Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.12 No.4, Issue of October 15, 2009

Received July 10, 2008 / Accepted September 21, 2009
DOI: 10.2225/vol12-issue4-fulltext-10
RESEARCH ARTICLE

Expression of SOD transgene in pepper confer stress tolerance and improve shoot regeneration

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Financial support: Grant from Greek Secretary for Research and Technology to A.S.T.

Keywords: *Capsicum annum* L., SOD, MV, drought stress, regeneration.

Abbreviations: CAT: catalase
MV: methyl viologen
RWC: relative water content
ROS: reactive oxygen species
SOD: superoxide dismutase

The objective of this work was to study the stress tolerance and regeneration capability of transgenic pepper plants carrying a *sod* gene, encoding a tomato chloroplast-localized Cu/Zn SOD protein. The expression of the *sod* gene was confirmed by enzymatic staining following polyacrylamide gel electrophoresis (PAGE), revealing a 'novel' band, which could represent a heterodimeric enzyme. Transgenic T_1 and T_2 progeny plants were exposed to different oxidative stresses including Methyl viologen (MV) and drought and found to have an increased resistance to oxidative damage. Furthermore, the SOD carrying transgenic pepper plants showed increased levels of regeneration efficiency compared to the wild type pepper plants. Pepper is a recalcitrant species in terms of its *in vitro* regeneration ability but it could be extremely useful for the development of pharmaceuticals. This approach enables the extent use of pepper for genetic transformation and the production of high valuable products in plants particularly the large fruit varieties.

Active oxygen species were considered to be important damaging factors in plants exposed to stressful environmental conditions such as drought (Badawi et al. 2004), and pathogen attack (Tertivanidis et al. 2004) as well as to chemical treatment such as paraquat (Perl et al. 1993). Plant antioxidant defense systems include enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Apel and Hirt 2004; Mancini et al. 2006). The SOD enzyme constitutes a component of the first line of cellular defense against oxidative stress by early scavenging superoxide radicals and converting them

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to hydrogen peroxide (Perl-Treves and Galun, 1991). Transformation of many plant genera for useful traits, such as oxidant-resistance, is now routine (Perl-Treves and Galun, 1991; Perl et al. 1993; Tertivanidis et al. 2004). More recently the studies were extended in important vegetable crops where a *sod* tomato gene was cloned into a pepper plant (Zambounis et al. 2002).

The active oxygen species hydrogen peroxide (H_2O_2) was viewed mainly as a toxic cellular metabolite but, it became evident that it has multiple roles in plants. It can function as a signaling molecule that mediates responses to various stimuli in plants (Neill et al. 2002). Moreover, H_2O_2 modulates the expression of various genes, including those encoding antioxidant enzymes and modulators of H_2O_2 production (Neill et al. 2002). In addition, a microarray study showed that the expression of 1 - 2% of genes was altered in H_2O_2-treated *Arabidopsis* cultures and particular genes encoding antioxidant enzymes were upregulated (Desikan et al. 2001).

Recently the involvement of H_2O_2 and SOD in regeneration of plants has also been proposed (Cui et al. 1999; Luo et al. 2001; Papadakis et al. 2001; Tian et al. 2003; Libik et al. 2005; Zheng et al. 2005).

Active oxygen species may also have a positive role in plant growth and development (Tian et al. 2003). A dual role for H_2O_2 in the regeneration of protoplasts has also been shown (De Marco and Roubelakis-Angelakis, 1996). Furthermore, it has been reported that the cytosolic Cu/Zn SOD was induced in regenerating tobacco protoplasts but not in the recalcitrant grapevine protoplasts (Papadakis et al. 2001), which supports the hypothesis that SOD is involved in plant morphogenesis.

The aim of this work was to investigate the expression and behavior of transgenic red pepper type "Florinis" plants carrying a tomato Cu/Zn SOD gene under different stressesand investigate the potential regeneration efficiency of the SOD over-expressing transgenic plants.

MATERIALS AND METHODS

Plant material

The primary T_0 transgenic plants expressing tomato *chl*Cu/Zn SOD were produced by *Agrobacterium*-mediated transformation of *Capsicum annum L.*, red pepper type "Florinis" (Zambounis et al. 2002). T_0 plants were obtained and the T_1 were selected for further research.

