Ann. For. Sci. 63 (2006) 323–327
© INRA, EDP Sciences, 2006
DOI: 10.1051/forest:2006012

Original article

Impact of drought and leaf development stage on enzymatic antioxidant system of two *Populus deltoides* × *nigra* clones

Nicolas MARRON[a,b], Stéphane MAURY[a], Cécile RINALDI[a,c], Franck BRIGNOLAS[a]*

[a] Laboratoire de Biologie des Ligneux et des Grandes Cultures, UPRES EA 1207, UFR-Faculté des Sciences, Université d'Orléans, rue de Chartres, BP 6759, 45067 Orléans Cedex 02, France
[b] Present address: University of Antwerp (UA), Department of Biology, Campus Drie Eiken, Research Group of Plant and Vegetation Ecology, Universiteitsplein 1, 2610, Wilrijk, Belgium
[c] Present address: UMR INRA/UHP 1136 Interaction Arbres/Microorganismes, INRA, Centre de Recherches de Nancy, 54280 Champenoux, France

(Received 14 March 2005; accepted 12 September 2005)

Abstract – Impacts of mild and severe water constraints were investigated on leaf protein content and activities of superoxide dismutase (SOD), catalase and peroxidase in young cuttings of two *Populus deltoides* × *nigra* clones, 'Luisa_Avanzo' and 'Dorskamp', known to differ in their level of drought tolerance. Expanding and mature leaves were analyzed separately. The effect of water deficit on enzymatic antioxidant system depended on both clone and leaf development stage. For 'Dorskamp', which presents an higher drought tolerance than 'Luisa_Avanzo', activities of SOD and catalase increased in response to the severe water deficit in mature leaves only. For 'Luisa_Avanzo', peroxidase activity increased in response to the mild water deficit in expanding leaves merely. For both clones, three different SOD isoforms, Mn-SOD, Fe-SOD and Cu/Zn-SOD were detected in various amounts depending on drought intensity.

water deficit / leaf development stage / catalase / superoxide dismutase / peroxidase

Résumé – Impact de la sécheresse et du stade de développement des feuilles sur les systèmes antioxydants enzymatiques de deux clones de *Populus deltoides* × *nigra*. L'impact de sécheresses modérée et sévère sur le contenu en protéines des feuilles et sur les activités de la dismutase de superoxyde (SOD), de la catalase et de la peroxydase a été étudié chez de jeunes boutures de deux clones de *Populus deltoides* × *nigra*, 'Luisa_Avanzo' et 'Dorskamp', connus pour présenter des niveaux différents de tolérance au déficit hydrique. Les feuilles en croissance et matures ont été analysées séparément. La réponse à la sécheresse des systèmes de protection enzymatiques était différente selon le clone et le stade de développement des feuilles étudiées. Pour le clone 'Dorskamp', réputé plus tolérant que 'Luisa_Avanzo' à la sécheresse, les activités de la SOD et de la catalase augmentaient dans les feuilles matures en réponse à un déficit hydrique sévère. Pour le clone 'Luisa_Avanzo', l'activité de la peroxydase augmentait essentiellement dans les feuilles en croissance en réponse à une sécheresse modérée. Pour les deux clones, trois isoformes différentes de la SOD, la Mn-SOD, la Fe-SOD et la Cu/Zn-SOD ont été détectées en quantités variables chez les deux clones en fonction de l'intensité de la contrainte hydrique.

déficit hydrique / stade de développement foliaire / catalase / dismutase de superoxyde / peroxydase

1. INTRODUCTION

Poplars (*Populus* L.) are the fastest growing trees in North America and Europe. However, their productivity is closely linked to water availability [21]. Even if poplars are among the most susceptible woody plants to drought, a large clonal variation in drought resistance within *Populus* species and hybrids has been described [10, 11]. A large number of responses occurs in trees under drought conditions, thus it is difficult to determine the mechanisms that contribute to explain diversity in drought tolerance among poplar clones [21].

