Antioxidant Enzyme Changes in Response to Drought Stress in Ten Cultivars of Oilseed Rape (*Brassica napus* L.)

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Abstract: The study was undertaken to identify the responses of antioxidant enzyme activities and their isozyme patterns in seedlings of 10 oilseed rape (*Brassica napus* L.) cultivars under drought stress conditions. Plants were grown under three irrigation regimes (FC; field capacity, 60% FC and 30% FC) in a greenhouse. Drought stress preferentially enhanced the activities of superoxide dismutase (SOD) and guaiacol peroxidase (POD) whereas it decreased catalase (CAT) activity. Licord with the highest level of enzyme activity under both optimum and limited irrigation regimes is reported as the most tolerant cultivar. Whereas Hyola 308 and Okapy, having the lowest enzymes activities, are mentioned as cultivars sensitive to drought stress. The native polyacrylamide gel electrophoresis (PAGE) analysis detected eight SOD isozymes. Oilseed rape leaves contained three isoforms of Mn-SOD and five isoforms of Cu/Zn-SOD. The expression of Mn-SOD was preferentially enhanced by drought stress. Five POD isoforms were detected in oilseed rape leaves. The intensities of POD-4 and -5 were enhanced under drought stress. According to the results, the appearance of new isozyme bands under drought stress conditions may be used as a biochemical marker to differentiate drought tolerant cultivars under drought stress.

Keywords: catalase; guaiacol peroxidase; isozymes; oilseed rape; superoxide dismutase; water stress

Drought, one of the environmental stresses, is the most significant factor restricting plant growth and crop productivity in the majority of agricultural fields of the world (Tas & Tas 2007). It inhibits the photosynthesis of plants, causes changes in chlorophyll contents and components and damage to the photosynthetic apparatus (Nayyar & Gupta 2006). In addition, it inhibits the photochemical activities and decreases the activities of enzymes in the Calvin Cycle in photosynthesis (Monakhova & Chernyadev 2002). Consequently, plants use some strategies to overcome this stress condition.

Abscisic acid (ABA) is central in the response to drought stress because it stimulates stomatal closure, thus reducing water loss, which limits CO_2 fixation and reduces $NADP^+$ regeneration by the Calvin Cycle. These adverse conditions increase the formation of reactive oxygen species (ROS) such as H_2O_2 (hydrogen peroxide), O_2^- (superoxide) and OH (hydroxyl) radicals, through enhanced leakage of electrons to molecular oxygen (Arora *et al.* 2002). ROS can act as second messengers involved in the stress signal transduction pathway (Chamnongpol *et al.* 1998), but excessive ROS production can cause oxidative stress, which damages plants by oxidizing photosynthetic pigments, membrane lipids, proteins and nucleic acids (Yordanov *et al.* 2000). To keep the levels of active oxygen species under control, plants have non-enzymatic and enzymatic antioxidant systems to protect cells from oxidative damage (Mittler 2002). Non-enzymatic antioxidants including β-carotenes, ascorbic acid (AA), α-tocopherol

(α-toc), reduced glutathione (GSH) and enzymes including: superoxide dismutase (SOD), guaiacol peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), polyphenol oxidase (PPO) and glutathione reductase (GR) (Xu *et al.* 2008). Superoxide dismutases (SODs), a group of metalloenzymes, are considered as the first defence against ROS, being responsible for the dismutation of O_2^- to H_2O_2 and O_2. CAT, APX, POD are enzymes that catalyze the conversion of H_2O_2 to water and O_2 (Gratao *et al.* 2005). The balance between ROS production and activities of antioxidative enzymes determines whether oxidative signalling and/or damage will occur (Moller *et al.* 2007). The capability of scavenging ROS and reducing their damaging effects may correlate with the drought tolerance of plants (Tsugane *et al.* 1999). Furthermore, the reactions of the plants to water stress differ significantly at various organizational levels depending upon intensity and duration of stress as well as plant species and its stage of development (Chaves *et al.* 2003; Jung 2004; Dacosta & Huang 2007). There are many reports in the literature that underline the intimate relationship between enhanced or constitutive antioxidant enzyme activities and increased resistance to environmental stresses in several plant species, such as rice (Guo *et al.* 2006), foxtail millet (Sreenivasulu *et al.* 2000), tomato (Mittova *et al.* 2000), sugar beet (Bor *et al.* 2003), wheat (Khanna-Chopra & Selote 2007) and barley (Acar *et al.* 2001).