T_1 and T_2 seeds were surface-sterilized by soaking in 2.5% NaOCI supplemented with a few drops of TritonX 100% (Merck)/100 ml solution for 10 - 15 min followed by 3 washes in sterile water. The seeds were dried and placed on half-strength MS medium (Murashige and Skoog, 1962) agar-solidified (0,8% w/v), the pH was adjusted at 5.8 with KOH or NaOH before autoclaving without antibiotics in order to determine the germination ability or with

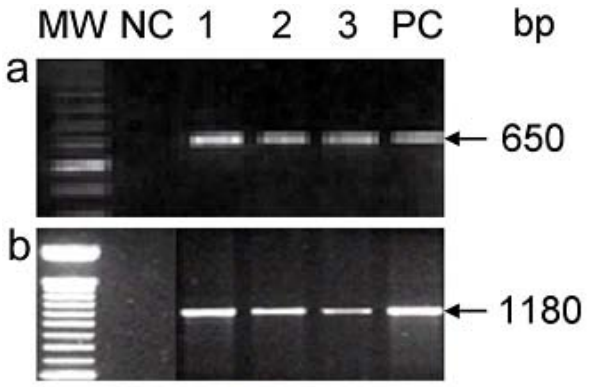

Figure 1. **PCR analysis of kanamycin resistant T1 and T2 progeny of transgenic pepper plants.** (a) PCR amplification of the *nptII* gene region. MW: marker, NC: negative control of non-transgenic pepper plant, 1-3: T_1 transgenic pepper plants, PC: positive control of pepper transgenic plant. (b) PCR amplification of the 35S-nos region. MW: marker, NC: negative control of non-transgenic pepper plant, 1-3: T_1 transgenic pepper plants, PC: positive control of pepper transgenic plant.

Kanamycin (100 $mg.l^{-1}$) for screening resistant (Kan^R) seedlings. The seeds were germinated under darkness at 25°C. All cultures were kept in growth chambers at 25 ± 2°C, with a photo period of 16 hrs / 8 hrs light (intensity of 2.500 - 4.000 Lux) / dark, respectively.

PCR verification of T_1 and T_2 transgenic pepper plants

The presence of the transgenes in kanamycin resistant plants was initially verified via PCR to confirm the presence of the *nptII* and the chimeric *sod* gene. DNA was extracted using the DNeasy Plant mini kit (Qiagen). For the PCR reaction 20 ng of DNA were added to the PCR mixed which consists of 1.5 mM $MgCl_2$, 1 X buffer (Gibco BRL), 0.2 mM deoxyribonucleotide triphosphate (dNTPs), 1 unit of Taq polymerase, 0.5 μM of each primer in a final volume of 12.5 μL.

Two rounds of PCR screening were performed. In the first round, plants were screened for the presence of a 650 bp band which corresponds to the *nptII* gene. The sequences of the primers used were Forward: 5'-GAG GCT ATT CGG CTA TGA CTG-3' and Reverse: 5'-ATC GGG AGC GGC GAT ACC GTA-3'. The PCR program included 4 min at 94°C, then 30 cycles of 94°C for 1 min, 59°C for 1 min, 1 min at 72°C, followed by a final extension at 72°C for 1 min.

In the second round, plants were screened for the amplification of a 1180 bp band, which corresponds to the 900 bp Cu/ Zn SOD gene and a fragment of Ca MV 35S promoter and nos terminator. The sequences of the primers used were Forward: 5'-GGA GCA TCG TGGA AAA AGA AGA C-3' and Reverse: 5'-TTA TCC TAG TTT GCG CGC TA-3'. The PCR reactions were run at 94°C for 5

