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Full Length Research Paper

Water stress induces overexpression of superoxide dismutases that contribute to the protection of cowpea plants against oxidative stress

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Water stress is known to induce active oxygen species in plants. The accumulation of these harmful species must be prevented by plants as rapidly as possible to maintain growth and productivity. The aim of this study was to determine the effect of water stress on superoxide dismutase isozymes (SOD, EC 1.15.1.1.) in two cowpea cultivars [*Vigna unguiculata* L. Walp., cv. Bambey 21 (B21) and cv. TN88-63]. Plants were submitted to water stress by withholding water supply and the expression of SOD was characterized during stress induction. In the same time, photosynthesis characteristics were determined through the measurement of the quantum yield of PS II photochemistry and the energy absorption rate per reaction centre. Results show how water stress regulates the synthesis and the activity of superoxide dismutase isoforms and how these enzymes contribute to protect photosynthesis against the damageable effects of superoxide radicals in cowpea. Increased MnSOD and FeSOD activity and concentration were shown to be induced by water stress and associated with protection of photosystem II photochemistry and whole plant growth against oxidative stress in these plants. On the contrary, plants unable to express high MnSOD and/or FeSOD isoforms showed more sensitivity to water stress.

Key words: Oxidative stress, superoxide dismutase, photochemistry, *Vigna unguiculata*, water stress.

INTRODUCTION

The most feared and widespread plant stress agents are active oxygen species. These include redox intermediates in the reduction and oxidation between dioxygen and water; superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$), and the electronically-excited oxygen species, singlet excited oxygen ($^1O_2$). These species are able to react with DNA, lipids, proteins, and almost any other constituent of plant or animal cells (Beauchamp and Fridovich, 1971; Demming and Björkman, 1987; Halliwell and Gutteridge, 1989). These reduced oxygen species are not only generated as by-products of endogenous biological reactions, but also their formation increases during biotic and abiotic stresses. Superoxide dismutase (SOD, EC 1.15.1.1) is a key enzyme which constitutes the first line of defence against oxygen toxicity and catalyses the dismutation of the superoxide anions to dioxygen and hydrogen peroxide (Elstner, 1982; Foyer et al., 1994). The three known types of this enzyme can be distinguished according to their metal cofactor made of manganese (MnSOD), iron (FeSOD) or copper and zinc (Cu/Zn SOD) (Bannister et al., 1987), and according to their be-

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Abbreviations: Cu/ZnSOD, Copper/Zinc-SOD; FeSOD, iron-SOD; MnSOD, manganese-SOD; mRNA, messenger ribonucleic acid; PS II, photosystem II; RNA, ribonucleic acid; SDS, sodium dodecyl sulphate; SOD, superoxide dismutase.

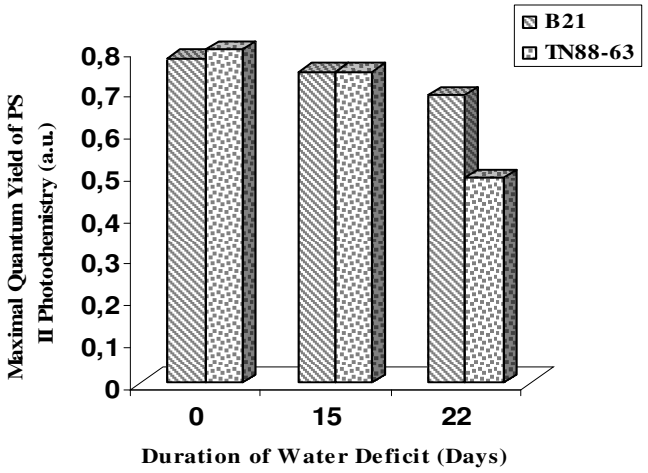

Figure 1A. . Effects of water deficit on the maximum quantum yield of PS II photochemistry (Fv/Fm) in *Vigna unguiculata* cv. B21 and cv. TN88-63 leaves. Plants were exposed to increasing water stress intensities. Chlorophyll *a* fluorescence and leaf water potentials (□were measured at the beginning of stress induction (□= -0.5 MPa for B21 and □= -0.55 MPa for TN88-63), after 15 days (□= -1.5 MPa for B21 and □= -1.8 MPa for TN88-63) and after 22 days (□= -2.5 MPa for B21 and □= -2.7 MPa for TN88-63). Data are means of five replicates.

