ISOENZYME PROFILES OF PEROXIDASE, CATALASE AND SUPEROXIDE DISMUTASE AS AFFECTED BY DEHYDRATION STRESS AND ABA DURING GERMINATION OF WHEAT SEEDS

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Summary. Isoenzyme profiles of peroxidase [EC 1.11.1.7], superoxide dismutase (SOD)[EC 1.15.1.1] and catalase [EC 1.11.1.6] in the endosperm and roots of wheat seedlings germinated under chronic stress conditions were investigated. Low and high temperature, 0.2 M NaCl and 0.5 M sucrose were applied as stress factors. Germination of seeds in the presence of ABA was used as a dehydration stress confirming standard. A strong inhibition of germination rate and growth of roots by all stress factors applied was established. Significant inhibitory effects of low temperature and sucrose on the activity of anionic peroxidase isoenzymes in the endosperm was observed. ABA activated moderate moving cationic peroxidases in the roots. The activity of fast moving SOD isoenzyme decreased significantly in the endosperm and roots of seedlings subjected to salt and osmotic stress. A strong inhibitory effect of H_2O_2 on catalase activity was observed in the endosperm, however, in the roots a stimulation was observed. The most sensitive among the antioxidant enzymes tested was catalase. The growth retardation in wheat seedlings correlated closely with a decrease in catalase activity. The low temperature was the strongest effector among the stress factors applied. The most specific isoenzyme profile was the profile of cationic peroxidase. Tissue-specific differences were found in the response of the antioxidant enzymes to the stress factors studied. The results were discussed in terms of the importance of the antioxidant enzymes tested for the protection of germinating wheat seeds against dehydration stress.

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Abbreviations: ABA- abscisic acid, CAT- catalase, NBT – nitrotetrazolium Blue Chloride, SOD- superoxide dismutase.

INTRODUCTION

Germinating seeds first meet unfavourable environmental conditions (water deficit, low and high temperature, pathogens, waterlogging) (Bewley and Black, 1982) thus leading to increased accumulation of damaging concentrations of reactive oxygen species (ROS) (Prassad et al., 1994; Srivalli et al., 2003; Apel and Hirt, 2004; Ashraf and Harris, 2004; Wang et al., 2004). At optimal concentrations, ROS play a positive role in the normal plant development and in plant responses to environmental stresses. Under normal conditions the production and destruction of ROS is regulated well in cell metabolism but under stress conditions the formation of ROS exceeds the amount present under normal physiological conditions, thus creating the oxidative stress (Larson, 1988; Scandalios 1993). Environmental stresses exert their effects directly or indirectly through the formation of ROS (Yu and Rengel, 1999) and ROS scavenging is a common response to most stresses (Scandalios, 1993; Srivalli et al. 2003). Plants cope with stress by activation of the cell antioxidant system (Gechev et al. 2002). The ability of plant tissues to mobilize enzymatic defense against uncontrolled production of ROS may be of great importance for plant survival under stress conditions (Dhindsa and Matowe 1981; Andarson et al. 1995).

It is well known that ABA levels increase in tissues subjected to dehydration stress (Xiong, Zhu, 2001). Under these conditions specific genes are expressed that can also be induced in unstressed tissues by exogenously applied ABA (Gomez et al. 1988). Therefore, the changes occurring in germinating wheat seeds in the presence of ABA can serve as a dehydration stress confirming standard.

Chilling (0-15^{o} C) causes elevated levels of hydrogen peroxide in leaves of winter wheat and in maize seedlings (Prassad et al., 1994; Anderson et al., 1995). In our study changes in seeds germinating in the presence of exogenously applied H_2O_2 were compared with those occurring in seeds germinated under low temperature (10^{o} C).

Plant peroxidases have been used as biochemical markers for various types of biotic and abiotic stresses due to their role in very important physiological processes, like control of growth by lignification, cross-linking of pectins and structural proteins in cell wall, catabolism of auxins (Gaspar et al., 1982).

