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Accumulation of osmolytes and osmotic adjustment in water-stressed wheat (*Triticum aestivum*) and maize (*Zea mays*) as affected by calcium and its antagonists

Harsh Nayyar *

Department of Botany, Panjab University, Chandigarh 160014, India

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Abstract

Maize (*Zea mays*) and wheat (*Triticum aestivum*) were water stressed for 4 days at early vegetative growth (15-day-old) using PEG-6000 (− 1.0 MPa), in the presence of 1 mM $CaSO_4$, 50 μM Verapamil (VP; calcium channel blocker); 50 μM Trifluoperazine (TFP; calmodulin antagonist) and then put to recovery in order to investigate the changes in osmoregulation in plants having C_3 and C_4 metabolism. Accumulation of proline (Pro) and quaternary ammonium compounds (QAC's), activities of pyrroline-5-carboxylate reductase (P5CR), proline dehydrogenase (PDH), water potential (Ψ_w), osmotic adjustment (OA), relative elongation rate (RER) and electrolyte leakage (EL) were examined during stress and recovery. Maize had significantly higher accumulation of Pro while wheat showed relatively more accumulation of QAC's. The activities of P5CR and PO were also significantly higher in maize than wheat. Maize shoots under stress showed higher Ψ_w, OA, RER and less EL than wheat shoots. Upon recovery from stress, maize regained its growth and water potential faster than wheat. Ca^{2+} elevated the accumulation of osmolytes in both the plants but OA was less sensitive to it. In the presence of Ca^{2+}, wheat showed significantly more accumulation of osmolytes, higher Ψ_w, RER than maize. Ca^{2+} inhibitors partially reversed the effects of calcium indicating its involvement in governing solute accumulation. The differential sensitivity of maize and wheat towards water stress may be related to variation in endogenous calcium expression and its function.

Keywords: Calcium; Drought stress; Osmoregulation; Proline; Quaternary ammonium compounds

1. Introduction

Osmotic adjustment (OA) is a part of drought avoidance mechanisms to counteract the loss of turgor by increasing and maintaining higher amount of intracellular compatible solutes in cytosol and vacuole and has been proved to be particularly significant among all the stress adap-

* Corresponding author: #1603, 44-B, Chandigarh 160014, India.
E-mail address: nayarbot@pu.ac.in (H. Nayyar).

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tation mechanisms (Cushman, 2001). Proline (Pro) and quaternary ammonium compounds (QAC's) e.g. glycinebetaine, choline, prolinebetaine are key osmolytes contributing towards OA (Naidu et al., 1992; Hare et al., 1999; Huang et al., 2000). Pro has been implicated in alleviation of cytoplasmic acidosis, maintenance of $NADP^+$/NADPH ratios at values compatible with metabolism, as antioxidant and protein-compatible hydrotrope besides having important role in plant development during non-stress conditions (Hare and Cress, 1997). QAC's like glycinebetaine in low concentration can improve salt and cold stress tolerance, possibly by protecting photosynthetic protein complexes (Holmstrom et al., 2000), reducing lipid peroxidation of the cell membranes (Chen et al., 2000), stabilizing enzymes and membranes during stress conditions (Sakamoto and Murata, 2000). The information on nature of signal transduction pathway linking the perception of the osmotic stress and accumulation of osmolytes is limited (Hare and Cress, 1997; Trotel-Aziz et al., 2000). Calcium (Ca^{2+}) has emerged as a key secondary messenger and signal transducer of various stress stimuli (Sanders et al., 1999). It has been unequivocally involved in linking stress perception and evocation of various adaptive cellular responses (Torrecilla et al., 2000). Ca^{2+} has also been implicated in stress protection by stabilizing membranes and reducing the oxidative damage (Larkindale and Knight, 2002). The increase in stress-induced cytosolic Ca^{2+} has been suggested to upregulate the proline biosynthesis since the induction of a transcript for proline biosynthetic enzyme, Δ^1-pyrroline-5-carboxylate (P5CS) synthetase (P5CS) in *Arabidopsis* (*AtP5CS1*) was inhibited in the presence of Ca^{2+} channel, blocker (Knight et al., 1997). C_3 and C_4 plants differ with respect to carbon fixation and water use efficiency with later being more tolerant to water stress due to higher stomatal resistance (Tissue et al., 1995; Ward et al., 1999). In the present study, wheat and maize having C_3 and C_4 metabolism, respectively, were compared for accumulation of osmolytes and growth during water stress in relation to Ca^{2+}. It was hypothesized that osmoregulation ability of these two types of the plants might be governed by differential functioning of endogenous calcium.

