Superoxide Dismutase and Arbuscular Mycorrhizal Fungi: Relationship between the Isoenzyme Pattern and the Colonizing Fungus

JULIA MARTIN[1], INMACULADA GARCIA-ROMERA[1*],
JUAN A. OCAMPO[1], and JOSE M. PALMA[2]
[1]*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, C.S.I.C., Apdo. 419, 18008 Granada, Spain, E-mail. inmaculada.garcia@eez.csic.es; and* [2]*Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, C.S.I.C., Apdo. 419, 18008 Granada, Spain, Tel. +34-58-121011, Fax. +34-58-129600*

Abstract

Total activity and the isoenzymatic pattern of superoxide dismutase (SOD; EC 1.15.1.1) were studied at different growth times in the following arbuscular-mycorrhizal (AM) symbioses: *Trifolium pratense-Glomus mosseae, T. pratense-Glomus intraradices, Allium cepa-G. mosseae,* and *A. cepa-G. intraradices.* In nonAM red clover, total SOD activity increased two-fold in 50- and in 80-day-old plants, compared to 15- and 30-day-old plants. However, in plants inoculated with AM fungi, the activity only rose to the same level after 80 d of growth. In contrast, no changes in SOD activity were detected in similar experiments carried out with onion. The results obtained from red clover are discussed in terms of a possible shift in the plant roots from control growth conditions to a senescent stage. Analysis of SOD isozymes in the symbioses studied showed the presence of one Mn-SOD and two CuZn-SODs (CuZn-SOD I and CuZn-SOD II) in each plant species at 15 d of growth. In both red clover and onion, two new CuZn-SODs (named CuZn-SOD 1 and CuZn-SOD 2) were detected in tissues older than 30 d, besides the constitutive isozymes indicated above. Both *T. pratense-G. mosseae,* and *A. cepa-G. mosseae*

*The author to whom correspondence should be sent.

symbioses also expressed a specific isozyme (mycCuZn-SOD), which was absent in the symbioses with *G. intraradices*. The expression of mycCuZn-SOD could only be detected in material older than 30 d. The fungal origin of this isozyme is discussed in terms of its specificity in the symbioses of plants with *G. mosseae*.

1. Introduction

Superoxide dismutases (SODs; EC 1.15.1.1) are a group of metalloenzymes which catalyze the disproportionation of the powerful toxic superoxide free radical (O_2^-) to H_2O_2 and O_2 (Fridovich, 1995). Therefore, SODs constitute a primary defense against the O_2^--derived deleterious effects which can be produced inside the cell (Fridovich, 1989; Bowler et al., 1992).

There are three types of superoxide dismutases depending on the prosthetic metal involved in the catalytic site of the enzyme, and they are designated as CuZn-SODs, Mn-SODs, and Fe-SODs. Copperzinc-containing superoxide dismutases were typically assigned to eukaryotic organisms (McCord, 1979; Steinman, 1982; Fridovich, 1983), although their presence in prokaryotes have also been reported (Parker et al., 1984; Langford et al., 1992). Manganese-containing superoxide dismutases have been broadly described in prokaryotes as well as in eukaryotes (McCord, 1979; Baum and Scandalios, 1981; Fridovich, 1989; 1995). Iron-containing superoxide dismutases were at first assigned to prokaryotic organisms, although the presence of these isozymes in several plant families have been reported during the last decade (Steinman, 1982; Fridovich, 1983; Salin and Lyon 1983; Asada, 1984; Sevilla et al., 1984).

The SOD isoenzymatic patterns in some symbioses of plants with several microorganisms have been reported earlier. In fact, different studies carried out on SOD in nodules of leguminous plants have indicated the importance of SOD as an antioxidant system in the N_2-fixation process in protecting nitrogenase (Puppo et al., 1982; Dimitrijevic et al., 1984; Becana et al., 1989). More recently, superoxide dismutase isozymes of the mycorrhizal symbiosis *Trifolium pratense-Glomus mosseae* were described (Palma et al., 1993). These authors found three new isozymes in AM roots in addition to the constitutive isozymes detected in nonAM plants. However, in the *Pisum sativum-G. mosseae* symbiosis, no qualitative differences among the isoenzyme patterns in both AM and nonAM roots were observed, although higher SOD activity was detected in AM pea roots (Arines et al., 1994a).

