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Effects of Na_2SO_3 on the activities of antioxidant enzymes in geranium seedlings

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Abstract

This study investigated the effects of Na_2SO_3, which releases SO_2 in apoplastic water, on the growth of geranium seedlings and on the activities of various antioxidant enzymes including peroxidase. Sodium sulfite (Na_2SO_3) addition both inhibited primary root growth and stimulated lateral root growth of the seedlings respectively. In addition, the contents of chlorophyll and Rubisco protein of the seedlings were greatly reduced with Na_2SO_3 treatment. Total peroxidase activities of the seedlings also increased proportionally with the amount of Na_2SO_3 , this presumably correlating with oxidative stress levels. Notably, about an 8-fold enhancement of total peroxidase activity occurred in seedlings treated with 60 nM Na_2SO_3 at pH 4.0. This enhancement of total peroxidase activity was mainly due to the increase of a strong cationic isoperoxidase, strong anionic isoperoxidase and neutral isoperoxidase activities. The strong cationic isoperoxidase from geranium seedlings was found to be the same enzyme as PC3 from geranium callus in terms of its physicochemical and catalytic properties. Moreover, the activities of superoxide dismutase and glutathione reductase were greatly enhanced with Na_2SO_3 treatments at pH 4.0 without significant alteration of catalase activity. These results suggest that Na_2SO_3 exposure, activities the plants defense mechanism against the reactive oxygen species generated. © 2002 Published by Elsevier Science Ltd.

Keywords: Pelargonium graveolens; Geraniaceae; Geranium seedlings; Sodium sulfite; Antioxidant enzymes; Peroxidase

1. Introduction

Major airborne pollutants that have an important impact on plants include CO_2, CO, SO_2, NO_X and ozone. Oxides of sulfur, carbon and nitrogen generated through combustion of fossil fuels ultimately become major components of acid rainfall. Gaseous pollutants such as SO_2 gain entry into the plant leaves through open stomata; accordingly, plants are most susceptible to these pollutants during daylight hours under conditions conductive to stomatal opening. Once inside the leaf, SO_2 readily dissolves in the apoplastic water to produce mainly sulfite (SO_3^{-2}) and bisulfite ions (HSO_3^{-}). When either sulfur species dissolves in aqueous solution, a pH-dependent equilibrium is established predominantly, and hydrogen ions may be released (Fine et al., 1987). The primary site of SO_2 injury appears to be the chloroplast and the photosynthetic machinery (Pfanz et al., 1993). Concentrations of SO_2 as low as 0.035 μ/l of air will cause disruption of chloroplast membranes, and higher concentrations will damage enzymes including Rubisco and PEP carboxylase, and generally disrupt metabolism (Pfanz et al., 1993).

During plant reactions to various environmental stresses, the role of reactive oxygen species (ROS) including superoxide and H_2O_2 is well known: i.e. as evidenced by the accumulation of ROS in the plant cells. Additionally, the importance of antioxidant enzyme is generally understouad in terms of preventing oxidative stresses by scavenging ROS (Donahue et al., 1997; Prasad, 1997). Peroxidase, superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) are also known to be the major constituents of the plant antioxidant enzyme system (Anderson et al., 1995; Lee et al., 1998d). The peroxidases are important in a wide range of cellular functions such as phenolic compound oxidation, indole-3-acetic acid oxidation and regulation of cell growth (Lee and Kim, 1994, 2000a; Lee et al., 1994; Lee and Kim, 1998b,c). Moreover, they are involved in lignification and crosslinking of cell wall proteins.

