rpm for 15 min. The clear supernatant was used as enzyme extract. To 2.5 cm^3 phosphate buffer, 2.5 cm^3 of starch solution was added. Then, 1 cm^3 of enzyme extract was added, mixed gently and kept at room temperature for 20 min. 1 cm^3 aliquot was taken in test tube, 1 cm^3 DNS reagent was added mixed well and

kept on boiling water bath for 5 min. The absorbance was measured at 540 nm. The αα-amylase activity was expressed as enzyme units on mass and time units.

Protease activity was assayed according to Nayak *et al.* (1979). Enzyme extraction was carried out in phosphate buffer 0.1 M, pH 6.0. The homogenate was centrifuged under cold conditions at 12 000 rpm for 20 min. The clear supernatant was used as enzyme extract. 20 mg casein and 1 cm^3 phosphate buffer (pH 6.0, 0.1 M) and 1 cm^3 enzyme extract was incubated at 37 °C for 2 h. The reaction was stopped by addition of 0.3 cm^3 of 5 per cent trichloroacetic acid (TCA). Protein precipitate was removed by centrifugation at 4 000 rpm for 20 min. The supernatant was used for enzyme assay. 0.5 cm^3 aliquot was taken from the supernatant in a test tube and mixed with 5.0 cm^3 of reagent D* and allowed to stand for 10 min. Absorbance was measured at 750 nm.

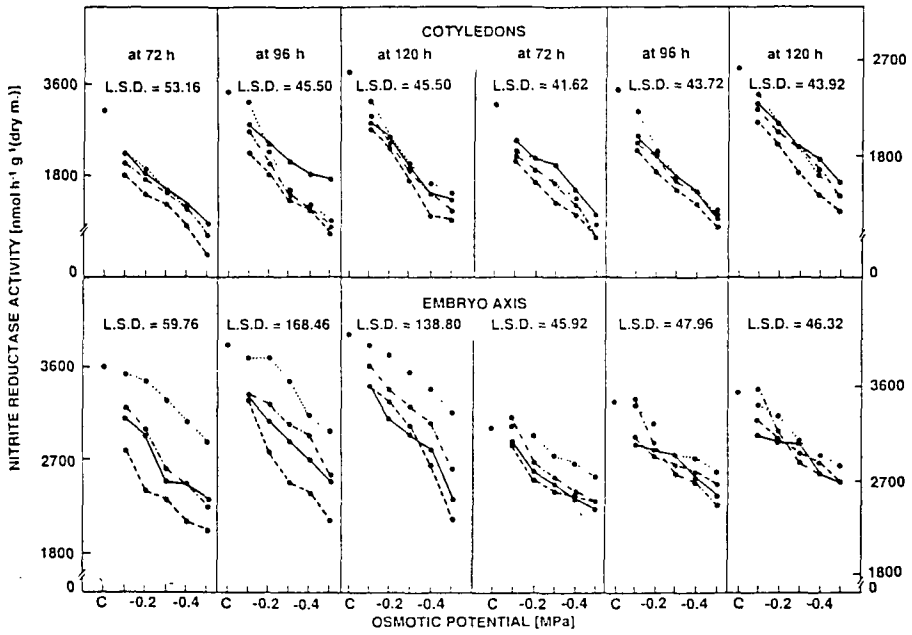
Results and discussion

The activities of nitrate reductase and nitrite reductase decreased progressively with increasing levels of salt and water stress. Higher activities of nitrate reductase and nitrite reductase have been observed in embryo axis under calcium chloride induced salt stress (Figs. 3 and 4). Sodium chloride on the other hand, had usually more depressive effect on the nitrate reductase and nitrite reductase activities at all the stages of seedling growth. The activities of nitrate reductase and nitrite reductase showed slight increase under low concentration of salt while higher amount of salt inhibited it. The activity of nitrate reductase and nitrite reductase in pea cotyledons decreased significantly with increasing levels of salt and water stress. Sodium chloride induced salt stress had more deleterious effect on nitrate reductase and nitrite reductase activities of pea cotyledons at all the stages of seedling growth. Higher activities of nitrate reductase and nitrite reductase in pea cotyledons (Fig. 1, 2) have been observed under water stress. Calcium chloride induced salt stress had almost similar effect as water stress on both enzymes at all the stages of seedling growth. In agreement with our results, Mali and Mehta (1977), Abdul-Kadir and Paulsen (1982), Bloom-Zandstra and Lampe (1983) and Khan and Varshney (1989) also reported reduction in nitrate reductase activity under water stress.