To date, there is neither strong evidence for a specific role for SOD in the symbiosis, nor are data available as to which symbiont (plant or fungus)

synthesizes the new SOD isozymes. A more thorough study using several plant and fungal species, as well as different growth conditions was needed, in order to clarify this aspect. The SOD isoenzyme patterns in several mycorrhizal symbioses, at several growth stages, are reported here. The specificity of the symbiosis on the pattern of the SOD isozymes is also discussed.

2. Materials and Methods

Biological material and growth conditions

Plants were grown in 0.3 l pots of a sand:vermiculite mixture (1:1, v:v). This mixture was autoclaved at 120°C for 20 min. Onion (*Allium cepa* L. cv. Babosa) and clover (*Trifolium pratense* L. cv. Huia) were used as test plants. Seeds were sown in moist sand, and after 2 weeks seedlings were transplanted to pots and grown under greenhouse conditions. Natural light was supplemented with Silvania incandescent and cool-white lamps, 400 mmol m^{-2} s^{-1}, 400–700 nm, with a 16/8 h light/dark cycle at 25/19°C and 50% relative humidity. Plants were regularly watered from below by capillarity, and fed with a diluted (1/2) Long-Ashton nutrient solution (Hewitt, 1952) plus 50 mg ml^{-1} K_2HPO_4 (pH 7.0).

The AM inoculum consisted of 5 g of rhizosphere soil from alfalfa pot culture of either an isolate of *G. mosseae* (Nicol. & Gerd.) Gerd. and Trappe, or *G. intraradices* (Nicol. & Gerd.) Gerd. and Trappe which contained spores (10 sporocarps per g with 1 to 5 spores per sporocarp of *G. mosseae*, and 100 spores per g of *G. intraradices*), mycelium and colonized root fragments. Uninoculated plants were given filtered leachings from the inoculum soils. Soil filtrate (Whatman No.1 filter paper) from the rhizosphere of AM plants was added to the nonAM treatment. The filtrate contained common soil microorganisms, but no AM propagules. Due to the nature of the filtrates used in our growth conditions (soil filtrates), both AM and nonAM plants were grown in the presence of natural *Rhizobium* strains; no other *Rhizobium* inoculation was carried out.

Mycorrhizal measurements

Plants were harvested after 15, 30, 50 and 80 d of growth. Roots were then washed and rinsed three times with sterilized distilled water and part of the material (2 g fresh weight) was cleared and stained (Phillips and Hayman, 1970). The percentage of total root length colonized by AM fungi was measured by the grid-line intersect method (Giovannetti and Mosse, 1980).

Preparation of extracts for enzyme assays

Roots (20 g, fresh weight) were frozen in liquid nitrogen and pulverized in a mortar. The resulting powder was homogenized for 2 h at 4°C in 0.1 M potassium phosphate buffer (PK buffer, pH 7.0), plus 2% (w/v) polyvinylpolypyrrolidone (PVPP), 0.15 mM NaCl, 3 mM KCl, 26 mM ß-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), at a ratio of 1:3 (w:v). The homogenate was filtered through several layers of cheesecloth, centrifuged at 20,000 g for 20 min, and the pellet resuspended and washed by centrifugation with the same buffer three times. The final supernatant was concentrated with an Omega membrane (10 kDa, Filtron Tech. Co.) and dialyzed overnight against several hundred volumes of diluted (1:9; v:v) PK buffer. The dialyzed solution was cleared by centrifugation at 25,000 g for 20 min and used as a crude extract for further analyses.