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Interestingly, Peroxidase activities and isoperoxidase patterns can be altered in response to a variety of physical, chemical, and biological stresses (Lee, 1997a,b; Lee et al., 2000b). Several plant defense-regulators such as methyl jasmonate and phosphatic acid (Lee and Kim, 1998a) were activators of the Korean radish peroxidase (KRCP) promoter, suggesting that the KRCP gene might be associated with wound healing (Espelie et al., 1986) and defense against pathogens (Lagrimini and Rothstein, 1987). It has also been reported that the activities of many antioxidant enzymes in Arabidopsis and pea (Pfanz et al., 1993; Madamanchi et al., 1994; Kubo et al., 1995) change in response to SO_2. For example, the levels of peroxidase in peas treated with SO_2 or air pollutant mixture were approximately twice than those found in filtered air (Mehlhorn et al., 1987). Notably, increases in extracellular and apoplastic peroxidase activities in barley were detected following application of SO_2, showing that sulfite oxidation could be regarded as a defense mechanism to protect symplastic reactions (Pfanz et al., 1993). GR, SOD and CAT were also suggested to act in relation with the defense mechanisms to remove ROS (Mehlhorn et al., 1987; Madamanchi et al., 1994). GR activity has also been shown to increase significantly in response to several types of stress (Madamanchi et al., 1992) and tobacco cultivars with high SOD or GR activities have been shown to be less ozone susceptible than those with lower antioxidant protection (Shaaltiel et al., 1988). Furthermore, endogenous GR activities were more abundant in ozone-tolerant Bel B tobacco than ozone-sensitive BelW3 tobacco (Pasqualini et al., 2001).

Pelargonium graveolense, referred to as scented-geranium, is an important ornamental plant worldwide, and has been studied extensively with respect to its conventional horticultural traits, although only a few reports have so far focused on its biochemical and physiological studies. There are multiple isoperoxidases in the geranium callus, which are distinguishable by starch gel electrophoresis. Among them, the PC3 cationic isoperoxidase was purified and characterized in a previous report (Lee et al., 2001). This study examined the effects of Na_2SO_3, which is known for release of SO_2 in apoplastic water, on the growth of the geranium seedlings and on the activities of various antioxidant enzymes including peroxidase.

2. Results and discussion

2.1. Effects of Na_2SO_3 on the growth of geranium seedlings

We examined the effects of various concentrations of Na_2SO_3, which releases SO_2 in apoplastic water, on the growth of geranium seedlings at pH 4.0. As shown in Fig. 1(A), the entire length of the primary root decreased proportionally with increasing amounts of Na_2SO_3. On the other hand, the lateral root numbers also increased proportionally. These results indicate that Na_2SO_3 can inhibit primary root growth, and stimulate lateral root growth. Phytohormones such as auxin also stimulated lateral root growth at concentrations where the primary root growth was inhibited (Muday and Haworth, 1994). Geranium seedlings grown in the presence of increasing concentrations of Na_2SO_3 for 4 weeks at pH 4.0 were shown in Fig. 1(B). In addition, 50% inhibition of primary root growth occurred at about 45 nM Na_2SO_3 at pH 4.0. By contrast 1.5 μM Na_2SO_3 at pH 6.0 and 6 μM Na_2SO_3 at pH 8.0 (data not shown) were required to achieve the same level of inhibition. The results indicated that physiological and biochemical responses toward Na_2SO_3 might occur in a pH dependent manner, and the responses might result from synergistic and multiple effects of Na_2SO_3 and pH. The roles of pH and ionic species were well examined in SO_2-induced human bronchoconstriction (Fine et al., 1987). The mean concentration of Na_2SO_3 solution calculated to increase specific airway resistance was significantly different at the various levels of pH: pH 4 (0.17 mg/ml) less than pH 6.6 (0.49 mg/ml) less than pH 9 (2.1 mg/ml).

2.2. Effects of Na_2SO_3 on the contents of chlorophyll and Rubisco protein in geranium seedlings

The primary sites of SO_2 injury have been reported to be in the chloroplast and to the photosynthetic machinery (Pfanz et al., 1993). In Table 1, the effects of Na_2SO_3 on the contents of chlorophyll and Rubisco protein were examined. Exposure of geranium seedlings to Na_2SO_3 at pH 4.0 resulted in a remarkable reduction of chlorophyll content and Rubisco protein as expected; the possibility exists that these reductions might reflect an enhancement of their susceptibility to proteolysis as shown in the cellular protein degradation by ROS in barley (Desimone et al., 1996).

