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Peroxidase activities of two rice cultivars differing in salinity tolerance as affected by proline and NaCl

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Abstract

Proline content, ion accumulation, cell wall and soluble peroxidase activities were determined in control and salt-treated calli (150 mM NaCl) and whole plants (30 mM NaCl) of two rice cultivars (salt sensitive cv. IKP and salt tolerant cv. Aiwu). Under salinity, the highest accumulation of Na^+, Cl^- and proline occurred in calli, roots and younger leaves of cv. IKP, coupled with the highest decrease in K^+ content; accumulations of Na^+ and Cl^- were restricted to older leaves in cv. Aiwu. Relative growth rates of calli and roots or shoots from both cultivars were not linked to peroxidase activities. High concentrations (1 M) of exogenously applied glycerol did not inhibit *in vitro* activities of soluble peroxidase extracted from control and salt-treated calli or plants. Conversely, 35 - 55 % (in cv. IKP) or 60 - 80 % (in cv. Aiwu) of soluble peroxidase activities were found in presence of isosmotic proline concentration. There were no differences between proline and glycerol effects on *in vitro* cell wall peroxidase activities.

Key words: callus culture, glycerol, *Oryza sativa*.

Introduction

The improved knowledge of physiological basis of the salt response provides useful informations in the understanding of salt tolerance at both cell and whole plant levels. While 50 mM NaCl is considered as a sub-lethal dose for *Oryza sativa* at the seedling stage (Yeo *et al.* 1990), a program of rice breeding for salt-tolerance using *in vitro* culture had previously defined a 300 mM NaCl concentration as the highest dose compatible with further regeneration of selected calli (Lutts and Bouharmont

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Abbreviations: IAA - indole acetic acid; IKP - I Kong Pao.
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1993). The mechanisms by which rice cells and whole plants respond to intermediate doses could afford us valuable indications on modalities of salt-stress effects on metabolism. Two biochemical markers of stresses were more particularly retained: peroxidase activities (Gaspar *et al.* 1985, Fieldes and Gerhardt 1994) and proline levels (Stewart and Lee 1974). Both markers are linked to the secondary effects of stresses, *i.e.* the accumulation of toxic reactive oxygen intermediates such as hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\bullet}$) and hydroxyl radicals ($OH^{\bullet}$), which are responsible of a wide range of cellular perturbations (Smirnoff 1993). The ubiquitous peroxidases, present in all cell compartments and in apoplasm, catalyse the breakdown of H_2O_2 (Siegel 1993), while proline has been reported as an hydroxyl radical scavenger (Smirnoff and Cumbes 1989), a cytocompatible solute and a protectant of enzyme activities (Stewart and Lee 1974, Pollard and Wyn-Jones 1979). Nevertheless, proline was found to accumulate in NaCl-sensitive plants (Pérez-Alfocea *et al.* 1993) and to inhibit some enzyme activities when added in reaction mixtures (Manetas 1990, Nikolopoulos and Manetas 1991). This suggests that proline was not always linked to osmotic adjustment and can exert a possible toxic role.

The aims of this study were to compare salt-stress effects on proline accumulation and peroxidase activities in calli and whole plants of two rice cultivars differing in salt tolerance and to analyse proline effects on soluble and cell-wall peroxidase activities.

Material and methods

Plant material and culture conditions: Seeds of rice (*Oryza sativa* L.) cvs. I Kong Pao (IKP; salt sensitive) and Aiwu (moderately tolerant) were obtained from International Rice Research Institute (Los Baños, Philippines). Calli were obtained from mature embryos on modified LS medium, pH 5.7, as previously described (Lutts and Bouharmont 1993) and maintained under dark at 25 °C with four weeks subculture periods. Salt stress (150 mM NaCl) was imposed two months after induction. Callus relative growth rate (RGR) was expressed as the $(m_f - m_i)/m_i$ ratio, where m_f is the fresh mass of calli after 30 d of stress and m_i the fresh mass at the time of stress imposition. Three replicates of 10 calli per treatments were performed.