Catalases and superoxide dismutases are the most efficient antioxidant enzymes (Scandalios, 1984). The expression of specific catalase isoenzymes is important and

critical against oxidative stress induced by a given environmental stress (Scandalios, 1994). Low temperature stress causes the accumulation of H_2O_2, which in turn may function in increasing the activity of the CAT 1 and CAT 2 isoenzymes to prevent high accumulation of H_2O_2 and other ROS in plant cells (Scandalios, 1984). Variable responses of SOD to dehydration stress have been reported in literature including decreased activity (Quatracci et al. 1994), lack of effect (Anderson, 1995; Bartoli et al. 1999) or increased activity (Srivali, 2003) depending on plant species, tissue and stage of development.

The aims of this study were: (a) to establish which of the antioxidant enzymes play a major role in protection of germinating wheat seeds against oxidative stress and (b) to study the tissue-specific differences in the response of the antioxidant enzymes to stress. For that reason we studied the stress-induced changes in the isoenzymes of peroxidase, SOD and catalase.

MATERIALS AND METHODS

Plant material

Wheat seeds (*Triticum aestivum*, Sadovo 1 cultivar) grown in the field were used throughout the experiments. Seeds were germinated in filter paper rolls wetted with tap water, in darkness under optimal (24ºC), low (10º C) or high (38ºC) temperature or in the presence of 0.2 M NaCl, 0.5 M sucrose, 30 μM ABA and 10 mM H_2O_2. All these stress factors cause dehydration stress. Endosperms and roots of wheat seedlings grown for 72 h were analysed.

Growth measurements

A batch of 100 seeds were used for FW determination of endosperm and roots of seedlings.

Enzyme extraction

Cell-free extracts from seedlings subjected to various treatments were analyzed for peroxidase, superoxide dismutase and catalase activities on nondenaturing gels. Fresh harvested endosperms and roots were ground in a mortar in 0.1 M tris-HCl buffer, pH 7.1. The material/buffer ratio was 1:3 for endosperm and 1:10 for roots. The homogenate was centrifuged at 12 000 x g for 30 min at 4^0 C. The supernatant was used as a crude enzyme extract. Aliquots of enzyme extracts mixed with equal volumes of 40% sucrose were prepared. All samples were stored at -20^0C until enzyme analysis.

Total protein content determination

Protein content in the crude extracts was determined after TCA precipitation according to the method of Lowry et al. (1951) using BSA as a standard.

Native polyacrylamide gel electrophoresis

Native PAGE in 7.5 % gel was carried out by the method of Davis (1964). A hundred μg of protein were loaded per tube. Two mA per tube was applied during electrophoresis. Cationic (basic) peroxidase isoenzymes were separated by the method of Reisfeld et al. (1962).

Enzyme visualization (after native PAGE)

Peroxidase isoenzymes were detected by incubating the gels for 5 – 20 min in a reaction mixture containing 0.5 mM benzidine hydrohloride and 10 mM H_2O_2 in 0.05 M acetate buffer, pH 4.9 according to the procedure of Ornstein (1964).

Superoxide dismutase isoenzymes were detected on the gels by the method of Greneche et al. (1991). The gels were incubated for 30 min in the dark in a mixture containing 10 mg NBT, 75 mg Na_2-EDTA and 3 mg riboflavin dissolved in 100 ml tris-HCl buffer, pH 8.2. After that gels were illuminated for 15 min.

Catalase isoenzymes were stained as described by Woodbury et al. (1971). The gels were incubated in the dark for 20 min in 10 mM H_2O_2 dissolved in K/Na phosphate buffer, pH 7.0, followed by incubation in the mixture of 1% $K_3Fe(CN)_6$ and $FeCl_3$ for 15 min.

All stained gels were fixed in a mixture containing H_2O:ethanol:acetic acid:glycerol (2:1:1:1). The stained isoenzyme patterns were scanned densitometrically.

We assessed the differences and identity between individual isoenzymes by their number and values of the relative electrophoretic mobility (Rm).

Results

Retardation of germination rate and reduction of seedling growth as estimated by the reduced FW were observed in all seedlings subjected to low and high temperature, sucrose, ABA, NaCl and H_2O_2 treatment (Fig.1). There was a 94 % decrease in growth of roots in low temperature stressed seedlings as compared to the control. The presence of sucrose, high temperature, ABA and NaCl suppressed root growth by 88%, 87%, 78% and 53 %, respectively. In addition, H_2O_2 suppressed root growth by 43 %. There were no significant differences between endosperms of seedlings subjected to the stress factors studied.