2.3. Effects of Na_2SO_3 on total peroxidase activities and isoperoxidase patterns from geranium seedlings

Alterations in total peroxidase activities and isoperoxidase patterns have been known to be related to environmental stresses such as salt, heavy metals, temperature and air pollutants (Kubo et al., 1995; Lee, 1997a,b; Yun et al., 2000). The effects of Na_2SO_3 at pH 4.0, a pH similar to acid rainfall, on total peroxidase activities in the roots of seedlings were thus next measured (Fig. 2). Total peroxidase activities increased remarkably with Na_2SO_3 treatment. In particular, about an 8-fold increase of total peroxidase activity was detected with 60 nM Na_2SO_3- treated seedlings compared to the control. On the other hand, when Na_2SO_3 was tested in

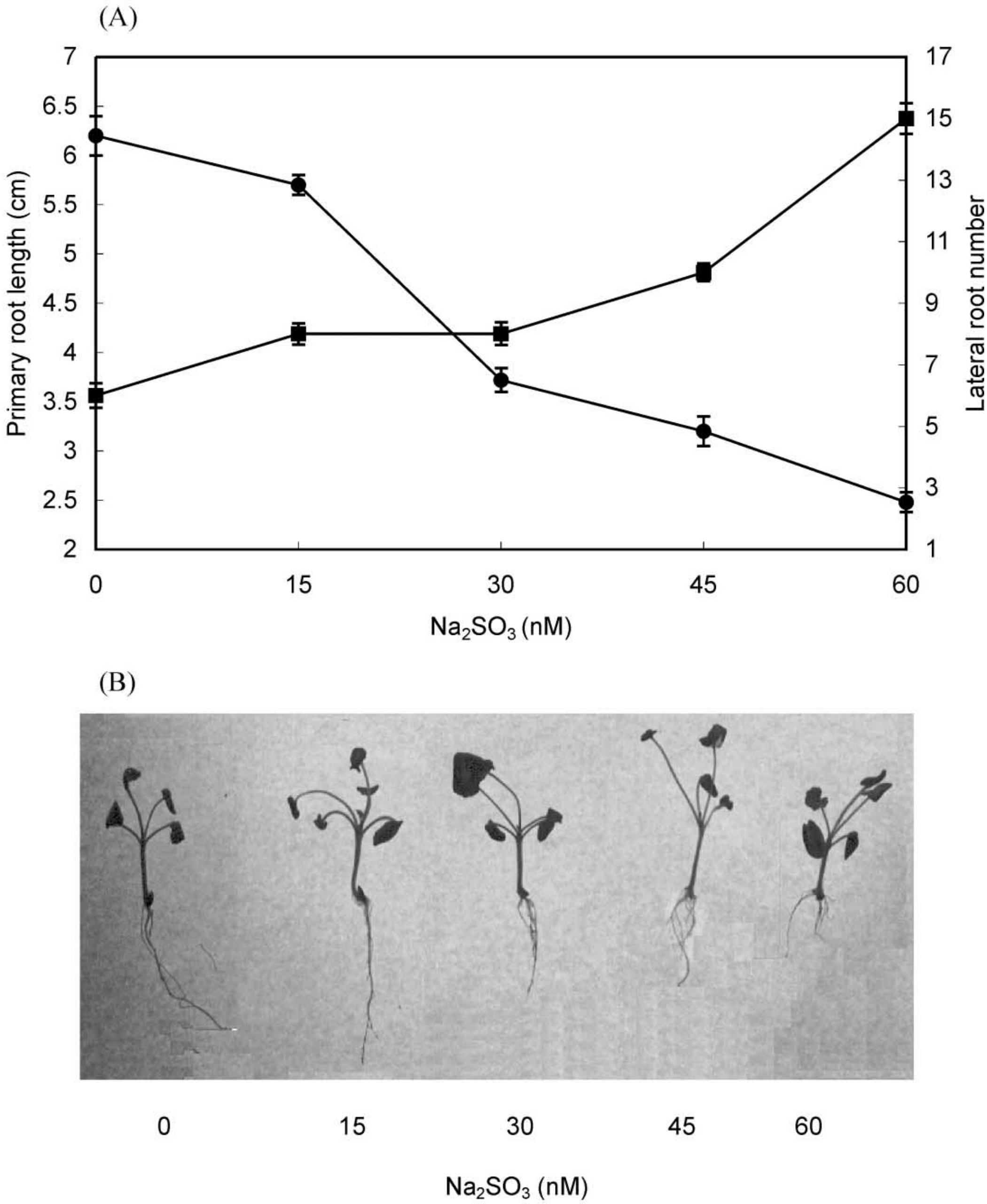

Fig. 1. (A) Effects of Na_2SO_3 on primary root length and lateral root numbers in geranium seedlings grown on media containing various concentrations of Na_2SO_3 at pH 4.0 for 4 weeks. All determinations are expressed as the mean ±s.e. of three separate experiments. ●–● Primary root length ; ■–■ lateral root numbers. (B) Geranium seedlings grown for 4 weeks in 0(A), 15(B), 30(C), 45(D) and 60(E) nM Na_2SO_3 at pH 4.0.