According to annual precipitation many regions in Iran suffer from water deficit. Oilseed rape is one of the best crops for rotation with wheat; therefore, its culture has been increasing in Iran in recent years. According to this fact that the seed yield of *Brassica napus* and *B. juncea* was decreased due to drought stress, the understanding of the physiological and biochemical mechanisms conferring drought tolerance of these species is very important in terms of developing selection and breeding strategies.

Comparatively there is little information on the accumulation of the antioxidant enzymes and their role in the drought tolerance of oilseed rape. Therefore, the goal of the present study was to examine the effect of drought stress on the activities and isoenzyme profiles of antioxidant enzymes in this plant, by use of 10 commercial cultivars of oilseed rape in Iran. This research will provide documentation for breeding/selection of higher drought resistant oilseed rape in arid regions and acquisition of good information for future molecular research.

MATERIALS AND METHODS

Plant material and growth conditions (drought treatment)

Ten cultivars of oilseed rape (*Brassica napus* L.) cvs. Hyola 401, Hyola 308, PF, R.G.S, Option 500, Talaie, Licord, Okapy, Opera and Zarfam were used in this study. A factorial experiment in a completely randomized design with four replications was performed in a greenhouse of the Agricultural College of Shiraz University in Iran during 2005–2006. After surface sterilization the seeds were sown into plastic pots filled with 5 kg soil. Water stress treatment was started after 3 weeks of seedling growth in optimal conditions. Irrigation treatment was carried out based on pot weight and soil water content. For drought treatment, one group of 3-week-old plants was maintained under optimum irrigation (field capacity; FC) and the other groups were subjected to 60% FC and 30% FC. Drought treatment continued for 21 days, and then leaves were collected and frozen in liquid N_2 immediately and stored at –20°C before analysis.

Enzyme extraction

For protein and antioxidant enzyme assays, frozen leaves were ground to a fine powder with liquid nitrogen and were extracted with ice-cold 0.1M Tris-HCl buffer (pH 7.5) containing 5% (w/v) sucrose and 0.1% 2-mercaptoethanol (3:1 buffer volume/FW). The homogenate was centrifuged at 10 000 g for 20 min, at 4°C, and the supernatant was used for enzyme activity and protein determinations. Preparations for enzyme extraction and enzyme assay were carried out at 4°C.

Protein determination

The concentration of protein was determined by the method of Bradford (1976) using BSA as a standard.

Enzymes assay

SOD activity assay was based on the method of Dhindsa *et al.* (1980) which is based on the measurement of inhibition in the photochemical

reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm. The reaction mixture contained 50mM K-phosphate buffer (pH 7.8), 13mM methionine, 75μM NBT, 0.1μM EDTA, 4μM riboflavin and required amount of enzyme extract. The reaction was started by adding riboflavin and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal colour, served as control. A non-irradiated complete reaction mixture served as a blank. One 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, which was measured according to the method of Giannopolitis and Ries (1977).

POD activity was determined at 436 nm by its ability to convert guaiacol to tetraguaiacol (ε = 26.6mM^{-1} cm^{-1}) according to the method of Polle *et al.* (1994). The reaction mixture contained 100mM K-phosphate buffer (pH 7.0), 20.1mM guaiacol, 10mM H_2O_2 and enzyme extract. The increase in absorbance was recorded by the addition of H_2O_2 at 436 nm for 5 min.

CAT activity was determined by monitoring the disappearance of H_2O_2 at 240 nm (ε = 40mM^{-1} cm^{-1}) according to the method of Aebi (1984). The reaction mixture contained 50mM K-phosphate buffer (pH 7.0), 33mM H_2O_2 and enzyme extract.

Native polyacrylamide gel electrophoresis (native PAGE) and activity staining

Plant extracts containing equal amounts of protein were subjected to discontinuous PAGE under non-denaturing and non-reducing conditions essentially, as described by Laemmli (1970), except that SDS was omitted. Native PAGE of SOD and POD was performed on a 10% resolving gel and 5% stacking gel at 140 V and 4°C. After electrophoretic separation the activity staining for SOD isozymes was performed as reported by Beauchamp and Fridovich (1971). Gels were incubated in 2.5M NBT for 20 min followed by incubation in 50mM K-phosphate buffer (pH 7.8), containing 28mM riboflavin and 28mM N,N,N,N-tetramethylethylenediamine (TEMED) in darkness for another 20 min, and then exposed to a light box until the SOD activity bands became visible. The enzymes appeared as colourless bands in a purple background. The isoenzymes were identified and characterized by selective inhibition with KCN or H_2O_2. The gel was incubated for 20 min in 50mM K-phosphate buffer, pH 7.8, containing either 3mM KCN or 5mM H_2O_2 before staining for activity. Cu/ ZnSODs were inhibited by KCN and H_2O_2; FeSODs were resistant to KCN but were inactivated by H_2O_2; MnSODs were resistant to both inhibitors.