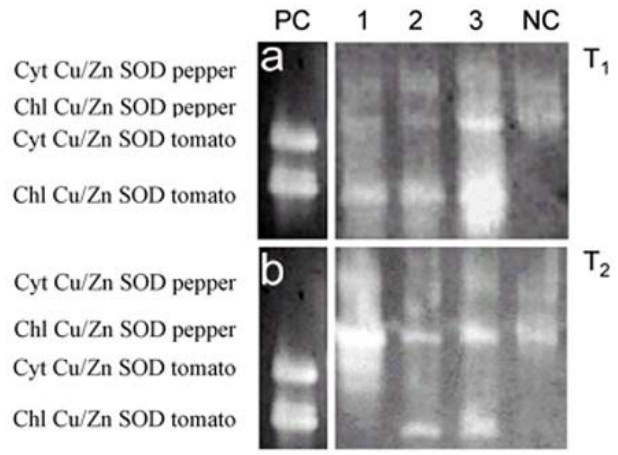

Figure 2. SOD isozyme patterns of protein extracts from non-transgenic pepper, tomato and transgenic T_1 (a) and T_2 (b) pepper plants. The non-denaturing polyacrylamide gel was loaded with 40 μg protein per slot and (negatively) stained with riboflavin-nitroblue tetrazolium. PC: control from non- transgenic tomato plant, 1-3: T_1 and T_2 transgenic pepper plants, NC: negative control of non -transgenic pepper plant.

min, then 30 cycles of 94°C for 40 sec, 57°C for 30 sec, 72°C for 1 min, followed by a final extension at 72°C for 3 min.

SOD enzyme electrophoresis

Leaves from T_1, T_2 transgenic and untransformed pepper plants (wt) were homogenized in a buffer consisting of a glycine buffer, pH 7.4, containing polyvinylpyrrolidone (p-6755). After centrifugation (5 min, 14,000 rpm), the clear supernatant was assayed for protein content (Bradford, 1976), and 40 μg of protein were loaded on a non-denaturing 9% polyacrylamide gel with a 3% stacking gel (Laemmli, 1970). The gel was run at 4°C (100 Volt) and stained for SOD by the riboflavin-nitroblue tetrazolium as described (Beauchamp and Fridovich, 1971).

MV application

The herbicide Methyl viologen (MV) (1.1 dimethyl-4.4-bipyridinium chloride, Sigma, USA), was used to generate free radicals and oxidative stress in cells during illumination. Prior to the experiment, we tested untransformed pepper plants with a range of MV solutions 0 - 0.5 x 10^{-3}M and found 0.25 x 10^{-3}M MV to be the optimal concentration in order to test the transgenic plants for tolerance to the herbicide (data not shown). Stem cuttings from greenhouse-grown untransformed red pepper type "Florinis" and T_1 and T_2 transformed plants, with fully expanded mature leaves, were placed in tubes containing 50 ml of 0.25 x 10^{-3}M MV, according to the procedure of Perl et al. (1993). After 16 hrs incubation, the MV was discarded and the test-tubes were washed and filled with tap water. Clear paraquat-damage symptoms were noticed after 48 hrs exposure to constant illumination at light intensity of 45 $\mu Em^2 s^{-1}$. Oxidative damage was assessed visually. All experiments were repeated at least twice.

Water-deficit stress

In order to investigate the drought stress tolerance of the T_1 and T_2 transgenic pepper plants and untransformed control, plants were grown in 5 lt pots in a controlled environment. In the experiment, the fifth leaf from the top was sampled, taken from 3 independent transgenic plants and each generation T_1, T_2 and from control plants. The state of the drought - stressed plants was expressed by Relative Water Content (RWC) defined as: 100 x (actual leaf weight-dry weight) / (hydrated weight-dry weight) according to Perl-Treves and Galun (1991). From each leaf, 12 leaf discs were cut and the RWC was measured. In order to measure the leakage of electrolytes, 8 leaf discs were used. A normal irrigation regime was maintained until the plants were exposed to drought for fourteen days. Following this method, after sampling and weighing (actual weight), leaves were immersed for 20 hrs in distilled water, blotted and weighed (hydrated weight). For dry weight determination leaves were dried overnight in a 70°C oven. Leakage of electrolytes from leaf discs was used as a parameter of membrane damage. Leaf discs were cut as before from soil grown plants, rinsed and immersed for 22 hrs in 3 ml of distilled water. The electrolyte leakage (%) was measured with a Consort C831 (Belgium) conductivity metre and was defined as: 100 x (electrolyte leakage before boiling / electrolyte leakage after boiling). Three replications were used in every experiment. The data were tested by analysis of variance (ANOVA) at $p < 0.001$.