Table 1
Effects of Na_2SO_3 on contents of chlorophyll and Rubisco proteins in geranium seedlings grown on media containing various concentrations of Na_2SO_3 at pH 4.0 for 4 weeks

Na_2SO_3 (nM)	Chlorophyll (μg/ml)	Rubisco protein (A_{595})
0	40.7±0.24	0.8±0.02
30	37.7±0.37	0.67±0.02
45	25±0.09	0.55±0.01
60	11.3±0.04	0.51±0.03

vitro on total peroxidase activity in the enzyme extracts, no significant alteration in enzyme activity was observed (data not shown), suggesting that Na_2SO_3 had no effect on the total peroxidase activity itself in vitro.

The changes in isoperoxidase patterns in the presence of 30, 45 and 60 nM Na_2SO_3 at pH 4.0 were next analyzed by starch gel electrophoresis (Fig. 3), where it was found that the levels of strong anionic and strong cationic isoperoxidases increased proportionally with increasing Na_2SO_3 concentration i.e. as revealed by

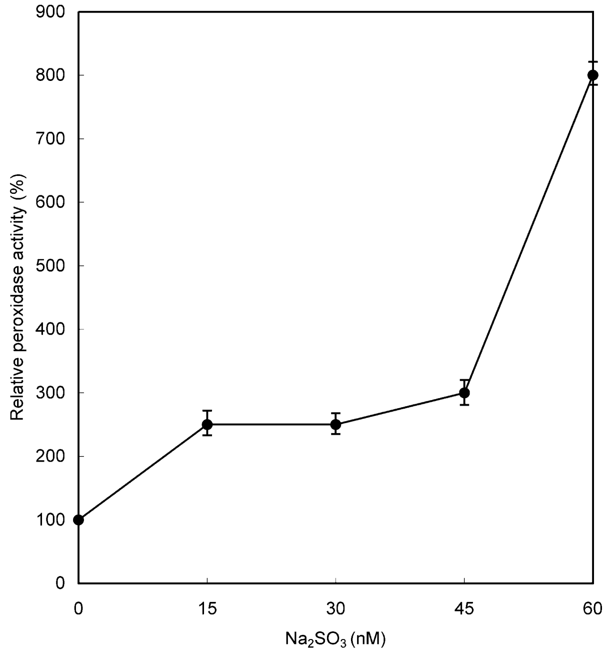

Fig. 2. Effects of Na_2SO_3 on total peroxidase activities in roots of geranium seedlings grown on media containing various concentrations of Na_2SO_3 at pH 4.0. All determinations were made in triplicate and the results are expressed as mean ±s.e.

enhanced intensity of the activity-staining bands. Slight increases of neutral peroxidase bands were also found. These results indicate that the increases in total peroxidase activities with Na_2SO_3 at pH 4.0 were mainly due to the increases in amounts of these isoperoxidases. Increases in specific isoperoxidases were previously reported to be correlated with the defense signals responding to various stresses experienced (Kubo et al., 1995; Lee, 1997a,b; Kang et al., 1999; Yun et al., 2000). Therefore, the activity of strong cationic isoperoxidase, strong anionic isoperoxidase and neutral isoperoxidase seems to increase in order to protect the seedling against Na_2SO_3 exposure.

In order to clarify whether the strong cationic isoperoxidase from geranium seedling, which increased in response to Na_2SO_3, might be the same isoperoxidase as PC3 from geranium callus or not, the strong cationic isoperoxidase from geranium seedling was isolated. Purification was performed by the method of PC3 isolation as previously described (Lee et al., 2001) including $(NH_4)_2SO_4$ precipitation, DEAE-Sephacel chromatography, CM-cellulose chromatography and Sephacryl S-200 gel filtration. The MW, optimum pHs and K_m values for guaiacol and H_2O_2, and pI value of the enzyme were determined and compared with those of PC3 from geranium callus (Table 2). The results show that these two isoperoxidases have the same physicochemical and kinetic properties, indicating that they are the same isoperoxidase.