The effect of salt stress on nitrate reductase and nitrite reductase activities can be attributed to inhibition of enzyme induction. It has been reported that stress causes a shift of ribosomes from the polymeric to the monomeric form in maize seedlings (Hsiao 1970). Another possibility is enhanced degradation of nitrate reductase by an inactivating system which may lead to a decrease in activity (Plaut 1974). According to Pessarakli and Tucker (1985) small and medium addition of salt (50-150 mmol) stimulated the enzyme while higher amounts (250 mmol) inhibited it.

*Reagent D = 50 cm^3 of 2 % Na_2CO_3 was mixed with 1 cm^3 of 0.5 % $CuSO_4 . H_2O$ in 1 % sodium citrate.

Amylase: The results presented in Figs. 5, 6 and 7 indicate that the specific activity of amylase in the cotyledon of pea increases with time during germination and decreased significantly with increasing levels of salts and water stress. Salt stress had more deleterious effects on the amylase activity as compared to water stress induced



Figs. 1 to 4. Effect of iso-osmotic levels of salts and PEG-6000 on nitrate reductase (Figs.1 and 2) and nitrite reductase (Fig.3 and 4) activities of germinating pea cotyledons (Figs. 1 and 3) and embryo axes (Fig. 2 and 4) at 72, 95 and 120 h after sowing. *Full point* - control; *dotted lines* - $CaCl_2$; *dashed lines* - NaCl; *dash-and-dot lines* - Na_2SO_4; *full lines* - PEG-6000.

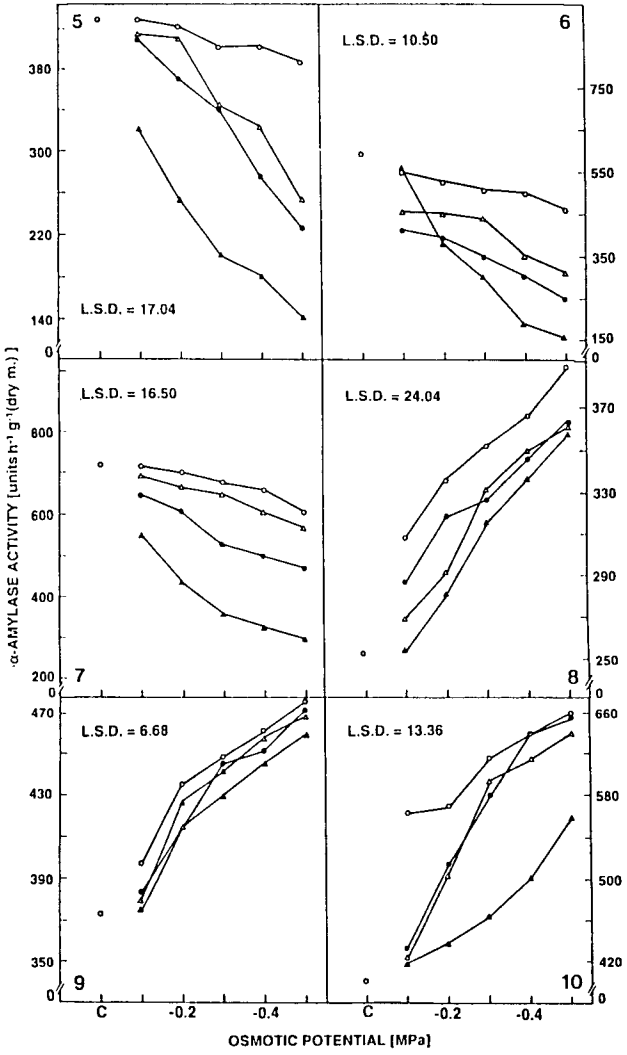
by PEG-6000. Maximum activity was observed under calcium chloride induced stress and minimum was recorded under stress induced by sodium chloride.

In contrast, an increase in the activity was observed in the embryo axis of pea under different levels of salt and water stress (Fig. 8, 9 and 10). the increase was higher with higher concentrations of the salt used. The maximum increase was observed under $CaCl_2$ followed by Na_2SO_4, PEG-6000 and NaCl at all the stages of seedling growth.

There is fairly extensive literature documenting a nutritive role of Ca^{2+} in seed germination. A number of reports have described that under specific conditions (temperature and salinity *etc.*) seeds germinate better if mM levels of Ca^{2+} are available in the surrounding soil or medium (Koller 1972). In this context, Ca^{2+} is probably playing mainly a nutritive role, sustaining certain basic functions such as membrane stability, needed for the growth events of germination.