2. Materials and methods

2.1. Growth of plants

The plants of maize (*Zea mays* L. cv. 'Sartaj') and wheat (*Triticum aestivum* L. cv. C306) were grown hydroponically in modified half strength Hoagland's basal salt mixture having macro- and micronutrients as described by Hoagland and Arnon (1950), in growth chamber (light/dark for 15/9 h at 28/25 C for maize; light/dark for 16/8 h at 23/20 C for wheat; irradiance 250 μmol m^{-2} s^{-1}). In the first experiment, 15-day-old plants of both the species were subjected to water stress of varying osmotic potential (−0.2 to −1.0 MPa) by incorporating PEG-6000 in the growth medium to evaluate the comparative sensitivity of these species towards different stress levels. The observations on growth and solute accumulation were recorded on fourth day. In the second experiment, the most inhibitory stress level (−1.0 MPa) was used to impose stress for 4 days and the plants were put to recovery thereafter for 3 days. The observations were taken daily during stress and recovery period. Calcium sulphate ($CaSO_4$; 1 mM), calcium channel blocker, verapamil (VP; 50 μM) and calmodulin antagonist, trifluoperazine (TFP; 50 μM) were supplemented in the growth medium of stressed plants. The concentrations of PEG, $CaSO_4$, VP and TFP were evaluated in an preliminary experiment on the basis of their effect on growth and electrolyte leakage (EL). The concentrations of $CaSO_4$ causing highest promotion and least EL while the concentration of PEG-6000, VP and TFP causing 50% growth inhibition were selected. The leaves of plants were investigated for growth, proline and some of the enzymes involved in proline metabolism, quaternary ammonium compounds (QAC's), water potential (Ψ_w), osmotic potential (Ψ_s), osmotic adjustment (OA), relative growth rate (RGR) during stress and recovery as follows.

2.2. Proline

The proline content was estimated by the method of Bates et al. (1973). The plant material was homogenized in 3% aqueous sulfosalicylic acid

and the homogenate was centrifuged at 10000 rpm. Supernatant was used for estimation of proline content. The reaction mixture consisted of 2 ml supernatant, 2 ml acid ninhydrin and 2 ml of glacial acetic acid, which was boiled at 100 °C for 1 h. After termination of reaction in ice bath, the reaction mixture was extracted with 4 ml of toluene and the absorbance was read at 520 nm.

2.3. Quaternary ammonium compounds (QAC's)

The amount of QAC's was estimated according to method of Grieve and Grattan (1983). The plant material (oven dried at 80 °C for 4 days) was finely ground, mechanically shaken with 20 ml deionised water for 24 h at 25 °C. The samples were then filtered and filtrates were diluted 1:1 with 2 N H_2SO_4. Aliquots were kept in centrifuge tubes and cooled in ice water for 1 h. Cold KI-I_2 reagent was added and the reactants were gently stirred with a vortex mixer. The tubes were stored at 4 °C for 16 h and then centrifuged at 10000 rpm for 15 min at 0 °C. The supernatant was carefully aspirated with a fine glass tube. The periodide crystals were dissolved in 9 ml of 1, 2-dichloroethane. After 2 h, the absorbance was measured at 365 nm using glycinebetaine as standard.

2.4. Pyrroline-5-carboxylate reductase (E.C. 1. 5. 1. 2)

The activity of pyrroline-5-carboxylate reductase (P5CR) was assayed according to Rena and Splittstoesser (1975). The tissue was homogenized in two volumes containing 1 mM cysteine and 0.1 mM EDTA. The homogenate was filtered and centrifuged at 18000 rpm in a refrigerated centrifuge for 15 min. The supernatant was passed through Sephadex G-100 column. Active fractions were collected and used as enzyme source. The reaction assay contained 128 μM NADH, 400 μM L-P5C and 0.1 M sodium phosphate buffer (pH 7.4) in a volume of 1 ml. The reference tube contained all except NADH. The reaction was carried out at 32 °C and was started by the addition of L-P5C and the enzyme activity was monitored for 3 min by measuring the decrease in absorbance at 340 nm. The enzyme activity was expressed as nmol NADH oxidized min^{-1} mg per protein.

2.5. Proline dehydrogenase (E.C. 1. 5. 99. 8)

The activity was determined according to the method of Huang and Cavalieri (1979). 1 g of plant tissue was homogenized with 5 ml of medium, and filtered through several layers of muslin cloth. The filtrate was centrifuged at 10000*g* for 10 min in a refrigerated centrifuge at 4 °C. The supernatant was re-centrifuged at 25000*g* for 25 min. The pellet thus obtained was mixed with 1 ml Tricine–KOH buffer (pH 7.5) containing 6 M sucrose. This extract was used for assaying the enzyme activity. The extraction was carried out at 4 °C. 3 ml of assay mixture containing 1.2 ml of 50 mM Tris–HCl buffer (pH 8.5), 1.2 ml of 5 mM $MgCl_2$, 0.1 ml of 0.5 mM NADP, 0.1 ml of 1 mM KCN, 0.1 ml of 1 mM PMS, 0.1 ml of 0.06 mM 2,6 dichlorophenol indophenol (DCPIP) and 0.2 ml of 0.1 M proline. The reaction was monitored at 600 nm at 25 °C using proline to initiate the reaction. The increase in absorbance was recorded. The rate of reduction of DCPIP was used to determine the enzyme activity expressed as nmol DCPIP reduced min^{-1} mg per protein.

2.6. Water potential (Ψ_w) and osmotic potential (Ψ_s)

The Ψ_w and Ψ_s of were measured with pressure chamber and osmometer, respectively. OA was calculated as difference between predawn turgid Ψ_s of control and stressed plants.

2.7. Electrolyte leakage (EL)

EL was measured as described by Lutts et al. (1996) using young leaf discs of five plants for each treatment. Samples were washed with deionized water to remove surface adhered electrolytes. Leaf discs were placed in closed vials containing 10 ml of deionized water and incubated at 25 °C on a rotary shaker for 24 h and subsequently electrical conductivity of the solution (L_1) was determined. Samples were then autoclaved at 120 °C for 20 min and the final electrical conductivity (L_2) was

obtained after equilibration at 25 °C. The EL was defined as follows: EL (%) = $(L_1/L_2) \times 100$.