Superoxide dismutase activity and protein assay

Total superoxide dismutase activity was determined by following the reduction of ferricytochrome c by superoxide radicals generated by a xanthine/xanthine oxidase system, and one unit of activity was defined as the amount of enzyme necessary to inhibit the reduction of cytochrome c in a ratio of 50% (McCord and Fridovich, 1969). SOD isozymes were separated by nondenaturing linear gradient electrophoresis on 10% acrylamide slab minigels (MiniProtean II, Bio-Rad), and detected by photochemical reduction of nitroblue tetrazolium, according to Beauchamp and Fridovich (1971). The different types of SOD were identified by performing the activity stain in gels previously incubated for 30 min at 25°C, in 50 mM K-phosphate buffer, pH 7.8, containing either 2 mM KCN, or 5 mM H_2O_2. CuZn-SODs are inhibited by KCN and H_2O_2; Fe-SODs are resistant to CN^- but inactivated by H_2O_2, and Mn-SODs are resistant to both inhibitors (Fridovich, 1989).

Protein content of samples was determined by the method described by Bradford (1976), using the Bio-Rad kit, and crystalline bovine serum albumin (Fraction V) to standardize the assay.

The results were evaluated statistically using Duncan's multiple range test (p = 0.05).

3. Results

Microscopic observations of stained roots showed no endophyte fungi in control plants and AM structures in the inoculated plants. Percentage AM root

Table 1.   Percentage AM root length colonization in onion and clover inoculated with *G. mosseae* and *G. intraradices*.

Plants	Endophyte	% Root length colonization after (days)			
		15	30	50	80
Onion	*G. mosseae*	11 a	35 b	51 c	62 d
	G. intraradices	10 a	32 b	53 c	61 d
Clover	*G. mosseae*	15 a	33 b	46 c	60 d
	G. intraradices	14 a	31b	47 c	65 d

Values of AM root length colonization of onion and clover sharing the same letter were not significantly different according to Duncan´s multiple range test (P = 0.05).

Table 2.   Total superoxide dismutase activity in crude extracts from uninoculated, *Glomus mosseae*-inoculated, and *Glomus intrarradices*-inoculated red clover plants at different ages. SOD activity is expressed as units mg^{-1} protein.

Treatment	Time (days)			
	15	30	50	80
Uninoculated	22.1 a	26.3 a	48.6 b	49.8 b
G. mosseae	25.2 a	27.9 a	27.3 a	46.9 b
G. intrarradices	26.0 a	26.3 a	27.8 a	49.2 b

Each value represents the mean of five pots. Data followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

Table 3.   Total superoxide dismutase activity in crude extracts from uninoculated, *Glomus mosseae*-inoculated, and *Glomus intrarradices*-inoculated onion plants at different ages. SOD activity is expressed as units mg^{-1} protein.

Treatment	Time (days)			
	15	30	50	80
Uninoculated	18.4 a	16.2 a	21.4 a	14.6 a
G. mosseae	17.1 a	17.1 a	19.5 a	20.6 a
G. intrarradices	17.1 a	16.0 a	14.6 a	17.6 a

Each value represents the mean of five pots. Data followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

length colonization was similar in onion and clover after 15, 30, 50 and 80 d of plant growth, and from 15 d after transplanting, AM colonization showed the usual logarithmic growth pattern until 80 d (Table 1).

In red clover plants, no differences in total SOD activity were detected when AM and nonAM roots were analyzed after 15 and 30 d of growth. Neither symbiosis of this plant with *G. mosseae* or with *G. intraradices* gave rise to changes in SOD activity at these growth stages (Table 2). However, 50- and 80-day-old nonAM roots showed total SOD activity two-times higher than that found at 15 and 30 d of growth. By contrast, both AM roots (*T. pratense-G. mosseae* and *T. pratense-G. intraradices*) showed the higher SOD activity only after 80 d of growth of plants, and the values obtained were similar to those determined in nonAM roots at that time (Table 2).