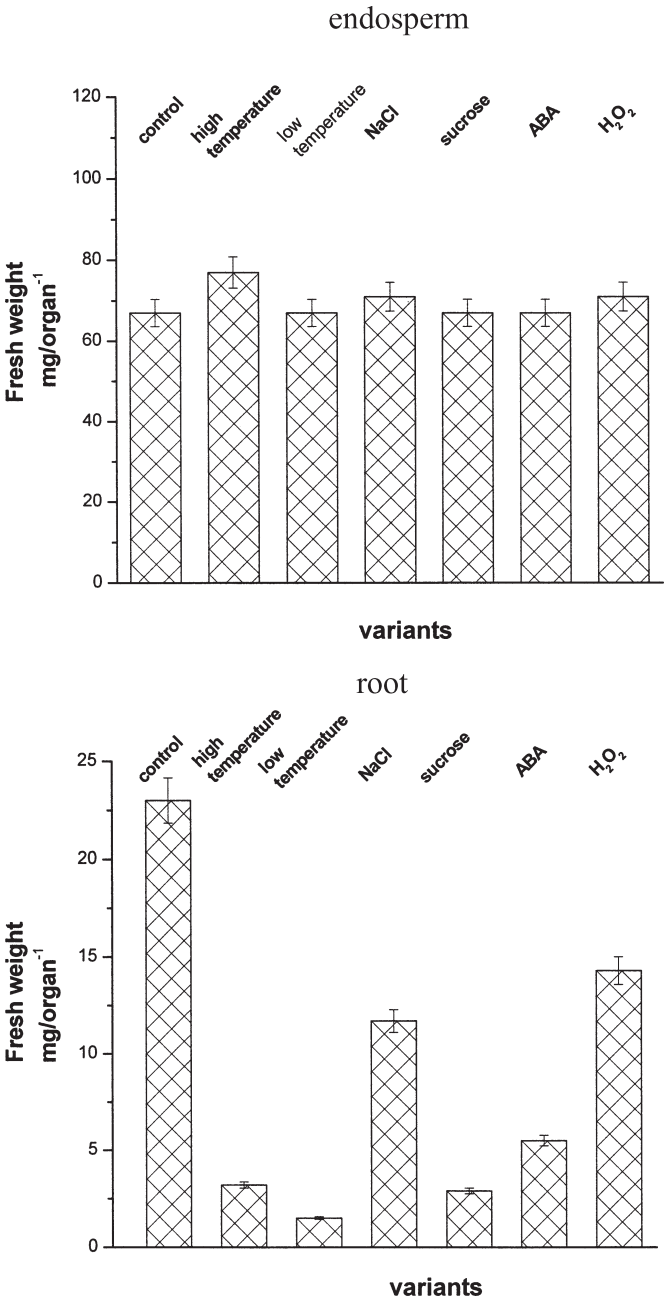

Fig.1. Changes in fresh weight of endosperms (A) and roots (B) of germinating wheat seeds. A hundred seeds were used for determination of FW.

In the spectrum of anionic peroxidase in the endosperm 7-10 isoenzymes were revealed (Fig. 2-A). A strong inhibition of peroxidase activity was detected in the endosperms of low temperature and sucrose germinated seedlings. In the spectrum of low temperature germinated seeds only two low active isoenzymes were visible (N 6 and 7) whereas the moderate and fast moving isoenzymes had disappeared. Similar trends could be seen for sucrose-germinated seeds but in this case another pair of isoenzymes remained in the spectrum – isoenzymes N 10 (Rm 0.52) and isoenzyme N 11 (Rm 0.59). The activity of the fast moving isoenzyme (N 15 with Rm 0.8) enhanced significantly in seedlings germinated under the experimental conditions - high temperature, NaCl, ABA and H_2O_2. The activity of the slow (N 1 with Rm 0.03) and moderate (N 7 with Rm 0.4) migrating isoenzymes increased after ABA treatment. The activity of the anionic isoperoxidases was significantly lower in the roots as compared to the endosperms. The moderate and fast moving anionic peroxidase isoenzymes were significantly inhibited in the roots (Fig. 2-B) of seedlings germinated at low temperature. In this case three moderate moving isoenzymes (Rm 0.41,