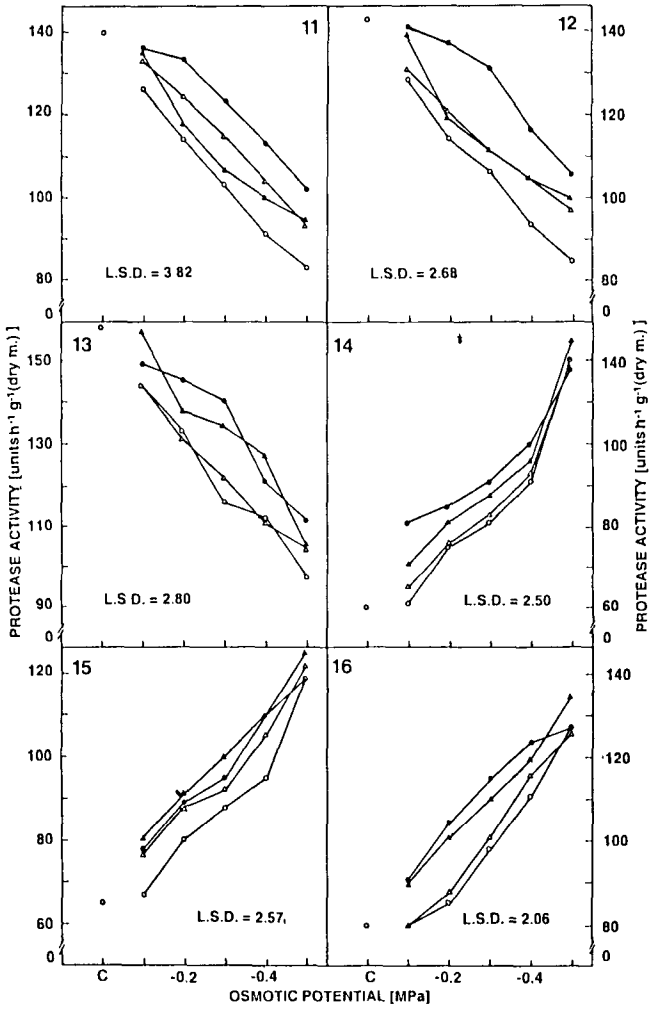
Sodium chloride salinity increases the activity of amylase in the embryo axis. Dixon and Webb (1957) reported that NaCl stimulates the activity of amylase when

applied in concentration of 5.40 nM. Strogonov (1964) also reported activation of this enzyme by NaCl and Na_2SO_4. However, Na^+ and Cl^- are not always the most effective ions. But our results indicate that Cl^- in combination with Na^+ was the most effective ion for increasing the amylase activity. The increase in amylase activity in embryo axis was also observed in mungbean by Sheoran (1980).



Figs. 5 to 10. Effect of iso-osmotic levels of salts and PEG-6000 on α-amylase activities in pea cotyledons (Figs. 5 to 7) and embryo axes (Figs. 8 to 10) after 72, 95 and 120 h after sowing. *Open point:* control; *Full lines* and *open points* - $CaCl_2$; *full points* - PEG-6000; *open triangles* - Na_2SO_4; *full triangles* - NaCl.

The reduction in growth of the embryo axis under salinity stress as reported by Sheoran (1980) and Ramana and Das (1978) may be attributed due to the inhibited hydrolysis of reserve from the cotyledons. Our results presented in Figs. 5, 6 and 7 reveal that the cotyledon of the treated seedlings had lesser amylase activity than those of control seedlings. Similar findings have also been reported by Kurumkar and Chavan (1987) and Sheoran (1980).



Figs. 11 to 16. Effect of iso-osmotic levels of salts and PEG-6000 on protease activities of pea cotyledons (Figs. 11 to 13) and embryo axes (Figs. 14 to 16) after 72, 95 and 120 h after sowing. *Open point:* control; *Full lines* and: *open points* - $CaCl_2$; *full points* - PEG-6000; *open triangles* - Na_2SO_4; *full triangles* - NaCl.

Protease: The specific activity of protease in the cotyledons of seedlings increased with advancement in growth while the activity was decreased progressively with increasing the salt and water stresses at all the stages of seedling growth (Fig. 11, 12 and 13). Salt stress had more deleterious effects on protease activity as compared to water stress induced by PEG-6000. Within the salts, calcium chloride had more harmful effect on protease activity followed by Na_2SO_4 and NaCl.