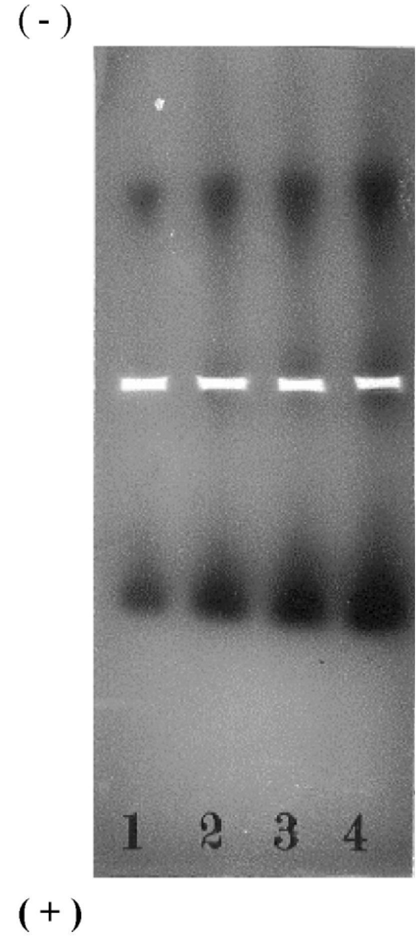

Fig. 3. Changes of isoperoxidase patterns in roots of geranium seedlings grown on media containing various concentrations of Na_2SO_3 at pH 4.0. Lane 1, control; lane 2, roots exposed to 30 nM Na_2SO_3; lane 3, roots exposed to 45 nM Na_2SO_3; lane 4, roots exposed to 60 nM Na_2SO_3. (−): cathode; (+): anode.

Table 2
Comparisons of physicochemical and catalytic properties of the strong cationic isoperoxidase from geranium seedling and PC3 isoperoxidase from geranium callus

Properties		PC3 (callus)[a]	Strong cationic isoperoxidase (seedling root)
MW	SDS-PAGE	58 kDa	58 kDa
	Sephadex G-150	58 kDa	59 kDa
Guaiacol	Optimum pH	6.0	6.0
	K_m	7.3 mM	7.4 mM
H_2O_2	Optimum pH	6.0	6.0
	K_m	3 mM	3 mM
pI		9.1	9.0

[a] Data were supplied from Lee et al. (2001).

Isoperoxidase PC3 from geranium callus and the strong cationic isoperoxidase from Na_2SO_3-treated seedlings had the same optimum pH of 6.0 toward guaiacol (Table 2), although the optimum pH range using guaiacol was between pH 5.0 and 6.5 (Lee and Kim, 1994). Therefore, the reason for the notable increases in total peroxidase and specific isoperoxidase levels at pH 4.0, might best be explained by the defense mechanism against ROS generated from Na_2SO_3 attack. Since this pH is far from the optimum pH for guaiacol oxidation. Furthermore Na_2SO_3 is known to release SO_2 in apoplastic water and free radical transfer might occur also in the hydrophobic regions (Player and Horton, 1981).