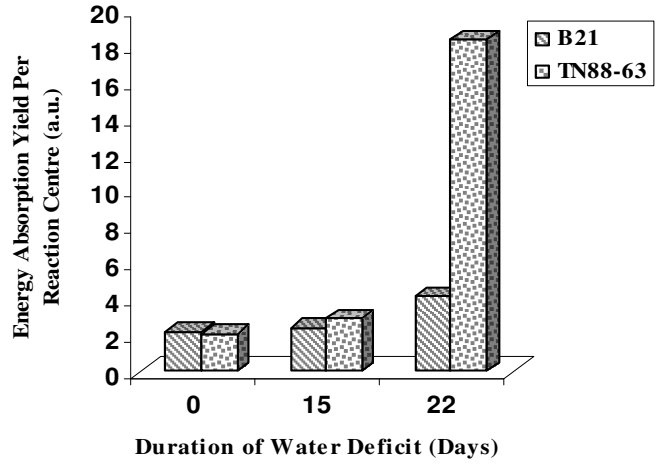

Figure 1B. Effects of water deficit on the energy absorption yield per photosystem II reaction centre, (ABS/RC) of Vigna unguiculata cv. B21 and cv. TN88-63 leaves.

behaviour towards specific inhibitors (Bowler et al., 1992). The identification of the three types of isozymes is based on the differential inhibition of SOD activity on polyacrylamide gels preincubated with KCN or H_2O_2 (Bowler et al., 1992; Rubio et al., 2001). H_2O_2 generated from the activity of SOD is scavenged by catalase in peroxisomes and by ascorbate peroxidase in Halliwell-Asada cycle (Elstner, 1982; Halliwell and Gutteridge, 1989).

Identification of resistance mechanisms to water stress is often difficult because resistance to drought stress is a quantitative trait. Induction of antioxidative enzymes can be assumed to reflect a general strategy required to overcome increased oxidative stress induced by water stress. In this paper, we point out SOD isoforms in cowpea cultivars, and we study their activity during water stress induction. Relationships between SOD activity and PS II photochemistry in these plants are discussed in order to understand the level of implication of these enzymes in the physiology of resistance to water stress.

MATERIALS AND METHODS

Experiments were undertaken on a drought-resistant cultivar (Bambey 21, B21) and a moderately drought-sensitive cultivar (TN88-63) of cowpea [*Vigna unguiculata* (L.) Walp.]. Plants were grown in a greenhouse at 27.7 ± 0.5°C, 35 ± 3.5% relative humidity, 505 ± 10.15 µmol.m^{-2}.s^{-1} light intensity and 14/10 light/dark photoperiod, on a sand-compost-vermiculite mixture (75.4% /19% /5.6%). Water deficit was imposed by withholding watering. Experiments started when cowpeas were 15 days old.

Biophysical studies

Chlorophyll *a* fluorescence was measured on the third attached leaf from the top, by using the plant efficiency analyser (PEA) (Hansatech Instruments LTD, King's Lynn, Norfolk, UK) according to Srivastava et al. (1995) and Strasser et al. (1996). The photon absorption efficiency per photosystem II (PS II) reaction centre (ABS/RC) and the maximum quantum yield of PS II primary photochemistry (F_v/F_m) were calculated and chosen to assess plant photochemistry during water stress imposition. Water stress intensity was evaluated by measuring leaf water potential with a pressure bomb (pms Instrument Co., Corvallis, Oregon, USA) (Peyrano et al., 1997) on the same leaf stage as previously.