The POD gel activity was detected using the method of Graham *et al.* (1964). Gels were incubated in 50mM acetate buffer (pH 5), containing 5% O-dianisidine and 30% H_2O_2 in darkness, till the POD activity-containing band visualized carefully.

Statistical analysis

Data were subjected to Duncan's multiple range tests using the SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

RESULTS

Antioxidant enzyme activities

The effect of drought stress on the activities of antioxidant enzymes participating in the scavenging of ROS is shown in Table 1. The results revealed an increase in SOD and POD activities and a decrease in CAT activity in leaves of oilseed rape under drought treatment.

A gradual increase was observed in SOD and POD activities under 60% FC in comparison with the control (10% and 6%, respectively). A significant increase ($P < 0.01$) was observed in SOD and POD activities under 30% FC (110% and 148%, respectively) when compared to the control. The interaction effect of cultivar × drought treatments was highly significant ($P < 0.01$) for SOD, POD and CAT (Figure 1). The maximum increase in the SOD activities was observed in the cultivars Licord and Zarfam (3.6 fold and 2.8 fold, respectively) while the minimum increase was indicated in the cultivar Hyola 308 (1.7 fold) compared to the control. Among the cultivars, Okapy showed a decrease in SOD activity at 30% FC (Figure 1A).

The cultivars Talaie and Opera showed the maximum increase in POD activity (6.7 fold and 5 fold, respectively). The cultivars Okapy and Hyola 308 showed a decrease in POD activity under 30% FC (Figure 1C).

Table 1. Effect of drought treatments on antioxidant enzymes of the leaves of *Brassica napus* cultivars (pooled data for all ten cultivars)

Drought treatment	SOD activity	CAT activity	POD activity
	unit/mg protein		
FC	3.13 ± 2.22[c]	2.91 ± 1.76[a]	1.57 ± 1.16[c]
60% FC	3.44 ± 2.25[b]	1.12 ± 5.02[b]	1.67 ± 1.27[b]
30% FC	6.60 ± 5.83[a]	0.02 ± 1.20[c]	3.90 ± 2.19[a]
CV	11.64	12.83	6.69

Data are the means ± SE of three different experiments with three replicated measurements; different letters within columns indicate significant differences ($P < 0.05$) according to Duncan's test; *CV* – coefficient of variation
SOD – superoxide dismutase; POD – guaiacol peroxidase; CAT – catalase

The cultivar analysis of total CAT activity indicated a reduction under drought stress except for the cultivars Licord and Zarfam, which had an increase in CAT activity (1.9 fold and 1.5 fold, respectively) (Figure 1B).

SOD and POD isozymes

After the native polyacrylamide gel electrophoresis (PAGE) analysis, eight SOD isozymes were detected (Figure 2A, B). The SOD bands were classified