For plants culture, 10-d-old plantlets were transferred to phytotrons (12 h photoperiod, irradiance 300 µmol(PAR) m^{-2} s^{-1} provided by *Sylvania* fluorescent tubes *F96T12/CW/VHO*, day/night temperature of 29/26 °C, air humidity 60 to 80 %). Plants were fixed on polystyrene plate floating on Yoshida *et al.* (1976) nutrient solution. For each cultivar, seedlings were distributed among 8 tanks containing 50 dm^3 of solution homogenised by an *Eheim* ultra small filter (*2007*) circulating pump. 25-d-old plantlets were salinized with 0 or 30 mM NaCl containing Yoshida solution (electrical conductivity of 94 and 474 mS m^{-1}; 4 tanks for each dose). Conductivity was readjusted every 2 d. Every week, solutions were renewed and tanks were randomly rearranged in the phytotron. Growth rate was determined

(four replicates of 3 plants per treatments) as previously described, separately for roots and shoots after 30 d of stress exposure.

Extraction of enzyme proteins: Crude extracts were prepared separately for callus, roots, older leaves and younger leaves of the main stem. For each sample, 5 g of fresh matter were ground in liquid nitrogen and then homogenised in 10 cm^3 of 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 g insoluble polyvinylpyrrolidone (*Sigma*), 2 mM $CaCl_2$, 7 mM $MgCl_2$ and 10 % glycerol. The homogenates were centrifuged at 39 000 *g* at 2 °C for 20 min and the supernatants were used for soluble peroxidase analysis. The pellets were resuspended during 2 h in 7 cm^3 of 50 mM Tris-HCl, pH 7.5, containing 10 mM EDTA, 0.6 M KCl and 10 % glycerol; after centrifugation for 10 min at 9 000 *g*, the supernatants were collected for cell-wall peroxidase assays. Proteins were assayed by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

Enzyme assays: Peroxidase (EC 1.11.1.7) activity was determined in triplicates at 25 °C using the guaiacol oxidation method (Maehly and Chance 1954). The reaction assay contained 2.2 cm^3 phosphate buffer, pH 6.0, 150 mm^3 0.1 M guaiacol and 0.1 cm^3 of extract. The guaiacol oxidation was followed spectrophotometrically at 436 nm, after initiating the reaction with 0.15 cm^3 H_2O_2. One unit of the enzyme activity was defined as the amount of enzyme that catalyses the oxidation of 1 nmol of guaiacol per s and per mg of protein.

In order to analyse proline effects on *in vitro* guaiacol peroxidase activities, assays were repeated in the same reaction mixture containing 0.1, 0.5, or 1 M proline or 1 M glycerol; the activities were expressed as percentages of activity previously determined in absence of proline or glycerol.

Endogenous levels of free proline and ion accumulation: Free proline (Bates *et al.* 1973) and Cl^- (Bourgeais-Chaillou and Guerrier 1992) were determined by colorimetry after extraction on fresh matter with boiling water under agitation for 1 h. K^+ and Na^+ were measured by an inductively coupled argon plasma emission spectrophotometer (*JY 48, Jobin-Yvon,* Longjumeau, France) after digestion of the dry matter with HNO_3.

Results

Calli and whole plant growth: The salt-tolerance of cv. Aiwu was clearly demonstrated at cell and whole plant levels: (*1*) RGR of salt-treated IKP and Aiwu calli decreased by 50 and 30 %, respectively; (*2*) root and shoot RGR of salt-treated plants decreased significantly ($P = 0.05$) compared to controls in cv. IKP but not in cv. Aiwu (Fig. 1). However, calli of both cultivars were more salt tolerant than the corresponding whole plants: the 50 % decrease of RGR in salinized IKP calli was reached for a concentration 5-fold higher than that necessary for the whole plants. Further assays (data not shown) indicated that root RGR in cv. Aiwu decreased at a 50 mM NaCl, being 46.5 % of the control.