In vitro regeneration ability

For the regeneration experiment, 7 - 10 days old seedlings were used as explant donors from three independent T_1, three independent T_2 *sod* transgenic plants and untransformed (wt) control pepper plants. Specifically, we used 45 hypocotyls per each independent transgenic plant and for each control. The shoot regeneration regime was as described before (Zambounis et al. 2002). The regeneration was estimated after 2 months. Regeneration frequency was calculated as the number of regeneration explants per total number of cultured explants. Three replications were used in the experiment. The results were analysed by analysis of variance (ANOVA), at $p < 0.02$.

RESULTS

PCR of T_1 and T_2 progenies

Figure 1 shows the results from the two PCR screening rounds using two different pairs of specific primers for each gene. All Kan^R T_1 plants were PCR- ositive while all Kan^S were negative (Figure 1a). Similar results were obtained from the PCR tests for the 1180 bp fragment, which included the *sod* gene (Figure 1b).

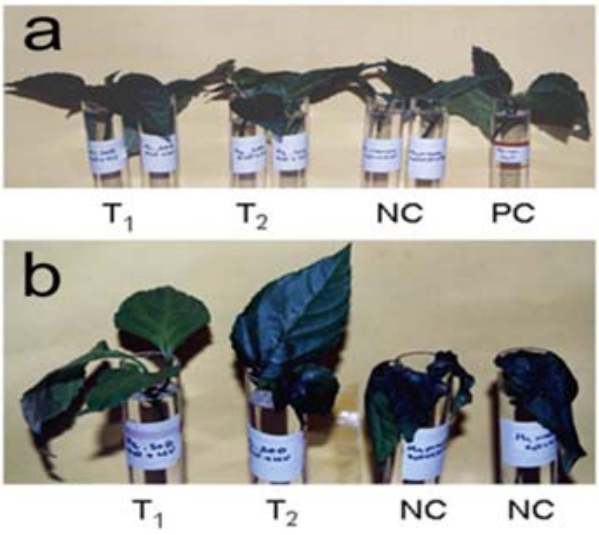

Figure 3. **Response of shoots from T_1 and T_2 transgenic pepper plants to MV**. (a) Stems with fully expanded mature leaves from greenhouse-grown plants were placed in tubes containing 50 ml 0.25 x 10^{-3} M MV. (b) 16 hrs after MV shoots were transferred to water and 48 hrs after exposure to constant illumination, they were photographed. T_1, T_2: transgenic pepper plants; NC: negative control of non-transgenic pepper plant, PC: positive control of transgenic pepper plant in H_2O.

SOD enzymatic activity in T_1 and T_2 progenies

To test the expression of the *sod* transgene, total protein extracts from leaves of transgenic and control pepper plants were electrophoresed in native polyacrylamide gels. Protein extracts were run on SOD activity gels, together with extracts of tomato plants and control untransformed red pepper type "Florinis" plants. All T_1 and T_2 plants revealed a "novel" faster moving band at a lower position than the pepper chl Cu/ Zn SOD bands (Figure 2). This novel band co-migrates at the same molecular weight as the lower tomato band as shown in Figure 2 and could represent a heterodimer enzyme. Obtaining an enzymatic band for a protein encoded by the transgene suggests that the respective gene was not only introduced into the transgenic plants (T_1 and T_2) but it was also functional thus, yielding a functional product.

The effect of MV on T_1 and T_2 progenies

Preliminary experiments with untransformed pepper plants indicated that 0.25 x 10^{-3} M of MV was an appropriate concentration to detect for damages caused by MV to pepper plants. The results presented in Figure 3 demonstrate that T_1 and T_2 transgenic pepper plants were less damaged from 0.25 x 10^{-3} M of MV compared to the control plants (NC) and were phenotypicaly similar to the transgenic plants in water (PC). The untransformed control shoots showed clear paraquat - damage symptoms. The leaves turned chlorotic and then wilted irreversibly. On the contrary, the transgenic T_1 and T_2 shoots exhibited only moderate symptoms, compared to the control plants, indicating a positive correlation between over-expressing Cu/Zn SOD protein in the chloroplasts and MV tolerance.