One of the earliest plant responses to drought is stomatal closure that reduces water losses but also the availability of CO_2 for photosynthesis. Limitation of CO_2 fixation provides an insufficient sink for electrons generated in the Electron-Transport-Chains (ETC) involving decreased NADPH utilization and over-reduction of the ETC. In this case, alternative outlets for electrons gain in importance and lead to over production of reactive oxygen species (ROS) and to oxidative damages [5]. Under such conditions, oxygen acts as an alternate accepter of electrons resulting first in the production of the superoxide radical ($O_2^{\bullet -}$), and then in the formation of various reactive oxygen species such as the hydroxyl free radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) [5]. Reactive oxygen species are highly toxic and can cause lipid peroxidation and consequently membrane injury, protein degradation, enzyme inactivation, pigment bleaching and disruption of DNA strands [19]. Allen (1995) [1] reported that much of the injury to plants caused by exposure to various constraints is associated with oxidative damage at the cellular level. Plant cells are normally protected against the detrimental effects of reactive oxygen by a complex antioxidant system; active oxy-free radicals can be scavenged by both

* Corresponding author: franck.brignolas@univ-orleans.fr

enzymatic, such as superoxide dismutase (SOD), ascorbate peroxidase, peroxidase, glutathione reductase, and catalase, and non enzymatic detoxification mechanisms, such as glutathione, ascorbic acid, α-tocopherol, carotenoids, and phenolic compounds [5, 19]. Oxidative stress can occur when the scavenging of reactive oxygen species is overwhelmed by the production. Hence, mechanisms that reduce oxidative stress, such as modulation of the activities of these enzymes, could contribute to explain diversity in drought tolerance [1, 5]. In drought adapted herbaceous species, increase in activities of antioxidant enzymes, such as SOD, catalase, and peroxidase, has been observed [6, 8]. In trees, it has been shown that protection against oxidative stress generated by elevated CO_2, paraquat and ozone mainly involved SOD, catalase and peroxidase [3, 18, 20].

Two *Populus deltoides* × *nigra* clones, 'Dorskamp' and 'Luisa_Avanzo', have been selected for their differences in drought tolerance levels based on field and greenhouse observations. For similar limitation of water availability, growth of 'Dorskamp' was less affected than 'Luisa_Avanzo' one [11]. In response to re-watering, 'Dorskamp' only displayed the ability to recover a similar level of biomass production than well watered plants [11]. In this context, we have previously shown that non enzymatic antioxidant activity in leaves of the two clones decreased in response to water deficit, suggesting a limited participation of this class of molecules during drought [10]. The objective of the present investigation was to focus on the response of some of the leaf enzymatic antioxidant system (SOD, catalase and peroxidase) in order to try to answer the following question: do differences in drought tolerance being related to differences in enzymatic antioxidant systems? Isoforms of superoxide dismutase (SOD) have been studied because SOD response represents the first line of defense against reactive oxygen species [2, 9]. Leaf development stage and drought intensity were taken into account by analyzing separately growing and recently mature leaves under mild and severe water deficits.

2. MATERIALS AND METHODS

2.1. Plant material and drought treatment

Three-month-old 20-cm woody stem cuttings, from 2-year-old stems of *Populus deltoides* (Bartr.) Marsh. × *P. nigra* L. cv 'Dorskamp' [male] and 'Luisa_Avanzo' [female], were used in all experiments. During January 2001, 24 one-month-old rooted cuttings of each clone were repotted into 4-l pots containing a mixture of blond peat, brown peat, horse manure, heather and bromide-disinfected compost (25:25:20:20:10, v/v, pH 5.8) (Falienor, Vivy, France). Cuttings were grown in a greenhouse heated to 20 °C and exposed to natural daylight.

In April 2001, water constraint was induced by withholding water from 12 cuttings per clone. Leaves of 6 control and 6 water-stressed cuttings of each clone were collected (i) at the onset of stomatal closure (mild water deficit) and (ii) three days later (severe water deficit). Predawn leaf water potential (Ψ_{wp}; MPa) was measured with a pressure chamber on a mature leaf. Leaves were numbered from the top to the bottom of each cutting (i.e., Foliar Index, FI) and two leaves per cutting, belonging to distinct development stages, i.e. growing leaves (FI = 3.12 ± 0.16) and recently mature leaves (FI = 10.40 ± 0.61), were collected, frozen in liquid nitrogen, and kept at –80 °C until analyzed.