2.8. Relative elongation rate (RER)

RER of the shoot was calculated by observing length (mm) as ln(L4 − L0) × 100/d, where L0 and L4 are lengths on day of recovery and 4 days after that, respectively, and d is the number of days beginning from day of recovery till final observation (4 in the present case).The experiments were conducted using 15 plants per treatment in three replications following completely randomized block design and the data was analyzed for ANOVA, test of significance ($*P = 0.05$) between treatments and species and standard errors according to Gomez and Gomez (1984) using MICROSTA software.

3. Results

3.1. Effect of varying water stress levels

The seedlings of maize and wheat were subjected to water deficit stress of −0.2 to −1.0 MPa to evaluate their comparative growth and accumulation of osmolytes. The accumulation of proline (Pro) and QAC's increased in shoots with increase in stress level. (Fig. 1A). Wheat shoots achieved marked increase in these osmolytes at −0.6 MPa while maize shoots began accumulation only after −0.4 MPa of stress. Pro content was slightly but significantly higher in maize than wheat at −1.0 MPa while the latter had significantly more QAC's content. A negative and significant correlation existed between shoot growth and Pro accumulation in maize ($r = -0.62$, $*P = 0.05$) as well as QAC's accumulation and shoot growth in wheat ($r = -0.67$, $*P = 0.05$) suggesting that accumulation of these osmolytes may mark the reduction or cessation of growth under stress. Maize shoots showed significantly higher water potential but lower osmotic potential than wheat at −1.0 MPa (Fig. 1B) pointing to its better ability to maintain turgor. Maize roots and shoots were inhibited by about 50% at −0.8 and −0.6 MPa, respectively, contrary to wheat, which showed similar inhibition at −0.6 and −0.4 MPa, respectively (Fig. 1C). The root growth was relatively less affected due to stress than shoot growth in both the plants.

3.2. Effect of water stress and recovery

In the subsequent experiment, −1.0 MPa stress level was opted to investigate the effect of calcium on growth, accumulation of osmolytes and OA in shoots of both the species during stress and recovery. The plants of both the species were subjected to stress for 4 days and then put to recovery thereafter. The response was observed as follows.

3.2.1. Proline

Maize (Fig. 2A) showed inherently higher Pro content than wheat (Fig. 2B) during unstressed conditions and also accumulated significantly more Pro than wheat during stress. Pro accumulation began relatively early in wheat and the rate of its accumulation was also considerably faster in wheat indicating its higher sensitivity towards stress. Interestingly, both species showed nearly similar extent of increase (2.7 times) in Pro content from the first day to fourth day of stress. Ca^{2+}-treated wheat plants accumulated significantly more Pro (37%) than untreated-stressed plants as compared with insignificant (6%) increase in maize suggesting higher sensitivity of wheat towards exogenous Ca^{2+}. Pro content of wheat plants treated with Ca^{2+} nearly matched with that of Ca^{2+} -treated maize plants, on fourth day. Pro was consumed rapidly in maize as compared with wheat during recovery from stress. But in the presence of Ca^{2+}, wheat showed significantly higher decline than maize. Ca^{2+} inhibitors markedly reduced the Pro accumulation in both the species but wheat was particularly more sensitive pointing to higher Ca^{2+} dependence.

3.2.2. QAC's

The content of QAC's increased by two and three times in stressed plants of maize (Fig. 2C) and wheat (Fig. 2D), respectively. Ca^{2+} further stimulated their accumulation to a significantly higher extent in wheat than maize while its