Free proline accumulation: Free proline significantly accumulated from 10th day in salt-treated calli and roots of the salt sensitive cv. IKP while it did not change significantly compared to controls and did not vary with length of stress exposure in the salt tolerant cv. Aiwu (Fig. 2). Proline content in leaves of salt sensitive cv. IKP mainly increased during the first 10 d of salt exposure while such an increase began between 10 and 20 d of stress in leaves of salt tolerant cv. Aiwu.

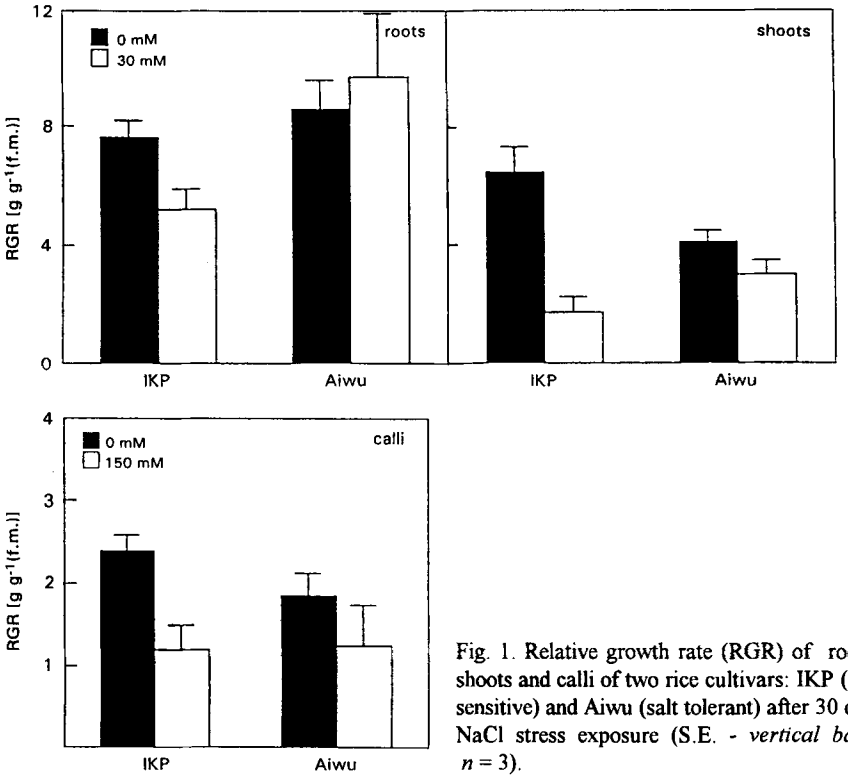

Fig. 1. Relative growth rate (RGR) of roots, shoots and calli of two rice cultivars: IKP (salt sensitive) and Aiwu (salt tolerant) after 30 d of NaCl stress exposure (S.E. - *vertical bars*; $n = 3$).

Table 1. Na^+, K^+ and Cl^- contents [mmol g^{-1}(d.m.)] of calli, roots, older and younger leaves in two rice cultivars(salt sensitive IKP and salt tolerant Aiwu) after 30 d exposure to salt stress (mean ± S.E.). Calli were exposed to 0 or 150 mM NaCl, whole plants to 0 or 30 mM NaCl.

Ion	Cv.	Calli		Roots		Older leaves		Younger leaves	
		0	150	0	30	0	30	0	30
Na^+	IKP	0.09±0.01	0.77±0.12	0.12±0.05	0.44±0.09	0.03±0.01	0.28±0.07	0.05±0.01	0.33±0.10
	Aiwu	0.08±0.01	0.72±0.08	0.13±0.02	0.22±0.10	0.04±0.02	0.38±0.09	0.06±0.01	0.17±0.03
K^+	IKP	0.73±0.11	0.59±0.08	0.83±0.03	0.48±0.05	1.08±0.13	0.71±0.12	0.94±0.09	0.73±0.11
	Aiwu	0.75±0.08	0.60±0.11	1.18±0.05	0.64±0.08	1.13±0.11	0.74±0.19	1.05±0.17	0.94±0.13
Cl^-	IKP	0.33±0.11	0.78±0.09	0.27±0.06	1.07±0.14	0.31±0.07	0.84±0.06	0.37±0.09	0.75±0.08
	Aiwu	0.29±0.08	0.52±0.10	0.43±0.11	0.67±0.09	0.25±0.09	0.90±0.09	0.45±0.13	0.60±0.03