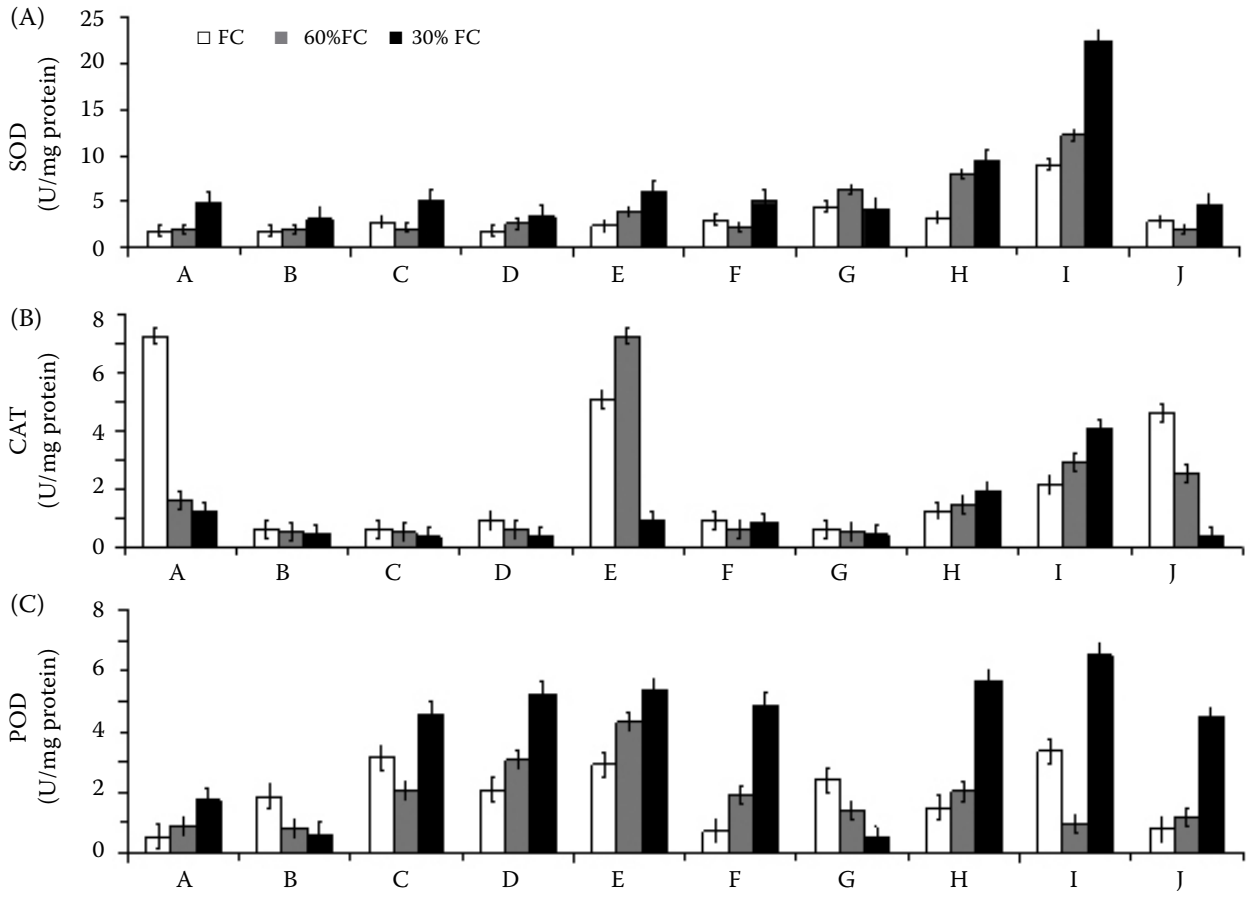

Figure 1. Activity of antioxidant enzymes: (A) superoxide dismutase (SOD), (B) guaiacol peroxidase (POD), and (C) catalase (CAT) in the leaves of ten oilseed rape cultivars subjected to drought stress treatments (FC, 60%FC and 30% FC)

A – Hyola 401; B – Hyola 308; C – PF; D – R.G.S; E – Option 500; F – Talaye; G – Okapy; H – Zarfam; I – Licord; J – Opera
Vertical bars indicate ± SE of three replications