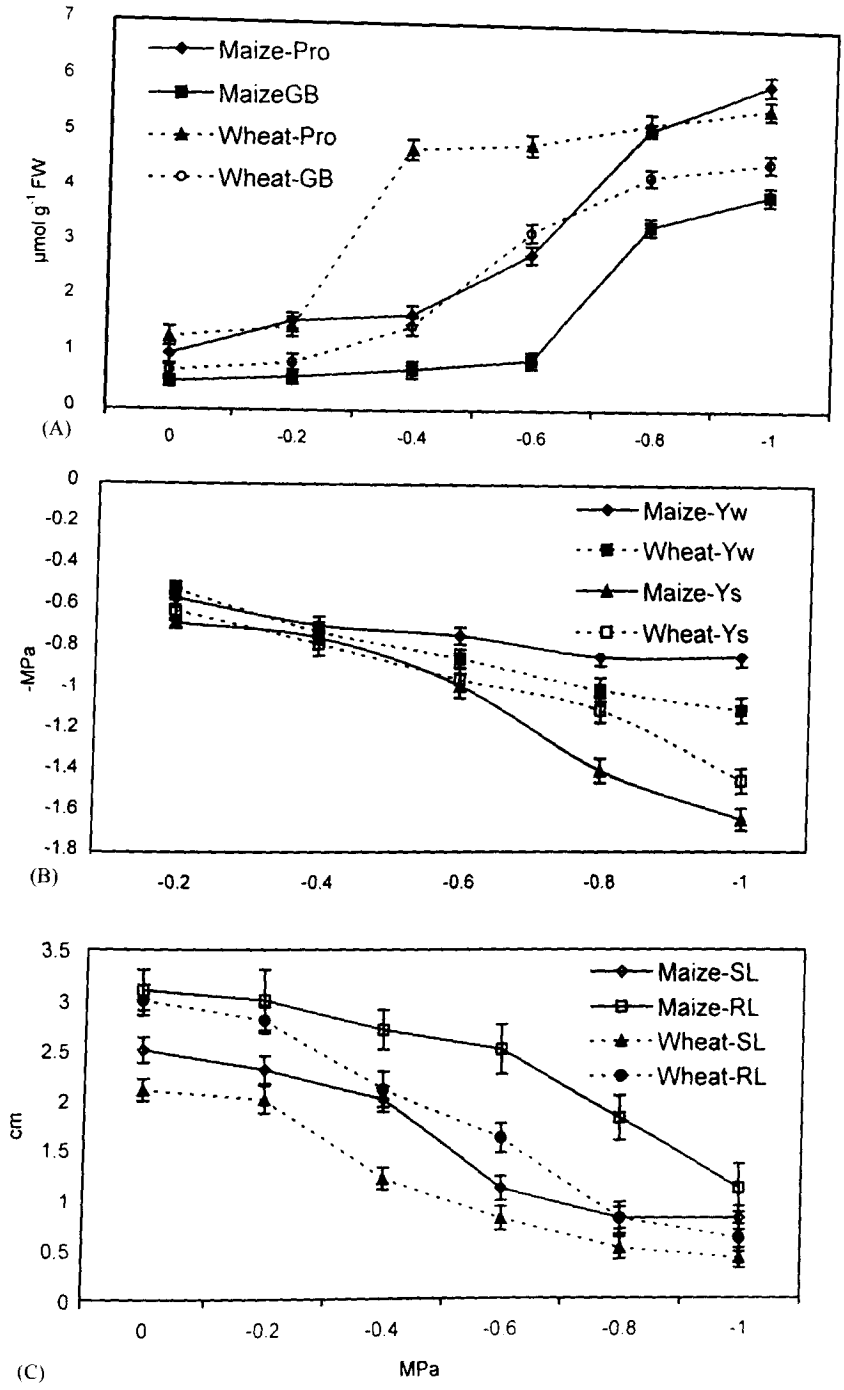

Fig. 1. Effect of different levels of water stress (− 0.2 to − 1.0 MPa on (A) proline (Pro), quaternary ammonium compounds (QAC's), (B) water potential Ψw and Ψs, (C) shoot length (SL) and root length (RL), in maize and wheat seedlings. Water stress was imposed using PEG-6000, on 15-day-old seedlings growing hydroponically for 4 days. Data represent mean ± S.E. of three replications. Results were significantly different at ($^*P < 0.05$) between treatments and genotypes.