Ion content: Salt stress induced similar Na^+ increase and K^+ decrease in calli derived from both cultivars; calli from cv. Aiwu accumulated less Cl^- than calli from cv. IKP (Table 1). Roots and younger leaves of cv. Aiwu accumulated less Na^+ and Cl^- and more K^+ than those of cv. IKP. Na^+ and Cl^- were accumulated mainly in the oldest leaves of the salt tolerant cv. Aiwu.

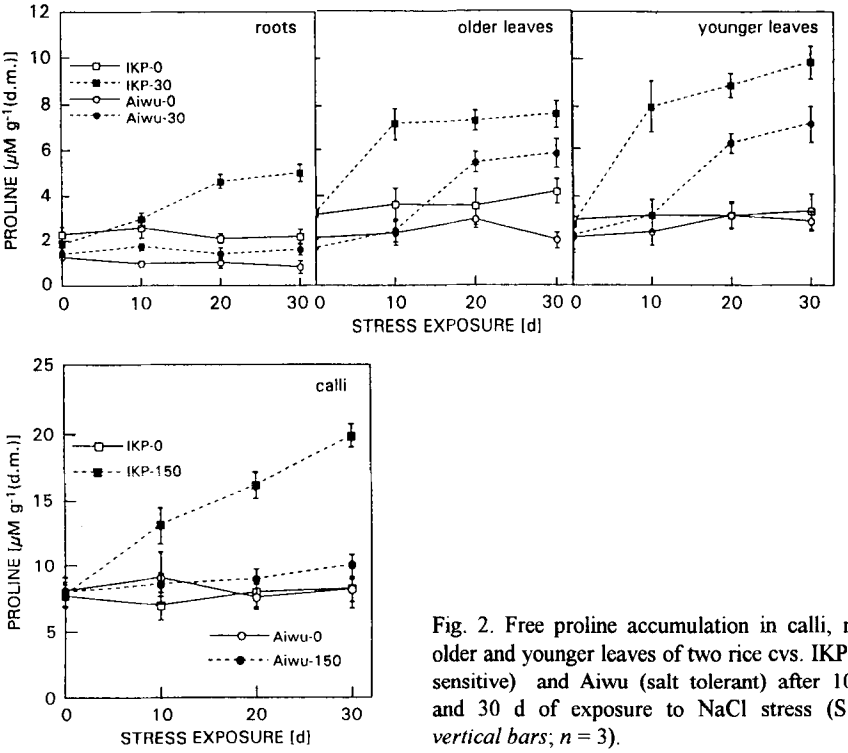

Fig. 2. Free proline accumulation in calli, roots, older and younger leaves of two rice cvs. IKP (salt sensitive) and Aiwu (salt tolerant) after 10, 20 and 30 d of exposure to NaCl stress (S.E. - *vertical bars*; $n = 3$).

Peroxidase activities: Under salinity, the calli of cv. IKP exhibited a higher decrease in RGR and soluble peroxidase activity compared to cv. Aiwu (Fig. 3). Conversely, no relationships did occur between RGR and peroxidase activities in the whole plants: soluble peroxidase activities were enhanced under salinity in roots of both cultivars, the root RGR either decreasing in cv. IKP or increasing in cv. Aiwu (see above). Soluble peroxidase activities increased in leaves of salt-treated cv. IKP and decreased in leaves of cv. Aiwu.

Cell wall peroxidase activities (Fig. 4) decreased in salt-stressed calli of both cultivars but were always higher in cv. Aiwu. Cell wall peroxidase activities increased in roots and decreased in leaves of salt-treated plants. In the presence of NaCl, activities of cell wall peroxidase, however, were clearly lower in older leaves of salt tolerant cv. Aiwu comparatively to salt-sensitive cv. IKP.