2.2. Extraction of enzymes and protein content

Frozen leaves (0.4 g) were ground in liquid nitrogen to a fine powder with a mortar and pestle. Powdered material was transferred into 2 mL of extraction buffer containing 50 mM potassium phosphate buffer (adjusted to pH 7.8), containing 100 mM EthylenDiamine-Tetraacetic Acid (EDTA), 0.4% (v/v) Triton X-100 and 400 mg insoluble polyvinyl-polypyrrolidone. This mixture was centrifuged at 14 000 *g* for 15 min at 4 °C. The supernatant was then collected for the determination of soluble protein content and enzymes activities. Protein content was determined with Bio-Rad Protein Assay reagent (Bio-Rad, France).

2.3. Antioxidant enzyme activities

For SOD activity (EC 1.15.1.1) assessment, the reduction of nitro blue tetrazolium (NBT) in formazan blue, by the anion $O_2^{\bullet -}$ produced by the xanthine/xanthine oxidase system, was measured by the decline in absorbance at 560 nm for 8 min (adapted from [7]). One SOD unit was taken as the amount of extract that gave 50% inhibition of reduction of tetrazolium blue. SOD isozymes were separated on non-denaturating (10%, v/v) polyacrylamide gel electrophoresis [12]. SOD isozymes were localized on the gels by the method of NBT reduction by superoxide radicals generated by riboflavin. Mn-SOD, Fe-SOD and Cu/Zn-SOD, were identified using specific inhibitors. Thus, before staining, zymograms were incubated at 25 °C for 45 min, separately, in solutions of 20 mM H_2O_2, 100 mM KCN, or 10 mM EDTA. The gels were covered with a solution containing nitro blue tetrazolium (0.25 mM NBT) and riboflavin (0.3 mM), and exposed to light. SOD activity in gels was visualized as achromatic bands after staining with NBT. The gels were pictured (PDR-M65 digital still camera, Toshiba) and the SOD activity was quantified using imaging software (Image-Tool for Windows version 3.00). For catalase activity (EC 1.11.1.6), the decomposition of H_2O_2 was measured by the decline in absorbance at 240 nm for 20 min (adapted from [6]). For peroxidase activity (EC 1.11.1.7) the oxidation of guaiacol was measured by the increase in absorbance at 420 nm during 100 seconds (adapted from [6]). All methods were adapted for microplate spectrophotometer (*μQuant*, supported with KC4 V3.0 software, BIO-TEK, USA). Six plants per clone and per treatment were analyzed and three replicates of each assay were realized.

2.4. Statistical analyses

Data management and statistical analyses were performed with SPSS software (SPSS, Chicago, IL, USA). Means are expressed with their standard error and were compared by two-way ANOVA (clone and treatment) with leaf development stage as covariate. All statistical tests were considered significant at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

In control conditions ($\Psi_{wp} > -0.59$ MPa), protein content, SOD, catalase and peroxidase activities of expanding leaves did not differ between both clones (Fig. 1). In contrast, mature leaves of 'Dorskamp' displayed a lower protein content than 'Luisa_Avanzo' ones, but exhibited a higher total SOD activity. Comparison of expanding and mature leaves revealed marked differences for 'Luisa_Avanzo' only, with a higher protein content and lower SOD and peroxidase activities for mature leaves than for expanding ones (Figs. 1D, 1F and 1J). Increase in the production of reactive oxygen species (ROS) with leaf ageing is a well-known phenomenon [15] and is