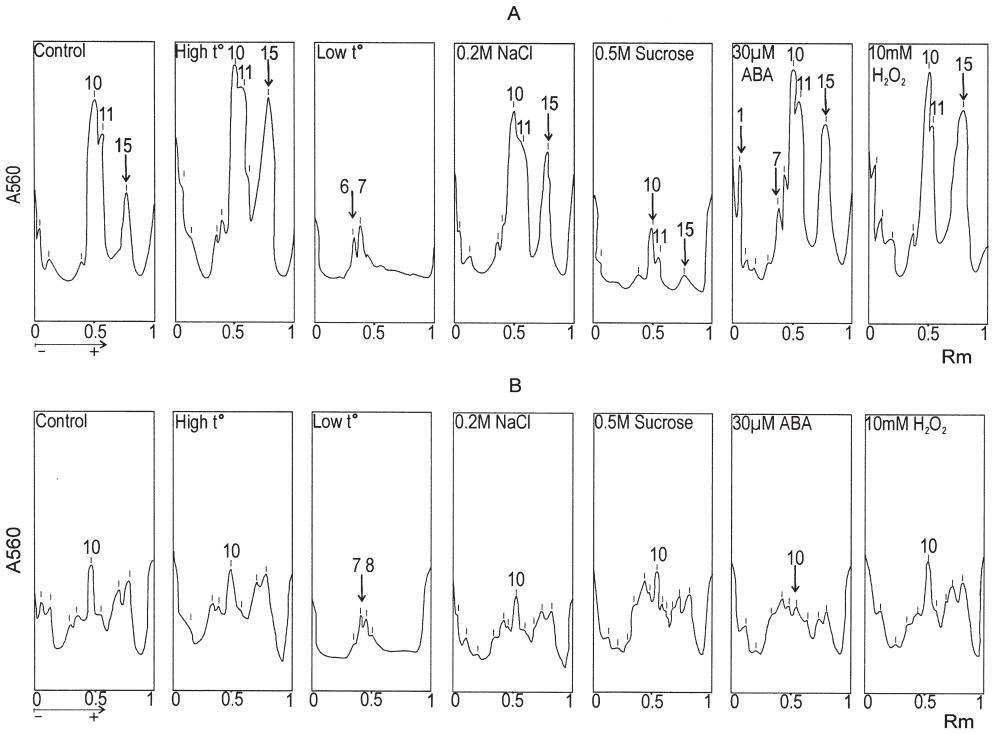

Fig.2. Densitometric scans of anionic peroxidase isoenzymes from endosperms (A) and roots (B) of wheat seedlings: Anionic isoperoxidases were separated in 7.5% PAGE by the method of Davis (1964). A hundred micrograms of total protein was loaded in each tube. Isoenzymes were visualized on gels with benzidine as H-donor. Brown bands with peroxidase activity appeared on the gels after 5 min of incubation for root and 20 min for endosperm isoperoxidases.

0.46 and 0.49) showed a very low activity. In the roots of sucrose, NaCl, and ABA germinated seedlings, the distribution of peroxidase activity among individual isoenzymes was altered. The tissue specificity of anionic peroxidase isolated from endosperms and roots of wheat seedlings was expressed as a difference in enzyme activity between the two seed organs, different distribution of total enzyme activity among individual isoenzymes and different manner of influence of the stress factors applied. In the endosperms low temperature and sucrose decreased drastically peroxidase activity but in the roots only low temperature had a similar effect. ABA and H_2O_2 increased the activity of the moderate and fast moving isoenzymes in the endosperm. In the roots of seedlings subjected to ABA the activity of these isoenzymes declined whereas H_2O_2 had no effect.

Only one highly active cationic peroxidase isoenzyme was observed in the endosperms (data not shown). Eight cationic peroxidase isoenzymes were detected in the roots (Fig. 3). High specifisity was established for isoenzyme patterns of basic peroxidases in the roots depending on the stress factor applied. The isoenzyme profile of cationic peroxidase was different for each of the stress factors studied. A specific set of highly active isoenzymes was detected in the roots of the treated seedlings. The most significant inhibition of the enzyme activity was observed in low temperature germinated seedlings where only one cationic isoenzyme (N5 with Rm 0.41) preserved its activity.