The effect of drought-stress on T_1 and T_2 progenies

The oxidative damage can also be quantitatively and statistically assessed by conductivity measurements of electrolyte leakage from leaf tissue. After about two weeks without watering (see Material and Methods for details), untransformed control plants exhibited all the stress symptoms such as permanent wilting, dark - green leaves and retarded growth, whereas T_1 and T_2 transgenic plants were less affected. For water stress tolerance, RWC and the leakage of electrolytes were conducted at days 0 and 14 (without watering) compared to normally watered wt and transgenic pepper plants. As shown in Figure 5, on the first

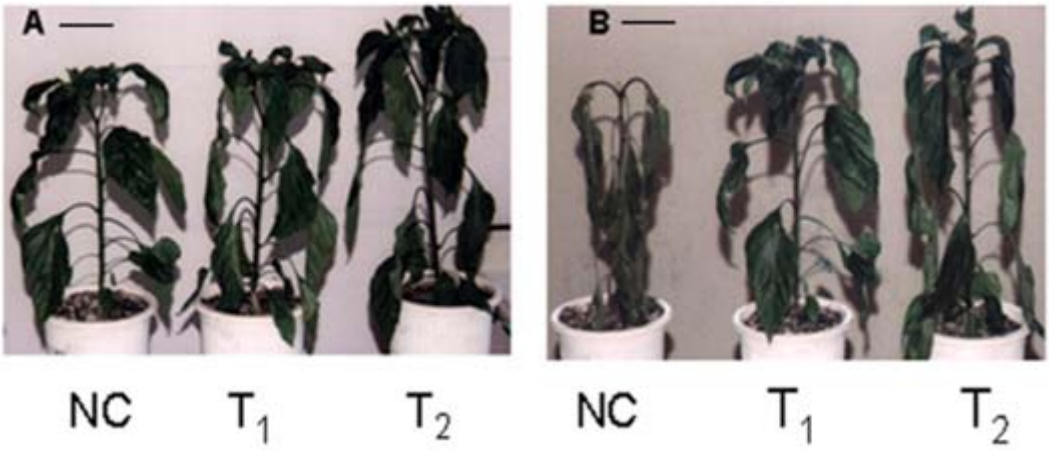

Figure 4. Response of T_1 and T_2 transgenic pepper plants to drought stress. (A): First days of water stress. (B): After 14 days without water. NC: negative control of non-transgenic pepper plant:transgenic pepper plants (bar = 28.6mm)