2.4. Effects of Na_2SO_3 on the various antioxidant enzymes in geranium seedlings

Many studies have been performed on the enhancement of plant tolerance to oxidative stress by modifying the plant antioxidant defense system (Allen, 1995; Vitoria et al., 2001). It has been shown that peroxidase, catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) are the major constituents of the plant antioxidant enzyme system, which operate by scavenging ROS (Donahue et al., 1997; Prasad, 1997). In addition, it is believed that the changes in antioxidant enzymes induced by oxidative stress might be due to synthesis of new isozymes or enhancement of activities of existing antioxidant enzymes for metabolism of ROS. In Fig. 4, the alterations of antioxidant enzymes such as CAT, SOD and GR following Na_2SO_3 application were thus also examined. In this regard the primary site of SO_2 injury was reported to be the chloroplast (Pfanz et al., 1993) and on this study it was found that an increase in SOD activity was proportional to the amount of Na_2SO_3, also about a 3.7-fold increase of SOD activity was found at 60 nM Na_2SO_3. Besides peroxidase and SOD, CAT and GR were also suggested to act in relation with removal of ROS (Mehlhorn et al., 1987; Rao et al., 1996; Donahue et al., 1997; Yun et al., 2000). CAT dismutates H_2O_2 into H_2O and O_2, whereas peroxidase decomposes H_2O_2 by peroxidation of co-substrates such as phenolic compounds. There were no significant alterations of CAT activities in Na_2SO_3 treated seedlings, while notable enhancements of GR activities were observed (Fig. 4), i.e. about 5.5-fold and 6-fold enhancement of GR activities were found in seedlings treated with 45 and 60 nM Na_2SO_3, respectively. On the other hand, the results obtained did not show alterations in CAT activity, which could have been the case if H_2O_2 levels were increased in the peroxisome. Instead increases in GR activity were observed, also being involved in H_2O_2 removal through the ascorbate-glutathione cycle in the chloroplast (Donahue et al., 1997). In this regard, sulfite pollutants were reported to increase the activities of ascorbate peroxidase and guaiacol peroxidase in *Arabidopsis* leaves, while having little effect on the activities of SOD, CAT, ascorbate reductase and GR (Kubo et al., 1995). On the other hand, the levels of peroxidase and GR were approximately twice in response to SO_2-treatment in peas (Mehlhorn et al., 1987). An increase in GR activity and new isozyme induction due to SO_2 and ozone exposure were also reported in several plants including wheat seedlings (Rao et al., 1995). Moreover, there are differential responses of SOD in two pea cultivars during exposure to SO_2. The cultivar progress showed an increased activity of SOD, whereas SOD activity decreased in the cultivar nugget in response to SO_2 (Madamanchi et al., 1994). Oxidative stresses such as engenderd methyl viologen treatment also markedly enhanced peroxidase activity in

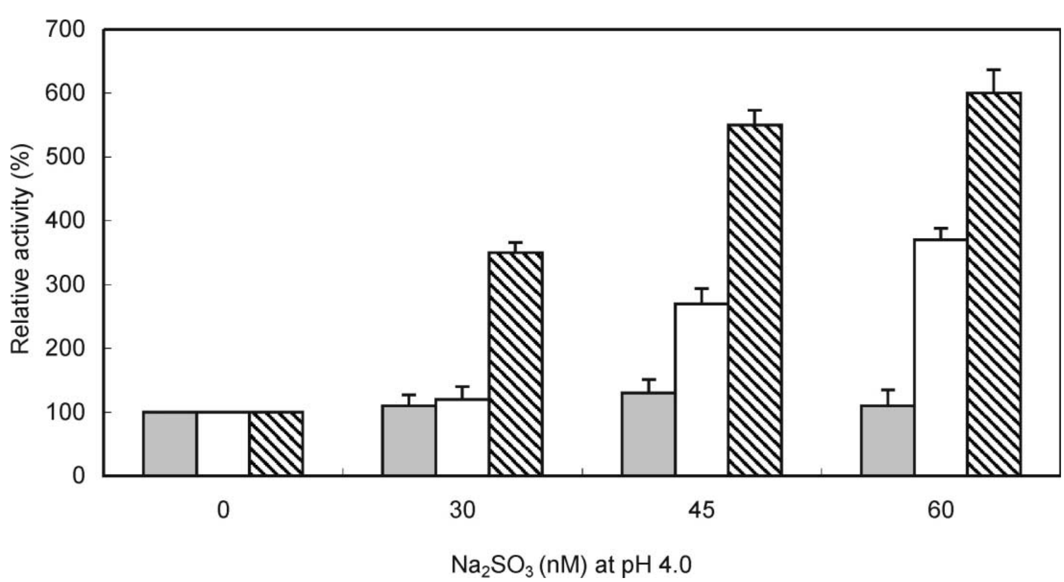

Fig. 4. Effects of Na_2SO_3 on activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) in geranium seedlings grown on media containing various concentrations of Na_2SO_3 at pH 4.0. Solid, open and hatched bars are, respectively, CAT, SOD and GR activities.

transgenic and nontransgenic tobacco; however, nontransgenic tobacco exhibited the greatest increase (about 12-fold) in SOD level compared with the transgenic plant by methyl viologen treatment. In contrast, CAT activity decreased after methyl viologen treatment in transgenic and nontransgeic tobacco (Yun et al., 2000). It remains to be seen whether these alterations in enzyme activities are due to alterations in gene transcription and de novo protein synthesis, or are due to posttranslational modification of pre-existing enzymes.