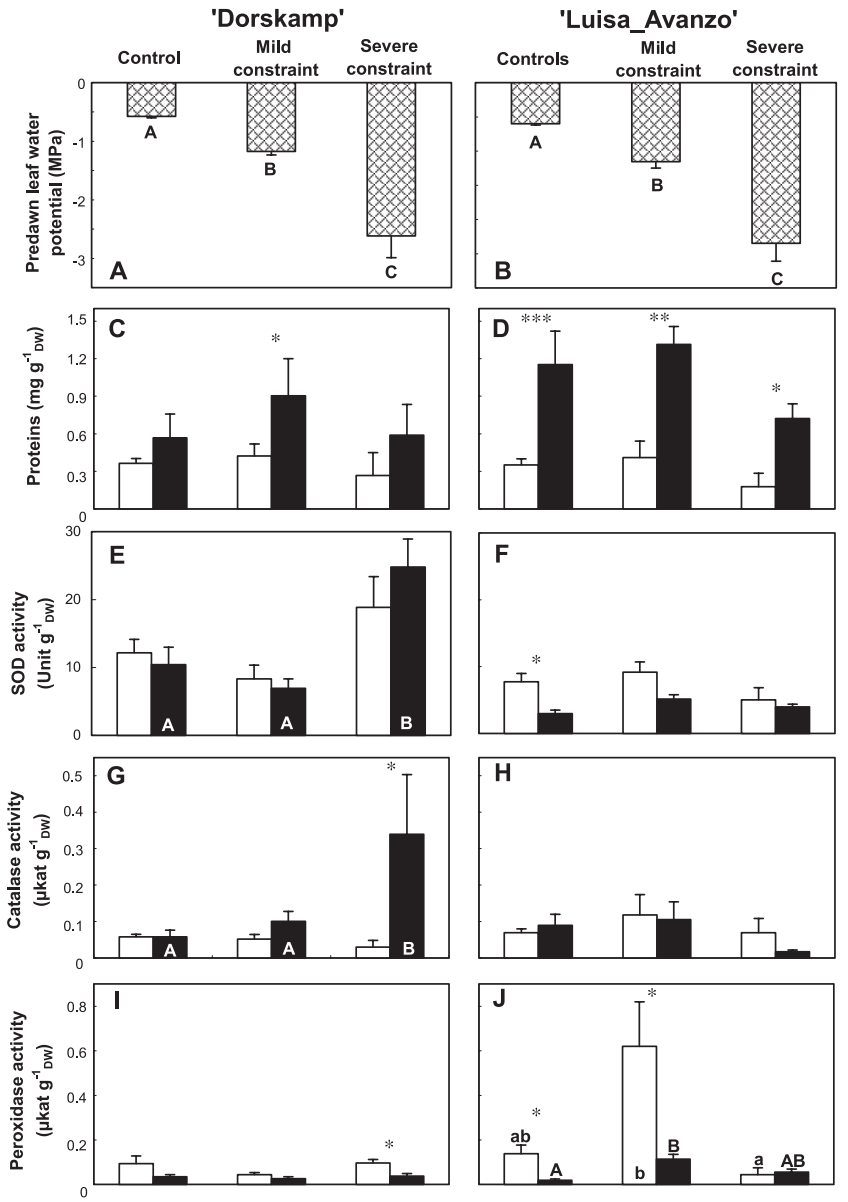

Figure 1. Predawn leaf water potential (Ψ_{wp}) of cuttings (**A** and **B**), and protein content (**C** and **D**), superoxide dismutase (SOD) activity (**E** and **F**), catalase activity (**G** and **H**), and peroxidase activity (**I** and **J**) of expanding (white) and recently mature leaves (black) of clones 'Dorskamp' (**A**, **C**, **E**, **G**, and **I**) and 'Luisa_Avanzo' (**B**, **D**, **F**, **H**, and **J**). Means (± SE), $n = 6$ plants for Ψ_{wp}, and $n = 6$ leaves for protein content and enzyme activities. Three replicates of each assay were realized. Significant differences between leaf ages are indicated by asterisks: * for $P \leq 0.05$, ** for $P \leq 0.01$, and *** for $P \leq 0.001$. Significant differences between water treatments are symbolized by different letters (from panels **C** to **J**, small letters for expanding leaves and capital letters for recently mature ones).

followed by a decrease of some of the antioxidant enzymatic activities in the case of 'Luisa_Avanzo' only.