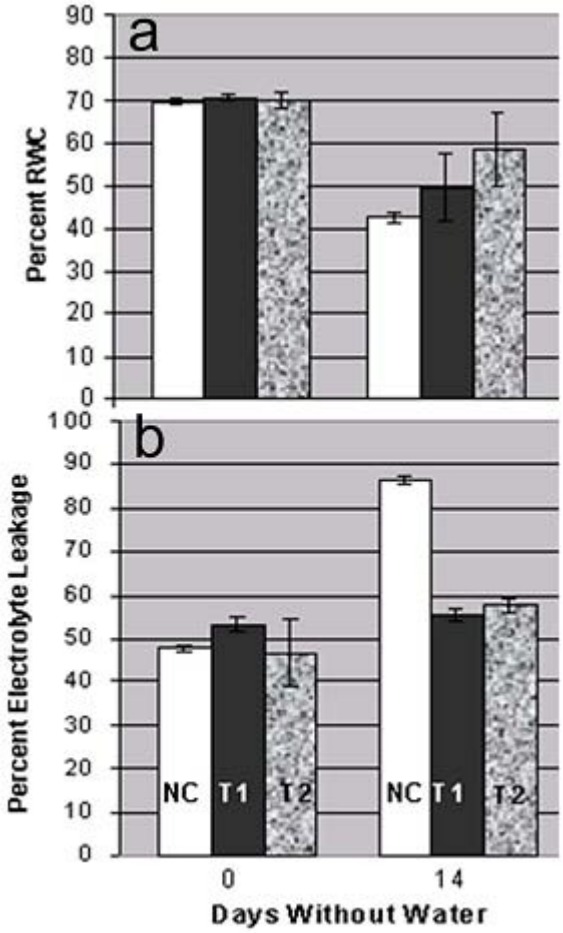

Figure 5. Water-deficit stress. The RWC (a) and the Electrolyte Leakage; (b) were measured from leaves of transgenic and wt pepper plant type "Florinis" at days 0 and 14 without water, using leaf disks from the fifth leaf of each plant. NC: negative control of non - transgenic pepper plant, T_1 and T_2: transgenic pepper plants. Values are means of 3 independent plants of the T_1, T_2 generation and untransformed plants similarly treated, p = 0,001.

day of the experiment there was no statistical difference and no change in RWC (70.57% and 69.69%) and the leakage of electrolytes (53.32% and 43.26%) in T_1 and T_2transformed plants, as compared to the control ones (69.54% and 47.65%).

Under long periods of water stress (14 days), the T_1 and T_2 transgenic plants showed reduced leakage (55.38% and 57.57%) and higher RWC (49.78% and 58.55%), respectively, compared to the control (86.65 and 42.49). We must note here that the transgenic plants recovered from the water stress while the wt plants showed clear symptoms of water deprivation after 14 days (Figure 5). The control plants showed reduced tolerance to water deficit as shown by the RWC (42.49%) and a higher percentage of leakage (86.65%), because the cellular damage reached nearly complete membrane disruption, whereas T_1 and T_2 *sod* transgenic plants exhibited significantly less damage (Figure 4).

The results presented in Figure 5a and Figure 5b suggest that he *sod* transgenic plants are highly resistant to water stress deficiency as shown by their the physiological response which corresponds to around 1.5 times lower electrolyte leakage compared to the wild type plants (NC).

Regeneration ability

Shoot regeneration from the hypocotyl explants of three independent T_1 and three T_2 transgenic plants and wt was assessed on regeneration medium (Zambounis et al. 2002). The frequency of shoot regeneration and the mean number of regenerated shoots are presented in Figure 6. Statistical analysis of the data (regeneration frequency/mean number) showed statistically significant differences between T_1 ($p < 0.02$) and T_2 ($p < 0.003$) generations of *sod* carrying transgenic pepper plants and the wt control plants. Differentiation of shoot buds was observed after 3 weeks of culture and most shoots were formed at the region of the cut. The transgenic plants have statistically significant higher regeneration ability (mean number of shoots) compared to the wt (20) both in the T_1 (29, 26, 25) and T_2 (28, 24, 27) generations (Figure 6). Similarly the transgenic plants have higher regeneration frequency. High percentage (%) of regeneration frequencies were obtained with the T_1 (55.33, 58.33, 65.21) and T_2 (53.33, 60.00, 62.22) generations, respectively, as compared to the wt (44.44) (data not shown). Taken together, all independent transgenic plants from two generations have a higher regeneration ability compared to the wt.

DISCUSSION

Plants possess various mechanisms in order to be protected from reduced oxygen species. Protection against oxidative stress is complex and includes both enzymatic and non-enzymatic components. Key enzyme in the defense mechanism is SOD, which is the first enzyme in the detoxifying process that converts O_2^- radicals to H_2O_2. In some abnormal conditions such as water stress (Synková and Valcke, 2001), drought and freezing stress (Wu et al. 1999; Li et al. 2006; Prashanth et al. 2008), heat stress (Sala and Lafuente, 1999) and preparation of tissue culture (Zheng et al. 2005), reactive oxygen species (ROS) production can be induced, leading to increased SOD and CAT activities in order to decrease the ROS concentration and protect the cell (Zheng et al. 2005). Moreover, ROS can cause oxidation of the cell membrane and destroy its function. Some biological macro-molecules such as chlorophyll, protein and DNA also are oxidized and could lead to plant death in stress conditions.