In contrast, an increase in the activity was observed in the embryo axis of pea under different salt and PEG-6000 induced water stress. The maximum increase was observed under water stress at 72 h and 120 h. The decrease in specific activity of protease was due to higher amount of protein during salt stress (Prisco and Fernades 1976).

References

Abdul-Kadir, S.M., Paulsen, G.M.: Effect of salinity on nitrogen metabolism in wheat. - J. Plant. Nutr. **5**: 1141-1151, 1982.

Balasubramaniam, V., Rajgopala, V., Sinha, S.K.: Stability of nitrate reductase under moisture and salt stress in some crops. - Indian J. Genet. **34**: 1155-1061, 1973.

Bernfield, P.: Amylases, α and β. - In : Colowick, S.P., Kaplan, N.O. (ed.): Methods in Enzymology. Pp. 149-158. Academic Press, New York 1955.

Bloom-Zandstra, G., Lampe, J.E.M.: The effects of chloride and sulphate salt on the nitrate contents in lettuce plant (*Lactuca sativa* L.). - J. Plant Nutr. **6**: 611-628, 1983.

Dixon, M., Webb, E.C.: Enzymes. - Longman, London 1957.

Ferari, T.E., Verner, J.E.: Intact tissue assay for nitrate reductase in barley aleuron layers. - Plant Physiol. **47**: 790-794, 1971.

Gaikwad, S.G., Karadge, B.A., Chavan, P.D.: Effect of salinity on nitrate reductase activity in four millet species. - Biovigyan **12**: 82-83, 1986.

Hsiao, T.C.: Rapid changes in levels of polyribosomes in *Zea mays* in response to water stress. - Plant Physiol. **46**: 281-285, 1970.

Jowarski, E.: Nitrate reductase assay in intact plant tissues. - Biochem. biophys. Res. Commun. **48**: 1274-1279, 1971.

Khan, M.G., Varshney, K.H.: *In vitro* nitrate reductase activity in soybean (*Glycine max* (L.) Mervill.) under salt stress. - In: Proceeding of National Seminar on Strategies in Physiological Regulation of Plant Productivity. Pp. 33. Bombay 1989.

Koller, D.: Environmetal control of seed germination. - In: Kozlowski, T.T. (ed.): Seed Biology. Academic Press, New York 1972.

Kurumkar, C.V., Chavan, P.D.: Salinity induced biochemical changes during germination of chickpea (*Cicer arietinum* L.). - Acta agron. hung. **36**: 43-46, 1987.

Mali, P.C., Mehta, S.L.I.: Effect of drought on enzyme and free proline in rice varieties. - Phytochemistry **16**: 643-646, 1977.

Nayak, B.R., Rao, N.M., Pattabhiram, J.N.: Studies on the plant gum proteases in neem (*Azadirachta indica*) gum. - J. Biosci. **1**: 393-405, 1979.

Pessarakli, M., Tucker, T.C.: Ammonium (NH_4) metabolism in cotton under salt stress. - J. Plant Nutr. **8**: 1025-1045, 1985.

Plaut, Z.: Nitrate reductase activity of wheat seedling during exposure to and recovery from water stress and salinity. - Physiol. Plant. **30**: 212-217, 1974.

Prisco, J., Pernandes, C.H.: Effect of NaCl salinity on nitrogenous compounds and protease activity during germination of *Vigna sinensis* seeds. - Physiol. Plant. **36**: 317-320, 1976.

Ramana, K.V.R., Das, R.: Physiological studies on the influence of salinity and alkalinity. I. Changes in growth, respiration, carbohydrates and fats during seedling growth of radish (*Raphanus sativa* L.). Indian J. Plant Physiol. 21: 93-105,1978.

Sheoran, I.S.: Changes in amylase during germination and early seedling growth of mungbean (*Vigna radiata* L.) under different salts. - Indian J. Plant Physiol. **13**: 169-173, 1980.

Sheoran, I.S., Garg, O.P.: Effect of salinity on the activities of RNAse, DNAse and protease during germination and early seedling growth of mungbean. - Physiol. Plant. **44**: 171-174, 1978.

Strogonov, B.P.: Physiological Basis of Salt Tolerance of Plants. - IPSI, Jerusalem 1964.

Tewari, T.N., Singh, B.B.: Sodicity-induced changes in chlorophyll, nitrate and nitrite reductase, nucleic acids, proline, yield and yield components in lentil. - Plant Soil **130**: 225-230, 1991.