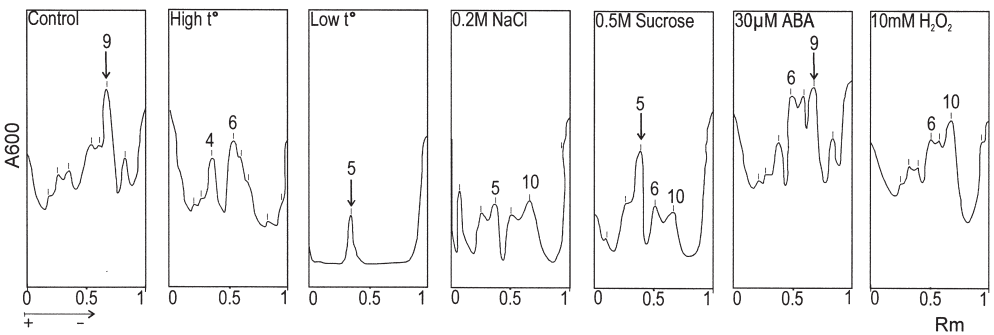

Fig. 3. Densitometric scans of cationic peroxidase isoenzymes from roots of wheat seedlings. Isoenzymes were separated in 7.5% PAGE by the method of Reisfeld et al. (1962). A hundred micrograms of total protein was loaded in each tube. Activity staining was carried out with benzidine as H-donor. Blue clored bands appeared after 5 min for root and 30 min for endosperm cationic isoforms.

Examination of SOD isoenzyme profiles in the endosperm revealed 7 isoforms (Fig. 4-A). The fastest moving SOD isoenzyme (N 10 with Rm 0.8) in the endosperm of high temperature germinated seeds was activated. Its activity strongly declined under high osmotic (NaCl and sucrose) conditions. In the roots six SOD isoenzymes

were found, three of them being most prominent (N 6, 9 and 10 with Rm values 0.4, 0.61 and 0.8) while the others were very faint bands (fig. 4-B). The activity of the fastest moving isoenzyme increased under high temperature, ABA and H_2O_2. In contrast, low temperature and osmotic stress caused a significant decrease in its activity.