Proline and glycerol effects on *in vitro* peroxidases activities: There was no difference in the influence of proline and glycerol on soluble and cell wall peroxidases of

control and salt-treated materials; thus, the results are presented only for control calliand whole plants. Compared to control or 1 M glycerol, the addition of 0.1 or 0.5 M proline to the assay media did not exert any significant effects on peroxidase

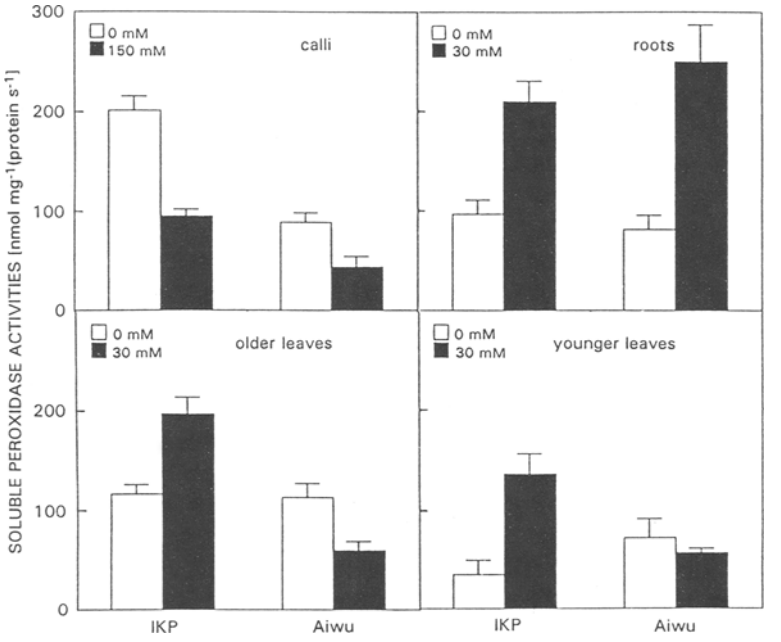

Fig. 3. Soluble peroxidase activities in calli, roots, older and younger leaves of cvs. IKP (salt sensitive) and Aiwu (salt tolerant) after 30 d of exposure to NaCl stress (S.E. - *vertical bars*; $n = 3$).

activities (Fig. 5). Conversely, 1 M proline exerted an inhibition of soluble peroxidase activity which was more pronounced in calli, roots and oldest leaves of the salt sensitive cv. IKP than of cv. Aiwu (Fig. 5A): 35 - 55 % of peroxidase activities were found in cv. IKP vs. 50 - 80 % in cv. Aiwu. Surprisingly, peroxidase activities of younger leaves of cv. Aiwu were inhibited in presence of 0.5 M proline and 1 M glycerol in the assay media.

Cell wall peroxidase activities of the overall materials (Fig. 5B) were less affected by proline in the assay media than soluble peroxidases. Proline even slightly stimulated cell wall peroxidase activities extracted from roots of salt sensitive cv. IKP. Furthermore, there were no major differences between isosmotic concentrations of proline and glycerol on *in vitro* inhibition of cell wall peroxidase activities. Cell wall peroxidases of older leaves were more affected by osmotic agent in salt tolerant cv. Aiwu than in salt sensitive cv. IKP while cell wall peroxidases of the younger leaves were more inhibited in the latter than in the former.

Discussion

Rice is very sensitive to salinity since, according to Yeo *et al.* (1990), a NaCl concentration as low as 50 mM could be considered as lethal during vegetative

growth. Cv. Aiwu behaved in our experiments as a moderately tolerant cultivar compared to the well known salt tolerant cvs. Nona Bokra or Pokkali. Compared to whole plant responses, a higher salt tolerance was found in calli of rice, similarly as in tomato and soybean (Bourgeais-Chaillou and Guerrier 1992, Bourgeais-Chaillou *et al.* 1992). When comparing RGR and ion contents of calli and whole plants, the salt-sensitivity appeared dependent on Na^+ and Cl^- accumulation. Salt tolerant cv. Aiwu accumulated more Na^+ and Cl^- in the older leaves, as previously shown by