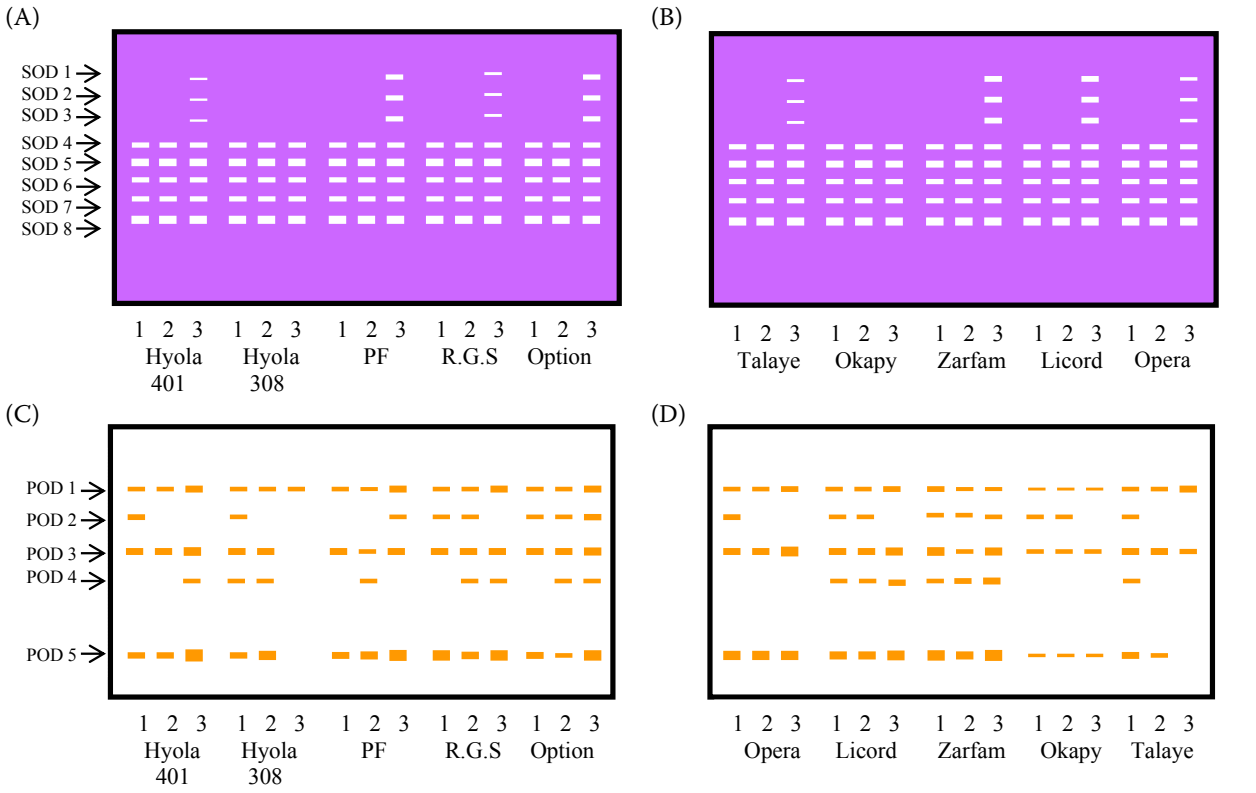

Figure 2. Effect of drought stress (vertical lanes 1, 2 and 3 represent FC, 60%FC and 30%FC, respectively) on isozyme patterns of SOD (A and B) and POD (C and D) in oilseed rape cultivars

according to their metal co-factor based on their inhibitory pattern to hydrogen peroxide and cyanide. SOD1 to SOD3 were identified as Mn-SOD by their insensitivity to KCN and H_2O_2, whereas SOD4 to SOD8 were inhibited by both KCN and H_2O_2, suggesting it represented Cu/Zn-SOD activity. The Fe-SOD isoform was not observed in the native gels (data not shown). The overall increase in total SOD activities in drought-stressed oilseed rape plants seemed to be mainly due to the appearance of Mn-SOD isozymes. The assessment of the POD isozyme profiles on 10% native polyacrylamide gel revealed the presence of five isoforms in oilseed rape plants under drought stress conditions (Figure 2C, D). The increase of band intensity and appearance of new bands may be an indication of an increase in POD activity under drought conditions.

DISCUSSION

Water stress is inevitably associated with increased oxidative stress due to enhanced accumulation of ROS, particularly O_2^- and H_2O_2 in chloroplasts, mitochondria, and peroxisomes. As a result, the induction of antioxidant enzyme activities is a general adaptation strategy which plants use to overcome oxidative stresses (Foyer & Noctor 2003).

The ability of plants to overcome oxidative stress partly relies on the induction of SOD activity and subsequently on the upregulation of other downstream antioxidant enzymes (Alscher *et al.* 2002). In our experiment, the results showed significantly enhanced SOD activity in seedlings exposed to water stress (Table 1). According to this fact that SOD processing is known to be substrate-inducible (Tsang *et al.* 1991), an increase in the SOD activity may be attributed to the increased production of active oxygen species as substrate that lead to increased expression of genes encoding SOD. Our results are consistent with other studies reporting the increased SOD activity in response to drought stress in sunflower (Gunes *et al.* 2008), poplar (Xiao *et al.* 2008), cowpea (Manivannan *et al.* 2007), liquorice (Pan *et al.* 2006), wheat (Bakalova *et al.* 2004; Csiszar *et al.* 2005) and pea (Malecka *et al.* 2001).

According to our results, the maximum increase in the SOD activity was observed in the Licord

and Zarfam cultivars, which might lead to their higher protection against water deficit. However, the Okapy showed a significant decrease in SOD activity at 30% FC (Figure 2), this may be related to the low potential of this cultivar to remove O_2^- under water deficit.

H_2O_2, which resulted from the action of SOD, is toxic to cells. Therefore, it is important that H_2O_2 be scavenged rapidly by the antioxidative defence system to water and oxygen (Guo *et al.* 2006). The overexpression of SOD, if accompanied by enhanced H_2O_2 scavenging mechanisms like CAT and POD enzyme activities, has been considered as an important antidrought mechanism to cope with oxidative stress during water deficit conditions (McKersie *et al.* 1999).