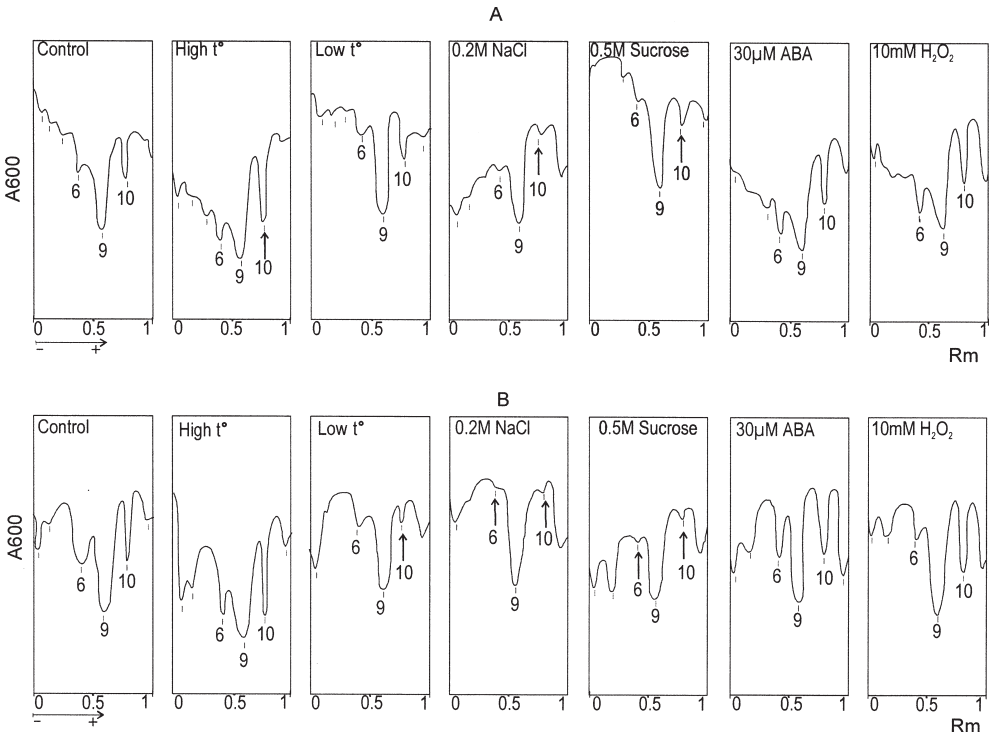

Fig. 4. Densitometric scans of superoxide dismutase isoenzymes from endosperms (A) and roots (B) of wheat seedlings. SOD isoenzymes were resolved in 7.5% PAGE according to Davis (1964). A hundred micrograms of total protein was loaded in each tube. Isoenzymes were detected on gels by staining procedure of Greneche et al.,(1991) with NBT, EDTA-Na_2 and riboflavin. SOD isoforms revealed as achromatic bands on dark blue background after 15 min of illumination.

Activity staining revealed 5-6 catalase isoforms in the endosperm of wheat seedlings but the bulk of catalase activity was localized in the group of slow migrating isoenzymes (N 1, 3, 5 with Rm values 0.04, 0.16 and 0.28 respectively) (Fig. 5-A). Low temperature and osmotic stress (NaCl and sucrose) strongly inhibited catalase activity of all three major isoenzymes. The effect of ABA and hydrogen peroxide on catalase electrophoretic profile was slightly expressed. In the roots an inhibition of catalase activity was observed upon low temperature, NaCl and sucrose treatments. On the other hand, H_2O_2 and ABA stimulated catalase activity of all isoenzymes detected in the roots (Fig. 5-B).

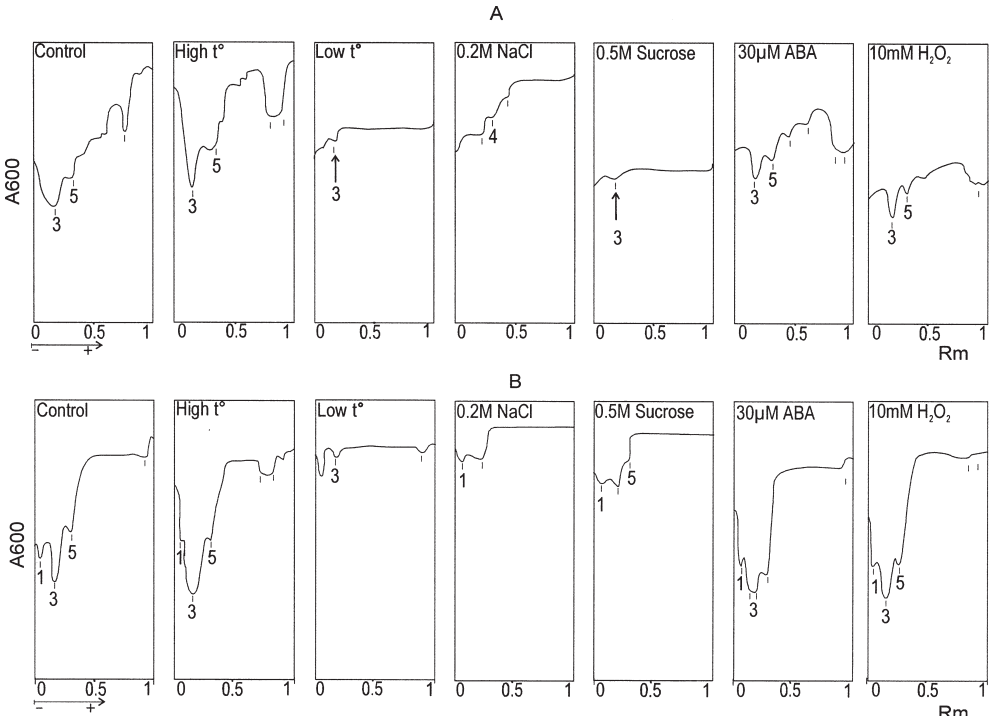

Fig. 5. Densitometric scans of catalase isoenzymes from endosperms (A) and roots (B) of wheat seedlings. Catalase isoenzymes were resolved in 7.5% PAGE according to Davis (1964). A hundred micrograms of total protein was loaded in each tube. The staining procedure of Woodbury et al. (1971) was used. The isoenzymes of catalase appeared as achromatic bands on dark green background 20 min after incubation of 1% solution consisting of $FeCl_3$ and $K_3Fe(CN)_6$.