Both clones were subjected to similar mild ($\Psi_{wp} = -1.17 \pm 0.05$ MPa) and severe ($\Psi_{wp} = -2.52 \pm 0.25$ MPa) water deficits (Figs. 1A and 1B). Protein content and enzyme activities of expanding leaves were not significantly affected by water deficit, except a significant increase of peroxidase activity in the case of mild water deficit for 'Luisa_Avanzo' ones (Fig. 1J). SOD and catalase activities of mature leaves increased significantly in response to the severe water deficit for 'Dorskamp' (Figs. 1E and G) while an increase of peroxidase activity and a slight decrease of protein content were observed for 'Luisa_Avanzo' in response to the mild and severe drought, respectively. (Figs. 1D and J). Thus, reaction to drought was clone, leaf age- and drought intensity-dependant: SOD and catalase activities were stimulated during severe drought in the mature leaves of the tolerant clone 'Dorskamp', while peroxidase seemed favored during mild drought in the expanding leaves of the more susceptible clone 'Luisa_Avanzo'. Stimulation of the antioxidant enzymatic activities of the Halliwell-Asada pathway has commonly been observed in response to drought [14, 16]. Nevertheless, the implication of these enzymes under drought conditions has been shown to be diverse according to species and/or to drought intensity [18]. Thus, increases, decreases as well as no change have been reported for the activities of H_2O_2-consumming enzymes, peroxidase and catalase, under drought according to the considered species [22, 25, 26]. For wheat and sorghum, SOD activity increases under moderate water deficit

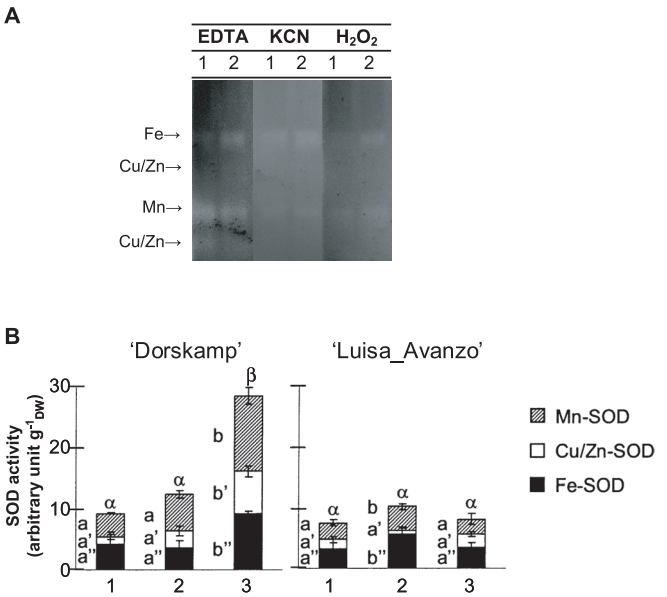

Figure 2. (**A**) Determination of superoxide dismutase (SOD) isoforms in poplar. One example of SOD zymogram corresponding to two replicates (1 and 2) for recently mature leaves of control cuttings of clone 'Luisa_Avanzo' with EDTA, KCN, or H_2O_2 is shown. (**B**) Relative contents of the three SOD isoforms, Fe-SOD, Cu/Zn-SOD, and Mn-SOD, in recently mature leaves of 'Dorskamp' and 'Luisa_Avanzo' under: (1), control (Ψ_{wp} = –0.59 ± 0.02 MPa); (2), mild water deficit (Ψ_{wp} = –1.17 ± 0.05 MPa), and (3), severe water deficit (Ψ_{wp} = –2.52 ± 0.25 MPa) conditions. Three replicates were realized. For total SOD activity of each clone, significant differences between treatments ($P \leq 0.05$) are indicated by different Greek letters (α and β). For each clone and each SOD isoform, significant differences between treatments ($P \leq 0.05$) are indicated by different small letters (a and b for Mn-SOD, a' and b' for Cu/Zn-SOD, a'' and b'' for Fe-SOD).

intensities and then stabilizes or decreases when constraint accentuates, while for rice, it decreases with osmotic constraint [17, 25, 26]. For bean and maize, increases in SOD activity were observed in drought-tolerant cultivars in response to drought, while no change was observed for drought-susceptible cultivars [8, 22].