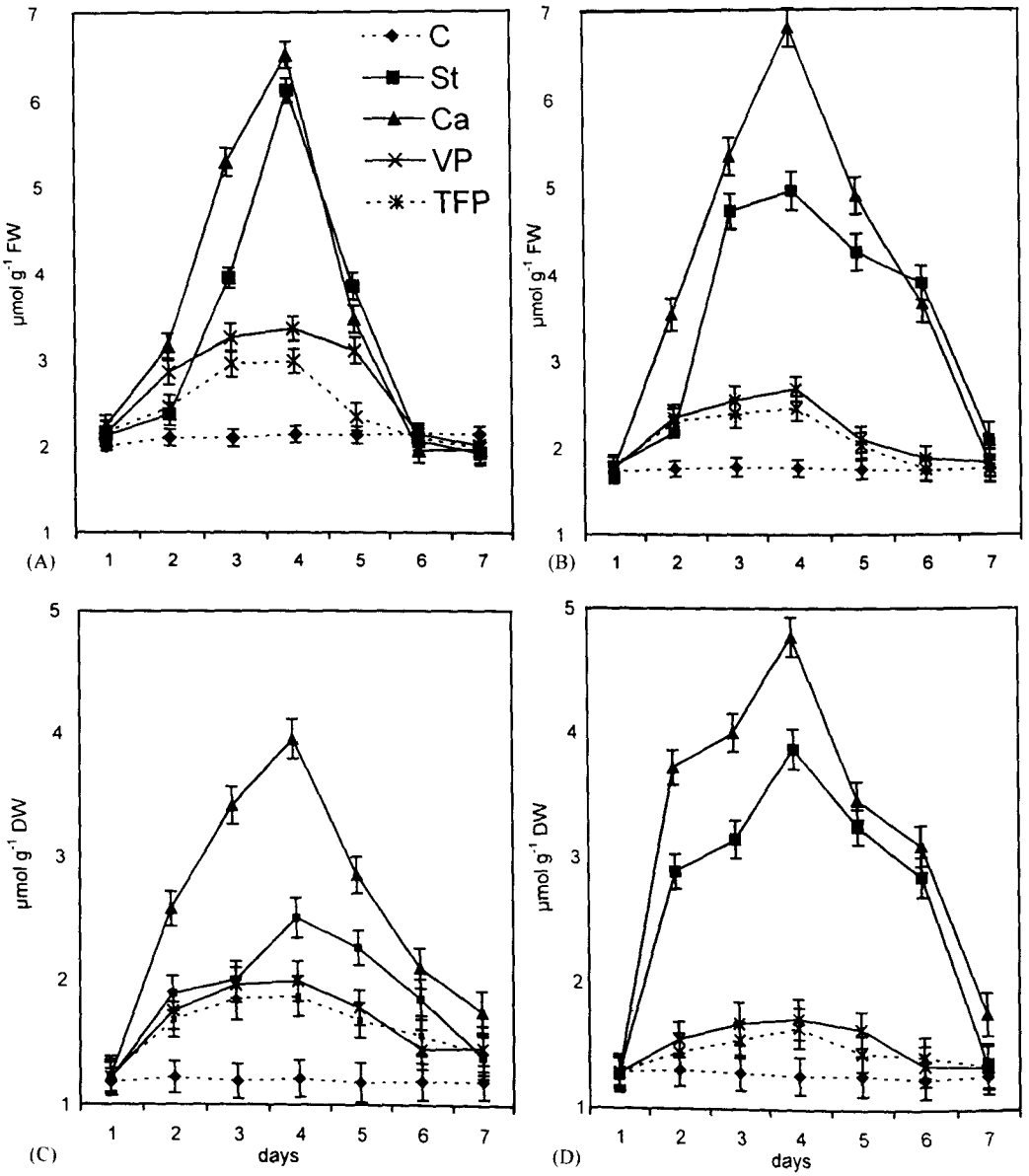

Fig. 2. Effect of water stress (till day 4) and recovery (from day 4 to day 7) on proline (Pro) in maize (A), wheat (B) and QAC's in maize (C) and wheat (D). Water stress (St) of −1.0 MPa was imposed using PEG-6000 on 15-days-old seedlings growing hydroponically in the presence of $CaSO_4$ (1 mM), calcium channel blocker, verapamil (VP; 50 µM) and calmodulin antagonist, trifluoperazine (TFP; 50 µM). Data represent mean ± S.E. of three replications. Results were significantly different at ($^*P < 0.05$) between treatments and genotypes.

inhibitors repressed it. During recovery, wheat reduced its QAC's levels faster than maize and Ca^{2+} promoted their decline, especially in wheat.

3.2.3. *Pyrroline-5-carboxylate reductase*

During stress, the enzyme activity increased by 92% in maize (Fig. 3A) and 72% in wheat (Fig.

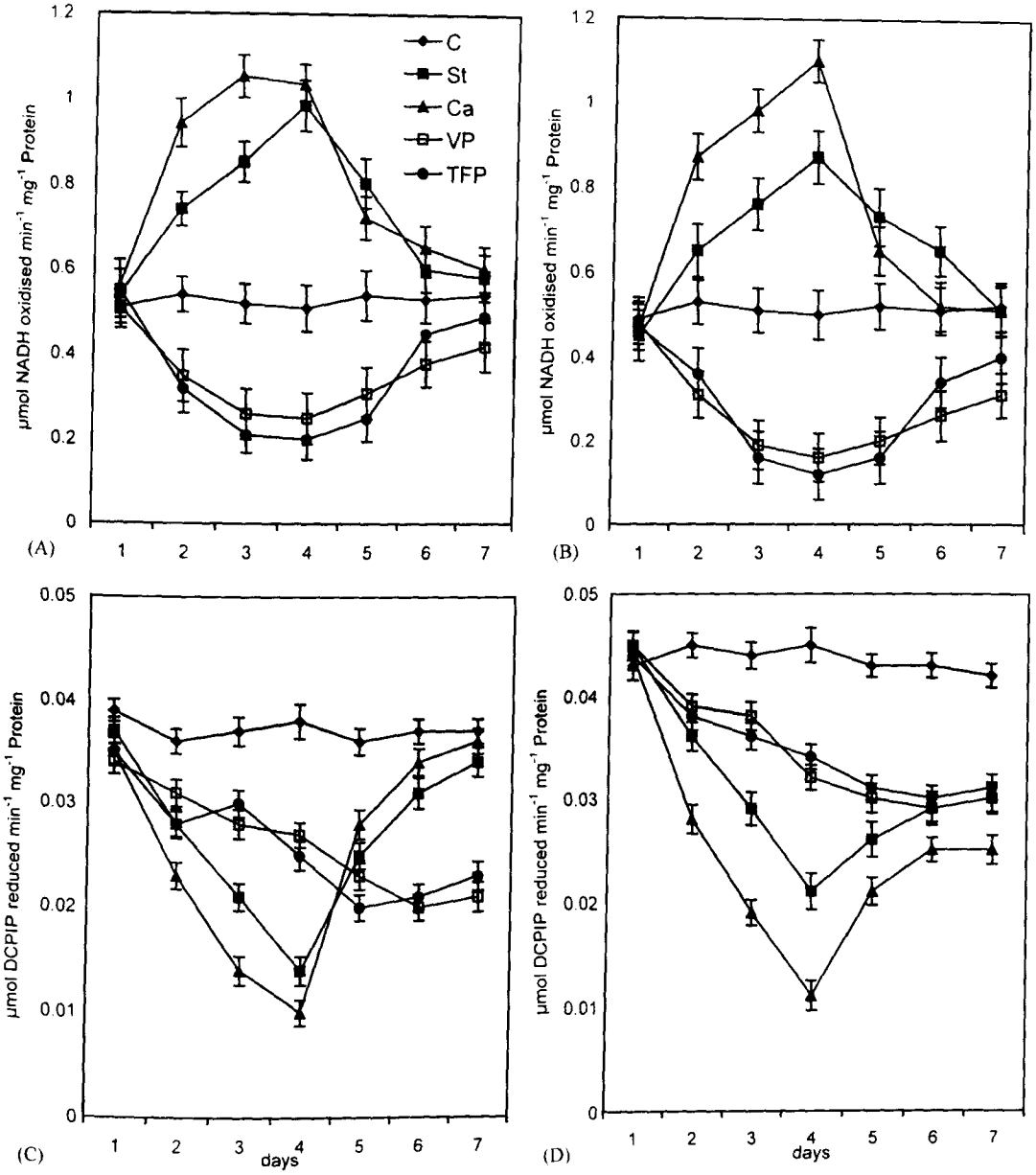

Fig. 3. Effect of water stress (till day 4) and recovery (from day 4 to day 7) on pyrroline-5-carboxylate reductase in maize (A), wheat (B) and proline dehydrogenase in maize (C), wheat (D). Details as in Fig. 2.