Biochemical studies

Parallel to biophysical studies, SOD isoforms were extracted from one gram of leaf (the same stage as previously) and from one gram of roots finely ground on ice by using a pre-cooled mortar and pestle, in 1 ml 50 mM potassium phosphate buffer (pH 7.8) containing 0.1% (w/v) bovine serum albumin (BSA), 0.1% (w/v) L-ascorbic acid and 10 mM dithiothreitol. The homogenate was centrifuged at 48,000 g for 30 min at 4°C. Fractions of 10 µl of the supernatant were used for protein estimation (Spector, 1987) with BSA as standard. SOD activity was evaluated on a native unidimensional bis-acrylamide (1:29) gel electrophoresis (16%) according to Beauchamp and Fridovich (1974), modified by Bowler et al. (1992), Rubio et al. (2001) and Moran et al. (2003). SOD isoforms were identified by using their specific inhibitors: 4 mM KCN for Cu/ZnSOD, 10 mM H_2O_2 for Cu/ZnSOD and FeSOD (Bowler et al., 1992). The addition of 2% SDS directly in the sample allowed confirming the identity of isoforms. The gels were then scanned.

RESULTS

Variations of PS II photochemistry under water stress

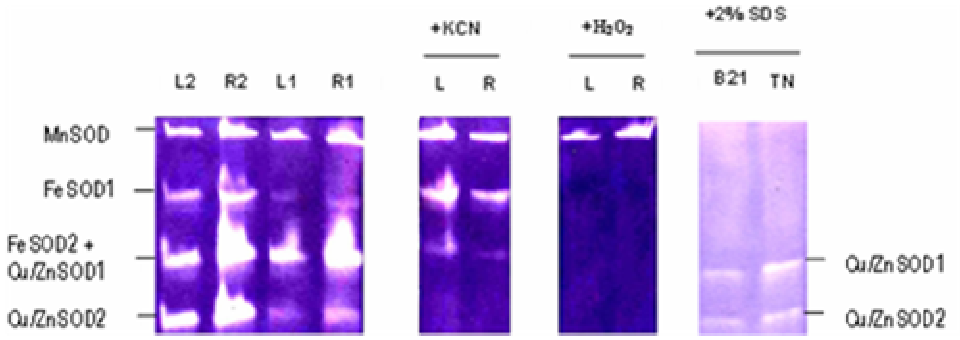

Figure 2A. Superoxide dismutase isoforms in well-watered *Vigna unguiculata* cv. B21 leaves (L) and roots (R). L1 and R1 bands were obtained with extracts containing 150 µg total soluble proteins and L2 and R2 bands, with extracts containing 200 µg total soluble proteins. The same result was observed in cv. TN88-63 leaves and roots. The use of 2% SDS in leaves extracts allowed to reveal the Cu/ZnSOD1 isoform that co-migrates with the FeSOD2 isoform.