SOD is a major scavenging enzyme acting as the first line of defense, and several isozymes have already been described and correspond to distinct subcellular localization [12, 23]: Cu/Zn-SOD is located in cytosol, peroxisome, and chloroplast, Mn-SOD in mitochondria and Fe-SOD in chloroplast. Due to the important increase of total SOD activity for the mature leaves of 'Dorskamp', protein electrophoresis and zymograms were realized from mature leaves of both clones. For the two clones, use of inhibitors allowed identification of one Fe-SOD, one Mn-SOD and two Cu/Zn-SOD (Fig. 2A). In control conditions, main isoforms were Fe-SOD and Mn-SOD for both clones (Figs. 2A and 2B). In response to water deficit, differential significant increases of isoforms were observed for both clones (Fig. 2B). The maximum SOD activities were reached for the three SOD-isoforms during the severe water deficit ($\Psi_{wp} < -2.5$ MPa) for 'Dorskamp' and for the chloroplastic Fe- and mitochondrial Mn-SOD only during the moderate water deficit ($\Psi_{wp} \approx -1$ MPa) for 'Luisa_Avanzo', in agreement with above results for total SOD activities (Figs. 1E and 1F). This observation fits well with chloroplasts and mitochondria as major sources of ROS in plants [6]. Such results, obtained on woody plants, are in agreement with previous publications for numerous herbaceous species where enhancement of these three isoforms was related to the level of drought tolerance [4, 12, 24]; these latter results have been confirmed by a transgenic approach [6, 13].

In conclusion, our results revealed that both clones did not present the same level of SOD activity in control conditions. Moreover, clonal differences in the nature of the stimulated enzymes as well as in the drought intensity at which the enzymes or isoenzymes are stimulated for a defined leaf development stage have also been shown. These results are in agreement with the respective levels of drought tolerance that have been previously reported for both poplar clones. Due to its early intervention within the Halliwell-Asada pathway and the particularly important toxicity of its substrate and derivative, respectively superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), the ability to stimulate SOD activity, SOD-isoenzymes in combination with one H_2O_2-consumming enzyme such as catalase seems to represent an advantage under drought conditions. This suggests that differences in drought tolerance could be related to differences in enzymatic antioxidant systems.

Acknowledgements: The authors thank R. Bénardeau, A. Delaunay and G. Moreau for technical assistance. N. Marron was supported by a Ph.D. grant from the Conseil Régional Région Centre, France.

REFERENCES

[1] Allen R.D., Dissection of oxidative stress tolerance using transgenic plants, Plant Physiol. 107 (1995) 1049–1054.

[2] Alscher R.G., Erturk N., Heath L.S., Role of superoxide dismutase (SODs) in controlling oxidative stress in plants, J. Exp. Bot. 53 (2002) 1331–1341.

[3] Arisi A.-C., Cornic G., Jouanin L., Foyer C.H., Overexpression of FeSOD in transformed poplar modifies the regulation of photosynthesis at low CO_2 partial pressures or following exposure to the prooxidant herbicide methyl viologen, Plant Physiol. 117 (1998) 565–574.

[4] Borsani O., Díaz P., Agius M.F., Valpuesta V., Monza J., Water stress generates an oxidative stress through the induction of a specific Cu/Zn superoxide dismutase in *Lotus corniculatus* leaves, Plant Sci. 161 (2001) 757–753.

[5] Edreva A., Generation and scavenging of reactive oxygen species in chloroplasts: a submolecular approach, Agr. Ecosyst. Environ. 106 (2005) 119–133.

[6] Fu J., Huang B., Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress, Environ. Exp. Bot. 45 (2001) 105–114.

[7] Hagège D., Kevers C., Salabert P., Gaspar T., Protective systems against activated oxygen species compared in normal and fully habituated non-organogenic sugarbeet calluses, In Vitro Cell Dev. Pl. 28 (1992) 143–147.

[8] Jagtap V., Bhargava S., Variation in the antioxidant metabolism of drought tolerant and drought susceptible varieties of *Sorghum bicolor* (L.) Moench. exposed to high light, low water and high temperature stress, J. Plant Physiol. 145 (1995) 195–197.