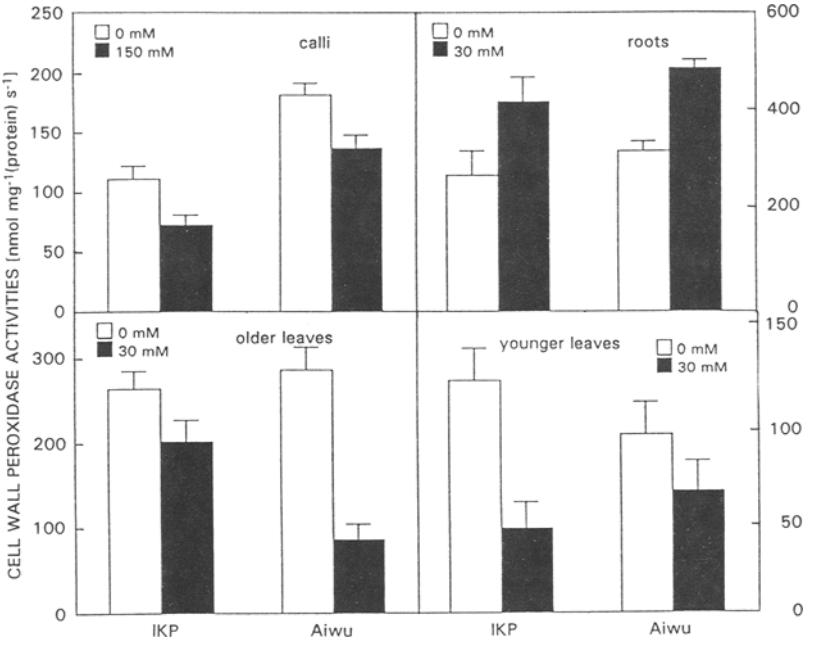

Fig. 4. Cell-wall peroxidase activities in calli, roots, older and younger leaves of two rice cvs IKP (salt sensitive) and Aiwu (salt tolerants) after 30 d of exposure to NaCl stress (S.E. - *vertical bars*; $n = 3$).

Yeo and Flowers (1986) for other salt tolerant rice genotypes. Thus the growing zones (roots, younger leaves) of cv. Aiwu exhibited higher K^+/Na^+ ratios. Soluble peroxidase activity, K^+/Na^+ ratio and RGR decreased together in calli derived from both cultivars. Nevertheless, growth response of rice to NaCl did not appear to be correlated to soluble or cell wall peroxidase activities, corroborating previous results obtained by Stevens *et al.* (1978) in salinized *Brassica* species. Salinity resulted in a similar decrease in peroxidase activities in roots of both cultivars while cv. Aiwu growing on 30 mM NaCl exhibited much higher root growth and K^+/Na^+ ratio. Moreover, shoot K^+/Na^+ ratio and shoot RGR of salt-treated cv. Aiwu were higher while soluble and cell wall peroxidase activities were lower than in cv. IKP. Therefore, no direct relationships between peroxidase activities and growth in the presence of salt were established, contrary to previous results obtained with germinating tomato, radish and red cabbage (Guerrier 1987) or with calli derived from various explants of soybean (Bourgeais-Chaillou *et al.* 1992).

According to Mittal and Dubey (1991), soluble peroxidase activities in the presence of NaCl were higher in the shoot of the salt sensitive rice cultivar than in the salt tolerant one. The decrease in root RGR could be linked to the stimulation of cell wall peroxidases which control the lignification process (MacAdam *et al.* 1992). The decreasing cell wall peroxidase in calli from both cultivars or in IKP salt-treated shoots together with RGR reduction remained yet unclear. On the other hand, considering that anionic cytosolic isoforms are involved in detoxification of H_2O_2 (Zheng and Van Huystee 1992), high soluble peroxidase activities in the shoot of cv. IKP might result from the overproduction of hydrogen peroxide often observed under stress conditions (Smirnoff 1993).