3B), over control, as noticed on fourth day. The rate of increase was, however, higher in wheat which was concomitant with accumulation rate of Pro. On the fourth day, wheat had significantly higher enzyme activity with Ca^{2+} treatment than maize. The antagonists of Ca^{2+} resulted in inhibition of activity and wheat showed more decline than maize. Upon recovery, maize attained 87% of its pre-stress activity within 48 h compared with 72% in wheat. With Ca^{2+} application, the

activity reached its control levels within 48 h in wheat relative to maize achieving the same in 72 h.

3.2.4. Proline dehydrogenase

Due to stress, a reduction of 63 and 48% was noticed in activity of proline dehydrogenase (PDH) in maize (Fig. 3C) and wheat (Fig. 3D), respectively. Ca^{2+} could reduce the activity to a significant extent in wheat (47%) than maize (21%). Upon recovery, the activity increased rapidly in maize than wheat. Ca^{2+} suppressed the increase in activity; significantly in wheat during recovery while Ca^{2+} antagonists elevated the activity.

3.2.5. Water potential (Ψ_w) and osmotic adjustment (OA)

Stress caused 55% reduction over control in Ψ_w of maize (Fig. 4A) relative to 77% reduction in wheat (Fig. 4B). Ca^{2+} ameliorated this decline by 44% in wheat and 24% in maize. Maize recovered its Ψ_w rapidly upon recovery than wheat but Ca^{2+} treated wheat plants regained their Ψ_w more quickly than maize. Maize also showed higher OA (Fig. 4C) than wheat (Fig. 4D). Ca^{2+} or its inhibitors did not affect the OA significantly in maize but wheat showed slight increase in OA with Ca^{2+}, which was partially reversed in the presence of its inhibitors.

3.2.6. Electrolyte leakage (EL)

The EL due to stress was significantly lesser in maize (Fig. 5A) than in wheat (Fig. 5B) shoots. In the presence of Ca^{2+}, EL was reduced by 46% in wheat and 16% in maize. The inhibitors elevated the EL, markedly over stress treatment in both the species.

3.2.7. Relative elongation rate (RER)

During stress, RER of wheat shoots was 50% lesser than maize shoots (Fig. 5C, D). In the presence of Ca^{2+}, growth rate of wheat reached at par with maize. Post-stress growth was also significantly higher with Ca^{2+} in wheat than maize. The antagonists of Ca^{2+} strongly inhibited RER of both the plants.

4. Discussion

It was evident from the present study that maize and wheat varied markedly in their sensitivity towards water stress. Maize was able to tolerate higher level of stress and could maintain the growth of its roots and shoots at stress levels that were inhibitory for wheat. The present study also indicated that maize appeared to have advantage over wheat during water stress with respect to accumulation of osmolytes and thus has a better capacity to osmoregulate, which was evident from its higher Ψ_w and OA than wheat. Higher proline accumulation by maize under stress was concomitant with higher activity of its biosynthetic enzyme, P5CR coupled with lower activity of PDH. Maize also recovered faster than wheat from stress as indicated by its higher growth rate, rapid decline in water potential and reduction in Pro due to decrease in P5CR and increase in PDH activities. Maize and wheat showed some distinction for accumulation for Pro and QAC's. While maize showed relatively higher Pro, wheat accumulated comparatively higher QAC's. Our findings are in contrast to those of Storey and Jones (1977) who found no difference for accumulation of pro and QAC's in roots of wheat and maize while shoots of maize had a low betaine content than wheat. The differences obtained in the present results could be related to use of different genotypes of wheat and maize in our experiments and may not be necessarily applicable to other genotypes of these plants. As reported earlier, the type of osmolytes and capacity to accumulate them during stress may vary between different types of plants (Rathinasabapathi, 2000) and specify different adaptive mechanisms. C_4 plants like maize are reported to be better adapted to water deficient conditions due to higher stomatal resistance, better water use efficiency and ability to carry on photosynthesis under low CO_2 concentration (Tissue et al., 1995; Matsuoka et al., 2001). C_4 pathway in maize is usually argued as conferring greater tolerance to water deficits, high temperature and nitrogen deficiencies while wheat shows contrasting response to these factors (Loomis and Connor, 1992). The present findings indicate that higher OA could be one of the