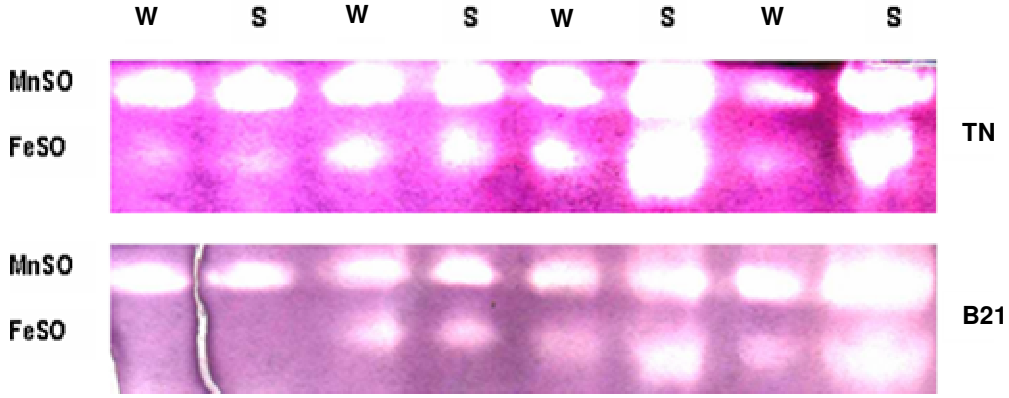

Figure 2B. Differential regulation of superoxide dismutase in *Vigna unguiculata* cv. B21 leaves during water deficit imposition. The same result was obtained from cv. TN88-63 leaves but in a lesser magnitude (not shown). Plants were exposed to increasing water stress intensities. SOD activity and leaf water potentials (ψ).were measured in watered (W) and stressed (S) plants. ψ = -0.8 MPa for B21 and ψ = -0.95 MPa for TN88-63 ten days after stopping water supply; ψ = -1.5 MPa for B21 and ψ = -1.8 MPa for TN88-63 after 15 days of stress imposition, and ψ = -2.5 MPa for B21 and ψ = -2.7 MPa for TN88-63 after 22 days. Enzymes were assayed parallel to biophysical studies.

are reported in Figures 1A and 1B. Water deficit induced a decrease in the maximum quantum yield of PSII photochemistry (F_v/F_m) (Figure 1A). The decrease in PSII photochemistry reactions was more pronounced in the sensitive cultivar (TN88-63) than in the resistant one (B21). On the contrary, water deficit effect resulted in an important increase in energy absorption yield per reaction centre (ABS/RC) in the sensitive cowpea cultivar TN88-63 whereas this parameter remained almost unchanged in the resistant cowpea cultivar (Figure 1B).

Superoxide dismutase isoforms were revealed in leaves (L) and roots (R) of the cowpea cultivars. The electrophoretic profile of the isoforms detected is given in Figure 2A. We revealed three types of SOD (MnSOD, FeSOD and Cu/ZnSOD) in the cowpea plants (in leaves as well as in roots). In these plants, FeSOD and Cu/Zn SOD were present in two forms ($FeSOD_1$, $FeSOD_2$, $Cu/ZnSOD_1$ and $Cu/ZnSOD_2$). $FeSOD_2$ co-migrates with the $Cu/ZnSOD_1$ form and these isoforms were clearly distinguished by using 2% sodium dodecyl sulphate (SDS). This product is known for the first time to inhibit FeSOD and MnSOD isoforms.

The study of these SOD isoforms during water stress induction revealed differential regulation of their activities (Figure 2B): MnSOD and $FeSOD_1$ activities increased rapidly while Cu/ZnSOD activities decreased in cowpea plants. The increase in MnSOD and $FeSOD_1$ activities was observed earlier in TN88-63 than in B21 and was slightly more pronounced in B21 (resistant) than in TN88-63 (sensitive). $FeSOD_2$ activity remained relatively constant (Figure 2B).

DISCUSSIONS

The decrease in the maximum quantum yield of PSII photochemistry (F_v/F_m) implies a decrease in the capture and conversion rate of excitation energy by PS II

reaction centres and so, a reduction in PS II photochemical efficiency (Flagella et al., 1995). These results indicate the disorganisation of PS II reaction centres under water stress conditions (Giardi et al., 1996). This disorder appeared to be highly pronounced in TN88-63 (the moderately sensitive cowpea) than in B21 (the resistant cowpea). Otherwise, the increase in ABS/RC would be a survival strategy consisting in over-activity of unaltered reaction centres to maintain some energy absorption rate without compensating the lost of maximum quantum yield of photosystem II (Flagella et al., 1995). Sharma et al. (2005) found that the overproduction of antioxidative enzymes (SOD and peroxidase) was accompanied by the reduction of the net photosynthetic rate, stomatal conductance and transpiration rate in wheat genotypes. This result is in concordance with ours as the biochemical phase of photosynthesis is determined by the efficiency of the photochemical step.