The present study indicates a significant increase in POD activity in oilseed rape plants under drought stress (Table 1). Some previous studies, as parallel with our results, reported the increased POD activity under drought stress conditions in various plants, like sunflower (Gunes *et al.* 2008), poplar (Xiao *et al.* 2008), liquorice (Pan *et al.* 2006), brassica species (Das & Uprety 2006), wheat (Csiszar *et al.* 2005).

Like the present results, SOD and POD in general show simultaneous induction and decline, which may be due to their co-regulation (Shigeoka *et al.* 2002). The important point here is a decrease in POD activity in Okapy and H.308 cultivars under severe drought stress (Figure 1B), which may reflect the low ROS scavenging capacity and increased damage in these cultivars under this condition.

In terms of our results, although the activities of SOD and POD were up-regulated by drought stress, CAT activity decreased in all experimental plant cultivars, except in Licord and Zarfam (Figure 1). The decline in CAT activity is regarded as a general response to many stresses (Herbinger *et al.* 2002; Bakalova *et al.* 2004; Jung 2004; Guo *et al.* 2006; Pan *et al.* 2006; Gunes *et al.* 2008; Liu *et al.* 2008). The reduction of CAT activity is supposedly due to the inhibition of enzyme synthesis or change in the assembly of enzyme subunits under stress conditions. It may also be associated with degradation caused by induced peroxisomal proteases or may be due to the photo-inactivation of the enzyme.

All in all, the increase in POD might be considered as a key point for the decomposition of H_2O_2, especially under CAT inactivation.

According to the results, we observed higher constitutive activity of SOD and POD and enhanced activity of SOD, CAT and POD in Licord under water stress (Figure 1). As a consequence, Licord may have a better protection mechanism against oxidative damage under water stress by maintaining the higher constitutive and induced activity of antioxidant enzymes, compared with other oilseed rape cultivars especially the sensitive ones, Okapy and Hyola 308.

Changes in the subcellular distribution of antioxidant enzyme activities, along with different isoenzyme sensitivity, might result in a more efficient protection strategy than an increase in the enzyme activity (Foyer *et al.* 1994). The analysis of individual SOD isozymes is important, because it can help to understand how each stress may affect the different subcellular compartments. While Cu/ZnSOD isozymes – the most abundant SOD in higher plants – are localized in both cytosol and chloroplasts, MnSOD is found in mitochondria and glyoxisomes, and FeSOD is located in chloroplasts and peroxisomes (Bowler *et al.* 1992).

The number of isoenzymes of each type of SOD varies greatly from plant to plant (Gratao *et al.* 2005). After the native polyacrylamide gel electrophoresis (PAGE) analysis, we were able to identify and classify up to eight distinct SOD isoenzymes in oilseed rape plants (Figure 2A, B). A remarkable increase in total SOD activities can be ascribed to a drought-induced increase in the activities of Mn-SOD and Cu/Zn-SOD, particularly the Mn-SOD isoform proved to be the most responsive reaction to drought (Figure 2A, B). The high activity of the Mn-SOD isoform during drought stress could be related to O_2^- generation by the mitochondrial electron transport chain. Our results suggest that mitochondrial and cytosolic compartments are crucial in the SOD enzyme protection against superoxide formation when plants deal with drought stress.

After the native polyacrylamide gel electrophoresis (PAGE) analysis, we identified up to five POD distinct isoenzymes in oilseed rape plants. The drought-stressed leaves were highly capable of increasing the number and intensity of POD isoforms. This could be considered as a response to drought-induced oxidative damage, suggesting the enzymatic removal of H_2O_2 by POD (Figure 2C, D).

In conclusion, the results described here confirm that the antioxidant defence capacity and the increase of individual enzymatic activities during

stress were thereby dependent on plant genotype. In other words, different oilseed rape genotypes clearly responded differently to soil water deficiency in terms of the activities of POD, SOD and CAT content. These results can be used as practical biochemical parameters for selection of drought tolerant oilseed rape genotypes when selecting drought tolerant cultivars for breeding in arid regions. In addition, Licord with a high level of antioxidant enzyme activities which are related to its capacity for better protection mechanisms against oxidative damage could be introduced to farmers as a drought tolerant cultivar to plant in arid regions.

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Received for publication September 28, 2009
Accepted after corrections February 3, 2010

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