Discussion

The response of growth and antioxidant enzymes to stress in various seed organs (endosperm and root) of dark-grown wheat seedlings was examined in relation to stress tolerance. Growth retardation was more pronounced in both shoots and roots of stressed seedlings as compared to the endosperm. The lengths of shoots and roots as well as the fresh weight of roots in seedlings subjected to high, low temperature and sucrose were significantly depressed. Similar trend was established also for other stages of seedling development (24, 48, 96 and 120 h of germination) (data not shown here). These results could be interpreted in view of the well known fact that developmental programme is initially established in seedlings. Shoots and roots are intensively growing organs through cell division and elongation. The negligible differences between fresh weights of endosperms in the control and treated seedlings could be explained by fact that endosperm consists mainly of dead cells and quickly degen-

erates following germination. It reaches maximum water content before 24 h of germination and no division or elongation of cells occurs throughout the postgerminative growth of seedlings. In wheat seeds mobilization of stored proteins and starch begins 96 h from the onset of germination (Bewley and Black, 1982; Scandalios, 1994).

The isoenzyme profiles of peroxidase, SOD and catalase were studied in order to examine whether the differences between various stress factors and stress intensity (as judged by growth retardation) can lead to the induction of new isoforms or only to quantitative changes in the existing isoforms.

No induction of any new isoenzymes was observed in the profile of endosperm anionic peroxidase. A strong activation of isoenzyme with Rm 0.8 was observed under high temperature, salt stress (NaCl), ABA and H_2O_2. Similar result were reported by Srivalli et al. (2003) for peroxidase in rice. Among the four peroxidase isoenzymes stained only POX III increased in the stressed plants whereas the other forms did not show any differences among treatments. In addition, our results indicate a strong inhibition of peroxidase isoenzymes both in the endosperm and roots upon low temperature treatment. Oidaira et al. (2000) suggest that peroxidase might be an important enzyme in the antioxidant defense mechanism of rice as a transient increase in its activity has also been observed in rice seedlings on exposure to low temperature stress.

We found 6-7 SOD isoenzymes in wheat seedlings and only one of them (isoenzyme N 10) responded to the stress applied. Similar results were reported by Srivalli et al (2003). These authors observed five SOD isoforms, four of them being Cu/Zn SODs. The activity of SODII increased during water stress in all cases. SOD III and IV did not show any differences among treatments. The enhancement of total SOD activity in stressed plants was due to the SODII isoenzyme. On the other hand, Bartoli et al. (1999) reported that SOD and CAT activity remained unchanged in water stressed wheat plants.

In the roots six SOD isoenzymes were found, three of them being most prominent (N 6, 9 and 10 with Rm values 0.4, 0.61 and 0.8), while the others were very faint bands (fig. 4-B). The activity of the fastest moving isoenzyme (N 10) increased upon high temperature, ABA and H_2O_2 treatments as compared to the control seedlings. In contrast, low temperature, NaCl and sucrose caused a significant decrease in its activity. The activity of SOD in response to the stress factors applied appeared to depend on its tissue localization. The fastest moving isoenzyme (N 10) in the endosperm was activated after high temperature treatment but in the roots its activity increased after ABA and H_2O_2 treatments, as well. It was shown that salinity stress enhanced the activity of Fe-SODI in the leaves of *Suaeda salsa* but osmotic stress did not change its activity (Wang et al., 2004). Yu and Rengel (1999) established that drought and salinity stress differentially influenced the activity of superoxide dismutase in lupine. Anderson et al. (1995) observed four SOD isoenzymes but none of them were strikingly affected by chilling. Tanida (1996) found no correlation between the

initial SOD level and germination rate at 15^0C. It could be seen that the levels of SOD were very high even in the control seedlings. These high levels of SOD could be explained by the very important role of the enzyme in scavenging the highly toxic O_2^- and the products of lipid peroxidation. Maximal levels of the appropriate SODs might be required at all times to provide adequate protection of the cells (Scandalios, 1993). The lack of strong effects of the stress factors applied in our study on the SOD isoenzyme profile could be likely due to the fact that the activity of SOD in the embryos is sufficiently high to convert O^-_2 to H_2O_2 or that O^-_2 accumulation in the embryos is not appreciable at the early stages of seedling growth.