3. Experimental

3.1. Plant material

Scented-geranium seeds were sterilized with 30% H_2O_2 for 20 min, and incubated for 1 h in sterile distilled water. Geranium seedlings were grown on MS0 media for 4 weeks in the presence of various concentrations of Na_2SO_3 at indicated pH under sterile conditions. The controlled conditions were as follows: 14 h photoperiod, day/night air temperature between 24 and 18 °C, and relative humidity 60–75%.

3.2. Preparation of enzyme extracts

Roots of geranium seedlings were homogenized in 50 mM sodium phosphate buffer (pH 6.0) containing sea sand. The homogenate was centrifuged at $10{,}000\times g$ for 30 min and the supernatant was stored in separate aliquots at −20 °C until used for enzyme analyses.

3.3. Effects of Na_2SO_3 on plant growth

The effects of Na_2SO_3 on the plant growth were investigated in seedlings grown on media containing increasing concentrations of Na_2SO_3 at pH 4.0. The primary root length and lateral root numbers were measured in the seedlings. All determinations are expressed as the mean ±s.e. of three separate experiments.

3.4. Determination of the contents of chlorophyll and Rubisco protein

Chlorophyll concentration was determined by the method of Agrawal and Agrawal (1991), with total chlorophyll concentration being determined by sum mation of chlorophylls a and b. Determination of Rubisco protein was performed using the method of Kang et al. (1999). The Rubisco protein bands bound to CBB-R dye on the SDS-PAGE gel were eluted with formamide, and the absorbance of the formamide-CBB-R band was measured at 595 nm. The amounts of Rubisco proteins were estimated as absorbance values.

3.5. Peroxidase activity assays

Peroxidase activity with guaiacol as substrate was assayed by a modified procedure of Lee and Kim (1994). The assay mixture contained 40 mM phosphate buffer, 15 mM guaiacol, 5 mM H_2O_2 and 50 µl of enzyme preparation in a total volume of 1 ml. The reaction was initiated by the addition of H_2O_2 and the increase in absorbance at 470 nm was measured for 1 min. All determinations were made in triplicate and the results are expressed as mean ±s.e.

3.6. Starch gel electrophoresis

Starch gel electrophoresis was performed as described by Lee and Kim (1994). Isoperoxidase bands were visualized by placing the gel in a solution of 100 mg of 3-amino-9-ethylcarbazole in 10 ml of DMF, 184 ml of NaOAc buffer (pH 5.0), 10 ml of 100 mM $CaCl_2$ and 0.2 ml of 30% H_2O_2.

3.7. Purification of the strong cationic isoperoxidase from geranium seedlings

The enzyme extract was adjusted to 90% saturation with $(NH_4)_2SO_4$. The pellet from $(NH_4)_2SO_4$ treatment was then dissolved in minimum volume of 5 mM Na-Pi buffer (pH 6.0) and dialyzed against the same buffer. The crude enzyme preparation was loaded on a DEAE-Sephacel ion exchange column (3.5×12 cm) pre-equilibrated with 5 mM Na–Pi buffer (pH 6.0). The column was washed with the same buffer until the absorbance of the eluant containing all cationic proteins at 280 nm became zero. Eluants from DEAE-Sephacel column containing all cationic proteins were dialyzed against 30 mM Na–Pi buffer (pH 6.0) overnight. The dialyzed sample was applied on a CM-cellulose ion exchange column (2.5×4 cm) preequilibrated with 30 mM Na–Pi buffer (pH 6.0), and the strong cationic isoperoxidase was obtained by step-wise elution with 50 mM Na–Pi buffer (pH 6.0). Fractions containing the strong cationic isoperoxidase were applied to a Sephacryl S-200 column (1.3×110 cm) preequilibrated with 50 mM Na–Pi buffer (pH 6.0), and the purified enzyme solution thus obtained was used in this study.

3.8. Antioxidant enzyme assays

Catalase (CAT) activity was determined spectrophotometrically by measuring the decline in A_{240} due to H_2O_2 decomposition (Rao et al., 1996). Superoxide dismutase (SOD) activity was determined by using a xanthine/xanthine oxidase/NBT system. The inhibition of cytochrome c reduction by SOD was measured by the reduction of NBT (Asada et al., 1974; Vitoria et al., 2001). Glutathione reductase (GR) activity was determined by the method of Polle et al. (1990). The rate of reduction of oxidized glutathione was monitored by measuring the increase in A_{412} over 2 min. All determinations were made in triplicate and the results are expressed as mean $\pm$s.e.

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