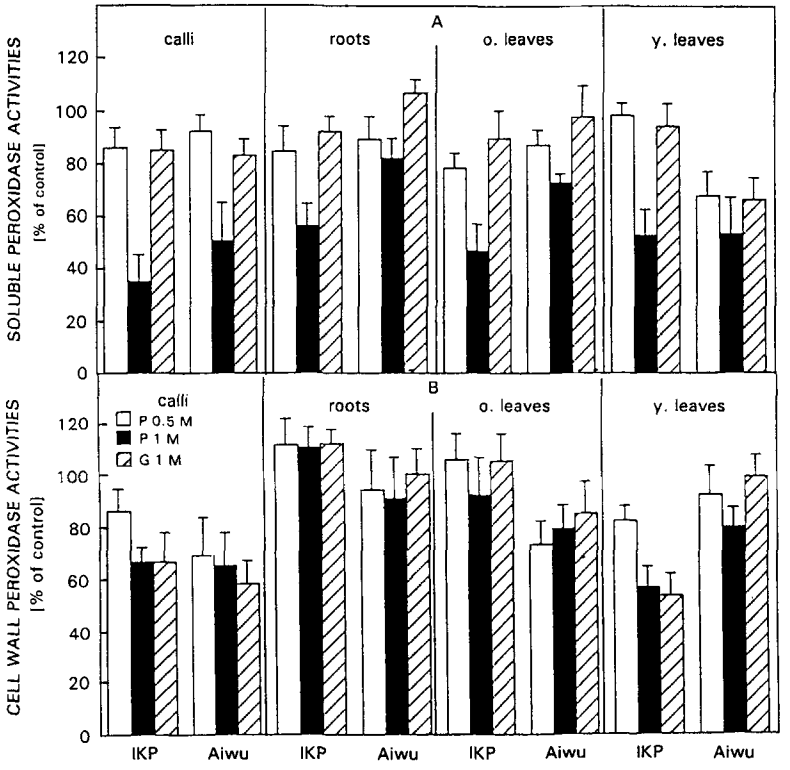

Fig. 5. Effects of proline (P; 0.5 and 1 M) and glycerol (G, 1 M) in the reaction mixture on soluble (*A*) and cell-wall (*B*) peroxidases of non-stressed calli, roots, older and younger leaves of rice cvs. IKP (salt sensitive) and Aiwu (salt tolerants). Activities are expressed as percentages of activities estimated in the absence of proline or glycerol (S.E. - *vertical bars*; $n = 3$).

Proline accumulation in rice, similarly as in *Lycopersicon* species (Bourgeais-Chaillou and Guerrier 1992, Pérez-Alfocea *et al.* 1993) is linked to growth decrease and therefore is likely to be a symptom of injury rather than the classical active osmoticum demonstrated in halophytic species (Stewart and Lee 1974). The similar proline accumulation in younger and older leaves of cv. Aiwu, which accumulated toxic ions mainly in the oldest leaves, support the hypothesis that proline did not play

a protective role. Moreover, the inhibition of *in vitro* soluble peroxidase activity by proline correspond to the deleterious effects of this amino acid on phosphoenolpyruvate carboxylase, malate dehydrogenase and nitrate reductase extracted from *Salsola soda* (Manetas 1990, Nikolopoulos and Manetas 1991). Soluble peroxidase activities in calli, roots and oldest leaves of salt sensitive cv. IKP which accumulates higher levels of proline in response to salt stress, were more inhibited by this amino acid than activities of soluble peroxidases extracted from salt tolerant cv. Aiwu. It can therefore be postulated that sensitivity to NaCl was not only due to the drastic effects of the fall in K^+/Na^+ ratios on some enzyme activities (Greenway and Munns 1980), but also to the possible negative effects of accumulated proline on enzyme activities. Even if the observed proline contents (Fig. 2) were lower than the concentration required for *in vitro* inhibition of peroxidase activity, it should be pointed out that glycerol, a well known compatible solute (Heimer 1973), had only slight effect on soluble peroxidase activities comparatively to isosmotic concentration of proline.

The distinctions between specific anionic and cationic peroxidases on one hand, guaiacol and ascorbate peroxidases on the other hand, could provide useful informations on involvement of those protective enzymes in salinity tolerance.

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