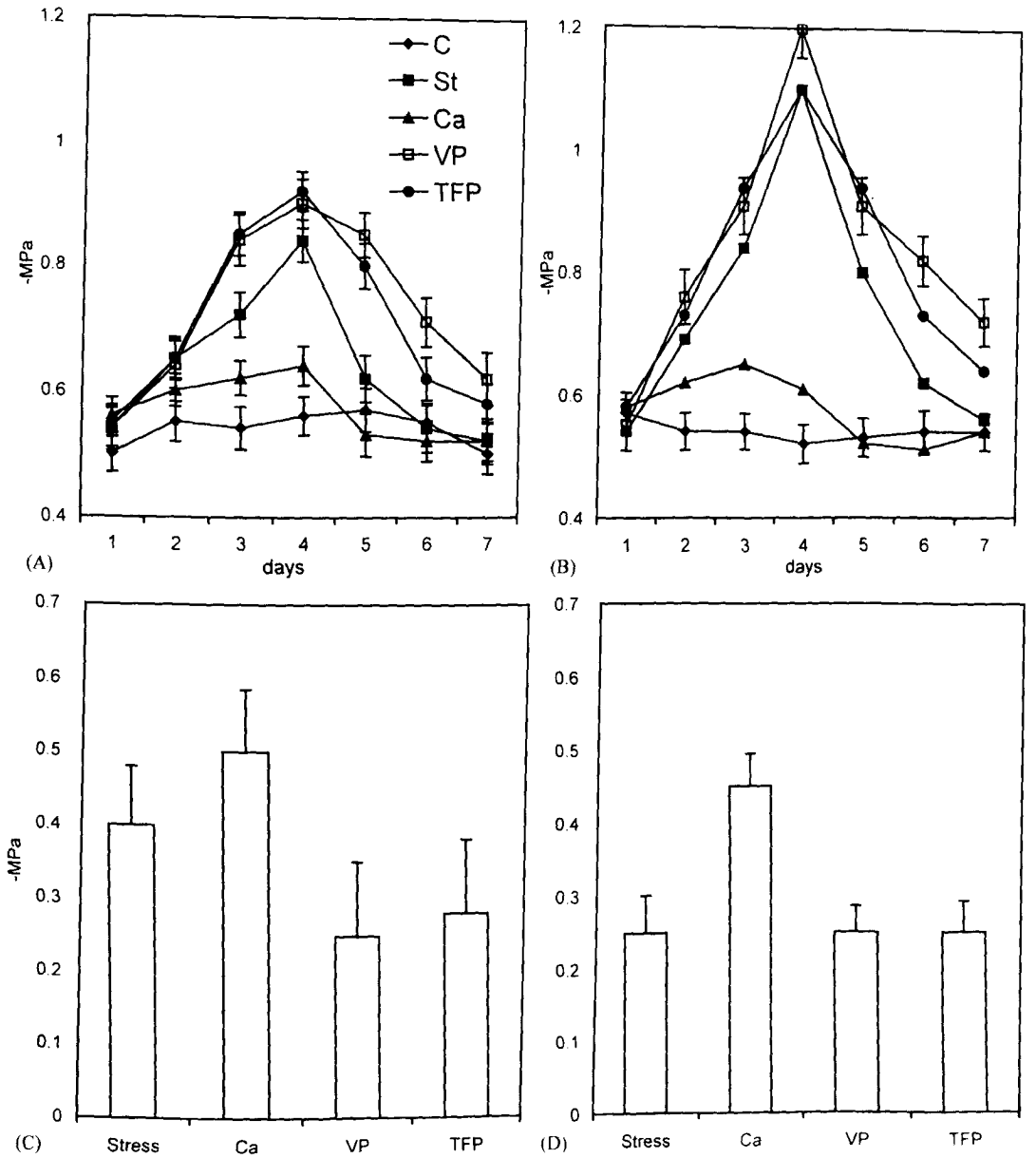

Fig. 4. Effect of water stress (till day 4) and recovery (from day 4 to day 7) on water potential of maize (A), wheat (B) and OA of maize (C) and wheat (D). Details as in Fig. 2.

deciding factors, which offers advantage to maize over wheat for superior performance under water deficit conditions.

In the present study, the stimulatory effect of Ca^{2+} could be observed on RGR, membrane integrity (as decrease in EL), accumulation of Pro and QAC's, activities of P5CR and PDH, which was reversed to a considerable extent by calcium inhibitors. Calcium inhibitors did not completely stop the accumulation of Pro indicat-