In this study we investigated T_1 and T_2 transgenic pepper plants expressing the *sod* gene in order to investigate whether they had enhanced resistance to various forms of oxidative stress.

Molecular data from PCR confirmed that the transgenic plants contained the *sod* gene (Figure 1). The *nptII* gene was detected using primers that amplified a 650 bp band, identical to the band amplified from the binary plasmid that was used for the transformation experiments (Figure 1a). T_1 and T_2 transgenic plants from *chl*Cu/Zn SOD constructs which were also confirmed to posses the 35S-SOD-nos fragment (1180 bp) were used for further experiments (Figure 1b).

SOD isozyme patterns of protein extracts from pepper, tomato and transgenic T_1 and T_2 pepper plants show that the transgenic plants are expressing the *sod* gene yielding a functional product. The *sod* gene is stably inherited from the T_1 to T_2 generation where it is also found to be expressed (Figure 2).

Shoots derived from 18 T_1 and T_2 transformed pepper plants were incubated in 0.25 x 10^{-3} M of MV. After the MV treatment, the maximum paraquat-damage symptoms were observed in control plants. All T_1 and T_2 transgenic pepper plants showed increase resistance to oxidative damage by exposure to relatively high concentration of MV. In Figure 3 we show a representative sample of the results obtained from the experiment above. It has been shown that over-expression of Cu/Zn SOD in rice leads to increased tolerance to MV mediated oxidative stress (Prashanth et al. 2008). Also, when SOD was over-expressed in tall fescue plants enhanced resistance to MV was observed (Lee et al. 2007). Moreover, potato plants expressing tomato Cu/Zn SOD showed increased MV tolerance (Perl et al. 1993). Furthermore, expression of Mn SOD from pea leads to enhanced drought tolerance to transgenic rice (Wang et al. 2005).

We have also measured ion leakage, (which reflects membrane damage), after 14 days of water deprivation. We observed an increased ion leakage in the control plants (86.65%) compared to the transgenic plants were the leakage was significantly reduced, in T_1 it was 55.38% and in T_2 57.57% (Figure 5). The membrane damage is attributed partially to oxidative stress, which is a secondary effect of drought stress. The reduction in membrane damage in transgenic plants after the drought treatment is probably caused by an increased SOD activity (Figure 4), which provides higher ROS scavenging efficiency. The increased SOD activity had a similar protective effect against oxidative stress caused by MV when T_1 and T_2 transgenic plants were challenged with the herbicide (Figure 3).

Furthermore, we tested whether the SOD expressing plants had increased regeneration ability, for that reason plant regeneration from hypocotyl explants of transgenic and control pepper was established. The transgenic plants T1 (29, 26, 25) and T2 (28, 24; 27) have statistically significant higher regeneration ability compared to the wt (20) (Figure 6).

The highest regeneration frequencies (%) were obtained from the transgenic plants T1:55.33 to 65.21% and T2:53.33 to 62.22% compared to the wt were the regeneration was only at 44.44%. All independent transgenic plants from two generations have statistically significant higher regeneration ability compared to the wt.

The last few years it became clear that H_2O_2 is not only a damaging agent. The role of H_2O_2 as cellular messenger capable of inducing gene expression and protein synthesis has been established (Vranová et al. 2002; Apel and Hirt, 2004). It has been shown that H_2O_2 may function as a development signaling molecule in the differentiation of secondary walls in cotton fibers (Apel and Hirt, 2004). Furthermore H_2O_2 is a membrane-permeable molecule that has been demonstrated to function as a diffusible intercellular signal (Levine et al. 1994). It is known to induce a number of genes and proteins involved in stress defenses (Scandalios, 2005). Recently, a link has been established between ROS production and plant developmental physiology. High ROS production was observed in the expansion zone of maize leaf and a certain concentration of H_2O_2 is necessary for leaf elongation (Rodriguez et al. 2002). The ability of H_2O_2 to diffuse through cell membranes and to be naturally produced makes it suitable to act as a signal molecule in cell function (Tian et al. 2003).