As the same concentration of substrate (0.24 mM nitro blue tetrazolium) was used for enzyme study in control as well as in stressed samples, fluctuations of bands intensity would reveal not only fluctuations of enzyme activity, but also analogous fluctuations of their concentrations. Furthermore, it is often assumed that SOD activity determines $O_2^{\cdot-}$ concentration (Schwanz et al., 1996). According to the relation:

$$[O_2^{\cdot-}] = \frac{K_1^{'}}{\sqrt{K_2 \cdot [E]}} \quad , \quad (K_1^{'} = \sqrt{K_1})$$

K_1, K_1' and K_2 are constants derived from $O_2^{\cdot-}$ production kinetic in photosystem I and its scavenging kinetic by SOD (Fridovich, 1974). Not only SOD activity, but also SOD concentration, determine $O_2^{\cdot-}$ concentration. Indeed, this relation implies the necessity for plants to increase SOD concentration to resist to increasing $O_2^{\cdot-}$ concentrations.

The increase in SOD activity may also confirm the increased production of superoxide radicals mediated by water stress. The differential regulation of SOD isoforms' concentrations induced by water deficit may be due to analogous regulation of their mRNA transcripts (Tsang et al., 1991). Analogous results were observed in tobacco lines where a wild-type revealed the same behaviour as the resistant cowpea cultivar until sixteen days of stress, and the mutant tobacco with MnSOD antisense-mRNA gave a similar response to TN88-63. However, the photochemistry of the wild-type tobacco line as well as the mutant line was altered at very severe water stress (after 18 days of withholding watering) (data not shown). The more elevated level of MnSOD and FeSOD1 concentrations and activities associated with the weaker disorganisation of PSII reaction centres in B21 than in TN88-63 on one hand, and the more increased energy absorption yield per unaltered reaction centres in TN88-63 (that proves a greater alteration rate) than in B21 on the other hand, suggest that these SOD isoforms contribute to the expression of B21 resistant trait to water stress. The decreased activities of the other isoforms would be due to their inhibition by hydrogen peroxide derived from MnSOD and $FeSOD_1$ activities.

Furthermore, at the whole plant level, the cultivar B21 appeared to be more tolerant to water stress than the cultivar TN88-63, though TN88-63 reacts earlier than B21 by over-expressing MnSOD and FeSOD1. The fact that B21 waited until 15 days after withholding watering before over-expressing MnSOD and FeSOD1, suggests that other resistance mechanisms were developed in B21 to allow supporting water deficit. Such mechanisms may be absent or expressed at low level in TN88-63. This resulted in the continuation of plant growth and elaboration of new leaves by B21; such observation was not perceptible in TN88-63 in our experimental conditions.

These results demonstrate that over-synthesized SOD isoforms contributed to maintain B21 photosynthetic apparatus in active form in spite of water stress pressure. Such increased resistance mechanism to oxidative stress was pointed out in transgenic tobacco plants that over-express pea chloroplastic Cu/ZnSOD under photoinhibitory stress (Sen et al., 1993a) and under methyl viologen-induced oxidative stress (Sen et al., 1993b). Overexpression of MnSOD in the chloroplasts of another transgenic tobacco transformed to over-express this isoform was shown to enhance the oxidative stress tolerance (Slooten et al., 1995). However, studying the rule of active oxygen species scavenging enzymes in salt-tolerance of cowpea, Cavalcanti et al. (2004) concluded that the ability of cowpea plants to survive under high levels of salinity is not caused by an operating antioxidant system involving SOD, peroxidase and catalase. This was not the case for Sharma et al. (2005) working on the effect of salinity in cowpea whose results are similar to ours.

Conclusion

Variations of PS II photochemistry and regulation of SOD activities were studied in two cowpea cultivars under water stress conditions. Photochemistry decreased in the sensitive cultivar and remained almost unchanged in the resistant one. We also show that increased synthesis of new SOD molecules associated with their increasing activities contributed to the resistance mechanisms of cowpea cultivars to water stress. However, these protective mechanisms appeared to reach a limit magnitude at higher water stress intensities, maybe due to the possible alteration of the expression of other genes associated with stress tolerance when SOD acti-

vity increases or to the involvement of excessive oxidative agents. This limit could also be due to the involvement of several complex genes with possible opposite effects. The differential regulation of SOD isoforms under water deficit conditions makes these enzymes as biochemical indicators for the study of oxidative stress induced by these unfavourable environmental conditions.

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