[9] Kliebenstein D.J., Monde R.-A., Last R.L., Superoxide dismutase in *Arabidopsis*: an eclectic enzyme family with disparate regulation and protein localization, Plant Physiol. 118 (1998) 637–650.

[10] Marron N., Delay D., Petit J.-M., Dreyer E., Kahlem G., Delmotte F.M., Brignolas F., Physiological traits of two *Populus* × *euramericana*, Luisa Avanzo and Dorskamp, during a water stress and rewatering cycle, Tree Physiol. 22 (2002) 849–858.

[11] Marron N., Dreyer E., Boudouresque E., Delay D., Petit J.-M., Delmotte F.M., Brignolas F., Impact of successive drought and rewatering cycles on growth and specific leaf area of two *Populus* × *canadensis* (Moench) clones, 'Dorskamp' and 'Luisa_Avanzo', Tree Physiol. 23 (2003) 1225–1235.

[12] Martinez C.A., Loureiro M.E., Oliva M.A., Maestri M., Differential responses of superoxide dismutase in freezing resistant *Solanum curtilobum* and freezing sensitive *Solanum tuberosum* subjected to oxidative and water stress, Plant Sci. 160 (2001) 505–515.

[13] McKersie B.D., Bowley S.R., Harjanto E., Leprince O., Water-deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase, Plant Physiol. 111 (1996) 1177–1181.

[14] Mittler R., Zilinskas B.A., Regulation of pea cytosolic ascorbate peroxidase and other antioxidant enzymes during the progression of drought stress and following recovery from drought, Plant J. 5 (1994) 397–405.

[15] Pastori G.M., del Rio L.A., Natural senescence of pea leaves: an activated oxygen-mediated function for peroxisomes, Plant Physiol. 113 (1997) 411–418.

[16] Perl-Treves R., Galun E., The tomato Cu/ Zn-superoxide dismutase genes are developmentally regulated and respond to light and stress, Plant Mol. Biol. 17 (1991) 745–760.

[17] Reddy P.C., Vajranabhaiah S.N., Drought induced lipid peroxidation: defensive mechanism in upland rice (*Oryza sativa* L.) seeds during germination, Adv. Plant Sci. 6 (1993) 229–236.

[18] Schwanz P., Polle A., Differential stress responses of antioxidative systems to drought in pendunculate oak (*Quercus robur*) and maritime pine (*Pinus pinaster*) grown under high CO_2 concentrations, J. Exp. Bot. 52 (2001) 133–143.

[19] Smirnoff N., The role of active oxygen in the response of plants to water deficit and desiccation, New Phytol. 125 (1993) 27–58.

[20] Tandy N.E., Di Giulio R.T., Richardson C.J., Assay and electrophoresis of superoxide dismutase from red spruce (*Picea rubens* Sarg.), loblolly pine (*Pinus taeda* L.), and scotch pine (*Pinus sylvestris* L.), Plant Physiol. 90 (1989) 742–748.

[21] Tschaplinski T.J. Tuskan G.A., Gebre G.M., Todd D.E., Drought resistance of two hybrid *Populus* clones grown in a large-scale plantation, Tree Physiol. 18 (1998) 653–658.

[22] Türkan I., Bor M., Özdemir F., Koca H., Differential responses of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress, Plant Sci. 168 (2005) 223–231.

[23] Wingsle G., Gardeström P., Hällgren J.-E., Karpinski S., Isolation, purification, and subcellular localization of isozymes of superoxide dismutase from Scots pine (*Pinus sylvestris* L.) needles, Plant Physiol. 95 (1991) 21–28.

[24] Wu G., Wilen R.W., Robertson A.J., Gusta L.V., Isolation, chromosomal localization, and differential expression of mitochondrial manganese superoxide dismutase and chloroplastic copper/zinc superoxide dismutase genes in wheat, Plant Physiol. 120 (1999) 513–520.

[25] Zhang J., Cui S., Li J., Kirkham M.B., Protoplasmic factors, antioxidant responses, and chilling resistance in maize, Plant Physiol. Biochem. 33 (1995) 567–575.

[26] Zhang J., Kirkham M.B., Antioxidant responses to drought in sunflower and sorghum seedlings, New Phytol. 132 (1996) 361–373.