In our study, 3 major bands with catalase activity were detected in wheat. In maize catalase is present as four isoenzymes (Scandalios et al. 1984), but only two of them (CAT1 and CAT3) are present in dark-grown seedlings. We found a close correlation between germination rate, seedling growth and a decline in catalase activity in endosperms of low temperature, sucrose and NaCl germinated seedlings. The same trend was observed also in roots upon low temperature, NaCl and sucrose treatments. The importance of such correlation has already been underlined by other authors (Anderson et al., 1995; Tanida, 1996) who considered that stand establishment in maize and rice is greatly affected by exposure of seedlings to low temperature during germination and early seedling growth. Such a close correlation between catalase activity of rice seed embryo and germination rate at a low temperature was established by Tanida (1996). He suggested that the tolerance of rice cultivars to chilling injury was closely linked to the cold stability of catalase and ascorbate peroxidase. In our experiments the roots and endosperms responded in a similar manner to low temperature, NaCl and sucrose treatments. Oidaira et al. (2000) reported that catalase in rice seedlings was not significantly changed under the influence of low temperature. The activity of all three isoenzymes decreased after low temperature, NaCl and sucrose treatments. However, different results were reported by Scandalios (1994) who observed that each of the three maize catalase genes responded differently to chilling stress. The activity of the CAT 1 and CAT 2 isoenzymes increased significantly, and a more prominent response occurred in the embryonic axis. Based on these results he considers that catalases might play a significant role during chilling stress through precise temporal and spatial regulation of the expression of the Cat gene products. It is possible that one of the effects of chilling stress is the oxidative inactivation of CAT. Srivalli et al. (2003) established an induction of a new catalase isoform in water stressed rice seedlings that reached a maximum at severe water stress. The highest intensity of all CAT isoenzymes was detected in plants grown under the most severe stress conditions. Water stress increased the activities of all scavenging enzymes with a differential response depending on stress intensity. Many authors consider that the ability of a plant to improve its active-oxygen-scavenging capacity may be a key element in stress tolerance (Anderson et al., 1995).

Our results showed no induction of new isoforms of the enzymes tested.The isoenzymes which were present in the spectra were either activated or inhibited. The activation of the existing isoenzymes suggests that the same isoenzymes detoxifying ROS produced under normal conditions function also under stress when an overproduction of ROS occurs. In agreement with many authors (Scandalios, 1994; Tsang et al., 1991; Wang et al. 2004) our results indicate that different isoforms of the antioxidant enzymes respond to different stress factors and only those isoforms which are needed to protect a particular cell compartment are expressed. It is well known that antioxidant isoenzymes are differentially compartmentalized depending on the tissue, likely to respond differently to biotic and abiotic stresses. Some authors reported that limitation of one of the components of the antioxidant system could be compensated through an up-regulation of other components (Wilekens et al., 1997). Our results showed no such dependence. For example, low temperature decreased the activity of catalase isoforms as well as the anionic and cationic isoperoxidases. In addition, the activity of SOD isoforms was also lowered to a certain extent in both seed organs studied. A close similarity was found between the enzyme profiles of peroxidase and catalase in seedlings subjected to low temperature and sucrose treatments as well as between the catalase, anionic peroxidase and SOD profiles in seedlings germinated in the presence of ABA and H_2O_2.

Conclusions

Based on the results presented in this study the following conclusions could be drawn:

1. Retardation of germination rate and reduction of seedling growth was a common feature for all seedlings subjected to the stress factors tested.
2. Catalase was the most susceptible antioxidant enzyme under the chronic stress conditions applied.
3. Catalase activity correlated closely with the decrease in growth retardation of wheat seedlings.
4. Low temperature was the strongest effector among the stress factors applied.
5. The cationic peroxidase profile was the most specific isoenzyme profile compared with the other enzymes studied in relation to the stress factors applied.

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