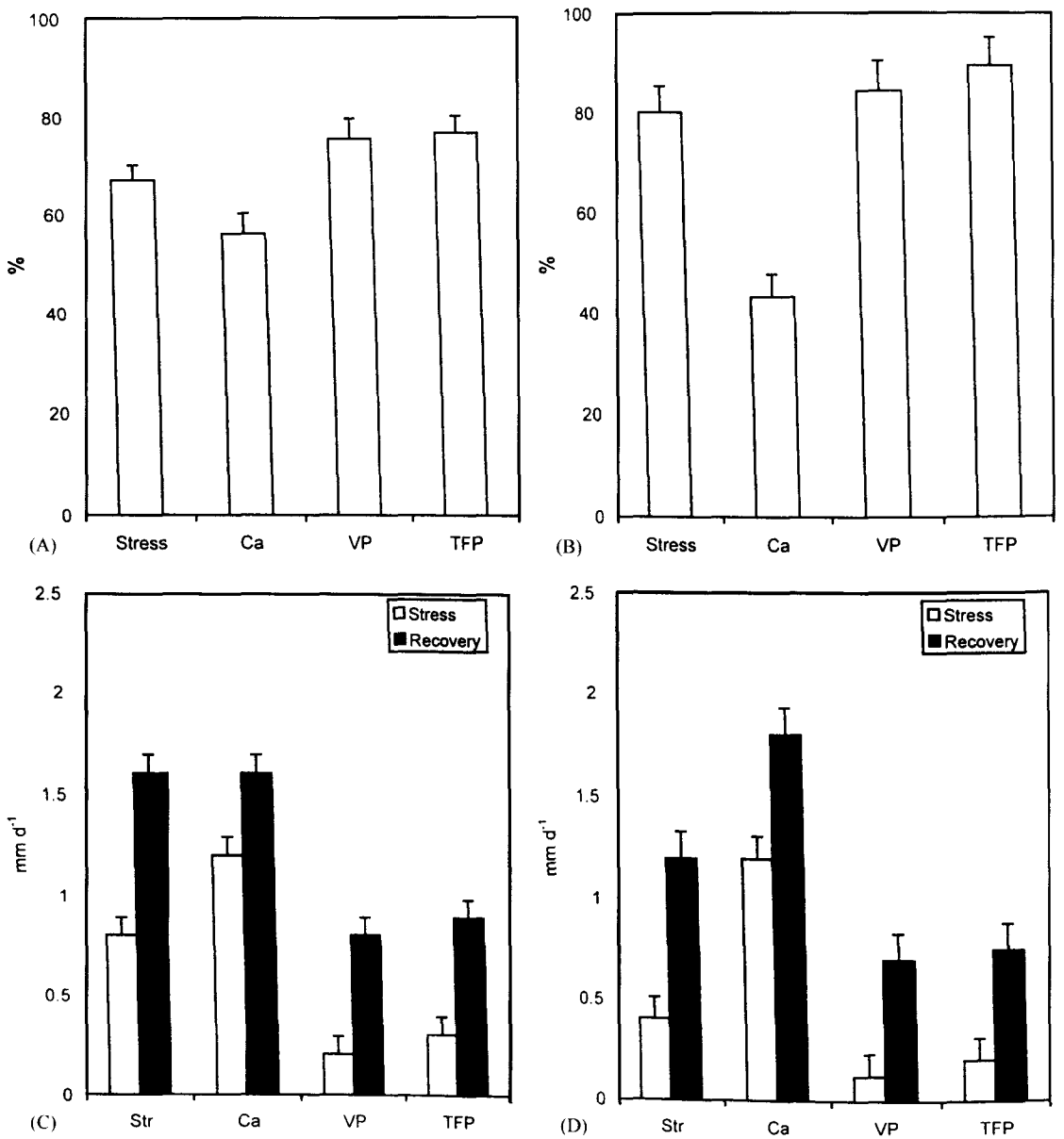

Fig. 5. Effect of water stress (till day 4) and recovery (from day 4 to day 7) on EL of maize (A), wheat (B) and RER of maize (C) and wheat (D). Details as in Fig. 2.

ing the involvement of some additional intracellular stores of Ca^{2+} in stress response (Knight et al., 1997). Ca^{2+} treated plants also recovered faster from stress than plants growing without it. Over-production of Pro and QAC'S due to Ca^{2+} application might increase the protection from stress as has been advocated in the previous studies (Huang et al., 2000). Ca^{2+} has been observed to confer protection to stress experiencing cells by several mechanisms like maintaining membrane

integrity, reducing the oxidative damage by elevation of antioxidants or reducing the activity of oxidative enzymes (Ming et al., 1998; Nayyar and Kaushal, 2002; Larkindale and Knight, 2002). Heat stressed cells of *Arabidopsis* were found to recover quickly in the presence of calcium while Ca^{2+} channel blockers accelerated the damage by stress (Larkindale and Knight, 2002). Ca^{2+} in growth medium of *Phaseolus vulgaris* plants experiencing osmotic stress decreased the initial loss of water from roots (Ortiz et al., 1994) which was attributed to regulation of level of organic metabolites related to OA by Ca^{2+}. Ca^{2+} in the stressed cells may also govern osmoregulation since increase in cytosolic Ca^{2+} has been suggested to upregulate the proline biosynthesis (Knight et al., 1997). Shah et al. (1990) also reported increase in Pro content of alfalfa cultures in the presence of exogenous Ca^{2+}. The present observations were, however, in contradiction with those of Girija et al. (2002) who reported decrease in activity of Pro biosynthetic enzyme and increase in activity of PDH and resulting in low Pro content in peanut plants growing under salt stress conditions in the presence of calcium. The difference could be related to type of the stress, type of Ca^{2+} salt and its concentration. While Ca^{2+} could significantly increase the level of Pro and QAC's in the present study but OA was relatively less affected. Dingkuhn et al. (1991) based upon his observations in diverse rice cultivars suggested that water stress induced proline accumulation and OA were independent of each other. This indicates that Ca^{2+} may differentially exercise its control on accumulation of osmotic solutes in cytosol and in vacuoles. Ca^{2+} has been reported to inhibit the influx of K^+ in guard cells during water stress by affecting inward rectifying K^+ channels (Berkowitz et al., 2000) and thus may inhibit the accumulation of ions in vacuole during stress.

A notable distinction between maize and wheat plants towards Ca^{2+} application was observed in the present study. Wheat showed more sensitivity towards calcium application than maize as indicated by response of accumulation of osmolytes, water potential, EL and growth rate in the presence of Ca^{2+}. This suggests that wheat may lack the ability to express some components of Ca^{2+} signaling like Ca^{2+} release mechanisms, Ca^{2+} channels or Ca^{2+} sensors (calmodulin, calcium dependent protein kinases), which may increase its sensitivity towards exogenous calcium application. An insight into the endogenous expression of Ca^{2+} in such cases may offer a better explanation for differential sensitivity towards external Ca^{2+} application. Ca^{2+} expression has been recently engineered genetically in tobacco to control one of its signaling components that has resulted in increased stress tolerance (Arazi et al., 1999).

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