Although the role of H_2O_2 in somatic embryogenesis is not

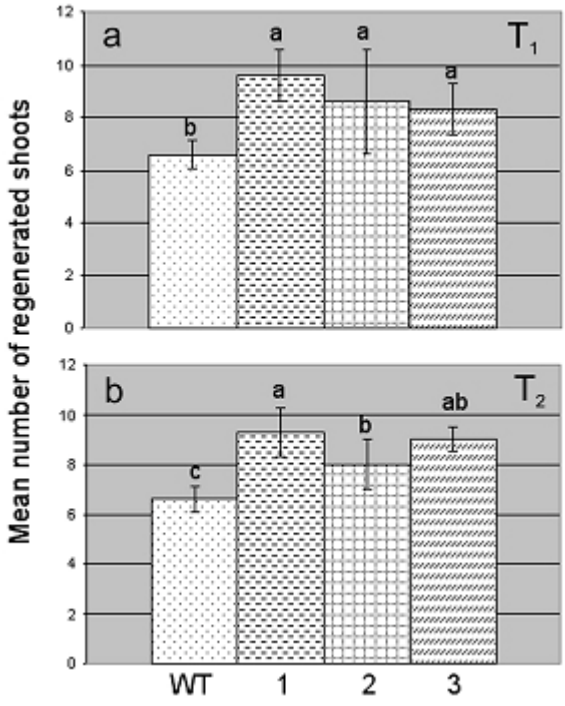

Figure 6. Mean number of regenerated shoots of T_1 (a) and T_2 (b) generations of *sod* carrying transgenic pepper compared to the control plants (WT), 1-3: T_1 and T_2 independent transgenic pepper plants.

clear, H_2O_2 has been considered to serve as secondary messenger to signal stress-induced responses in plants (Van Camp et al. 1998; Neill et al. 2002). Calli with low CAT activity and high H_2O_2 concentration displayed a regeneration potential which might support the hypothesis that the H_2O_2 produced in excess may promote the expression of some genes responsible for the induction of morphogenesis processes (Libik et al. 2005).

In peanuts (*Arachys hypogaea L.*) the use of antioxidants enhanced superoxide dismutase (SOD) and catalase (CAT) activities, thus increased the frequency of plant regeneration and transformation efficiency of peanut explants transformed via *Agrobacterium tumefaciens* (Zheng et al. 2005). Moreover, higher levels of intracellular H_2O_2 induce and promote somatic embryogenesis of *Lycium barbarum* L. callus (Cui et al. 1999). Furthermore during the differentiation and development of callus, SOD activity increased in the early regeneration culture and decreased thereafter and catalase activity constantly declined while peroxidase decreased during the 5 - days culture and gradually increased thereafter. No SOD and low H_2O_2 levels were detected in calli possessing low organogenesis capacity, suggesting that H_2O_2 is correlated with the morphogenetic process in strawberry callus and may actually serve as a messenger in the process of bud primordium formation (Tian et al. 2003). Gupta and Datta (2003) reported that the activity of SOD gradually increased during somatic embryogenesis and peaked when the somatic embryos were detected, while activities of catalase (CAT) and peroxidase (POX) decreased. In addition, recent research suggests that suppressed expression of totipotency in tobacco protoplasts was correlated with reduced activity of cellular antioxidant machinery (SOD) (Papadakis et al. 2001). In *Lycium barbarrum* (Cui et al. 1999) and *Astragalus adsurgens* (Luo et al. 2001) somatic embryogenesis can be enhanced by exogenous application of H_2O_2 or inhibition of H_2O_2 degrading enzymes such as catalase and ascorbate peroxidase.

Pepper is a recalcitrant species, so a line with enhanced regeneration efficiency could facilitate and increase the transformation efficiency of pepper. Pepper fruit could be useful as they could be used for the production of high valuable products, like oral vaccines. The results reported here where transgenic pepper plants over-expressing a tomato Cu/Zn SOD showed increased regeneration efficiency supports the hypothesis that H_2O_2 may play a role in plant morphogenesis.

ACKNOWLEDGEMENTS

Dr. Fotini Milona from NAGREF for her help with SOD gel analysis.

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