Analysis of the SOD isoenzymatic pattern of nonAM red clover roots by using inhibitors, revealed the presence of three isozymes in 15-day-old plants: one Mn-SOD and two CuZn-SODs, designated as CuZn-SOD I and CuZn-SOD II, according to their increasing electrophoretic mobility (Fig. 1A). The same isozyme pattern was also observed for the two plant-fungal symbioses at that age (Fig. 1A). After 30, 50, and 80 d of growth, all mycorrhizal *T. pratense-G. mosseae*, mycorrhizal *T. pratense-G. intraradices* and nonAM roots showed two new bands whose formation was inhibited by CN^-, and therefore, were assigned to the CuZn-SOD type (results not shown). The newly detected SOD isozymes were named CuZn-SOD 1 and CuZn-SOD 2. Fig. 1B shows the isoenzymatic pattern of 50-day-old plants, which is representative of the pattern also found for 30- and 80-day-old plants.

Interestingly, an additional band showing SOD activity, which was slower than CuZn-SOD I, appeared specifically in the red clover-*G. mosseae* symbiosis in 30-, 50- and 80-day-old plants (Fig. 1B). However, a similar isozyme was not found in the red clover-*G. intraradices* symbiosis at any of those ages. The new isozyme, only present in the symbiosis with *G. mosseae*, was designated as mycCuZn-SOD.

Similar analyses of total and isoenzymatic SOD activity were also carried out on AM onion plants. However, in this plant no changes in the total SOD activity were observed at any of the ages studied, whether they were symbiotic or not. Besides, no quantitative differences were found by inoculating plants with both mycorrhizal fungi (Table 3).

By analyzing the SOD isozyme pattern obtained by native PAGE, a parallelism among the results found in onion and those described above for red clover was observed. After 15 d of growth, one Mn-SOD and two CuZn-SODs (I and II) were found in nonAM and in both AM roots (Fig. 2A). As also observed in red clover, in 30-, 50-, and 80-day-old onion plants, two new isozymes (CuZn-SOD 1 and CuZn-SOD 2) appeared (Fig. 2B). Also, one isozyme of the CuZn-

SOD group was specifically detected in the onion-*G. mosseae* symbiosis (Fig. 2B). This new isozyme was also designated as mycCuZn-SOD, and had the same electrophoretic behaviour as the one determined for mycCuZn-SOD from the red clover-*G. mosseae* symbiosis.
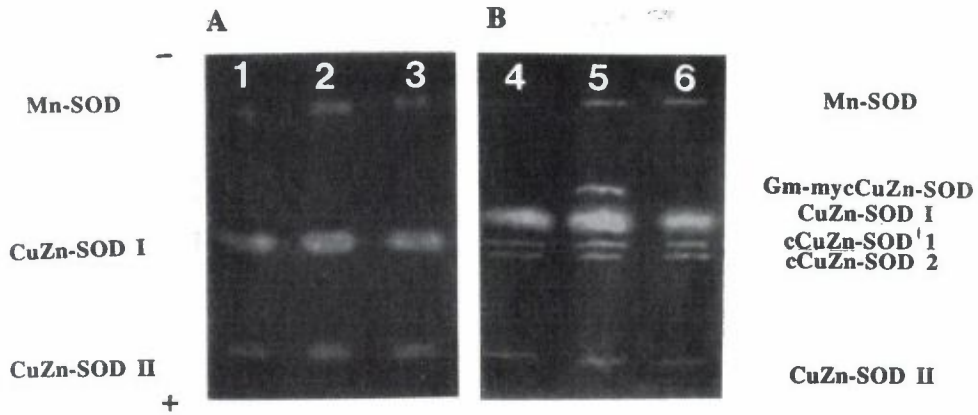

Figure 1.   Isozymes of SOD in crude extracts from uninoculated, *Glomus mosseae*-inoculated, and *Glomus intraradices*-inoculated red clover plants at 15 (A) and 50 (B) days of growth. Proteins (20–25 mg lane^{-1}) were loaded onto gels and activity in gels was detected by the NBT photochemical reduction method. Lanes 1 and 4, uninoculated roots. Lanes 2 and 5, *G. mosseae*-inoculated roots. Lanes 3 and 6, *G. intrarradices*-inoculated roots.
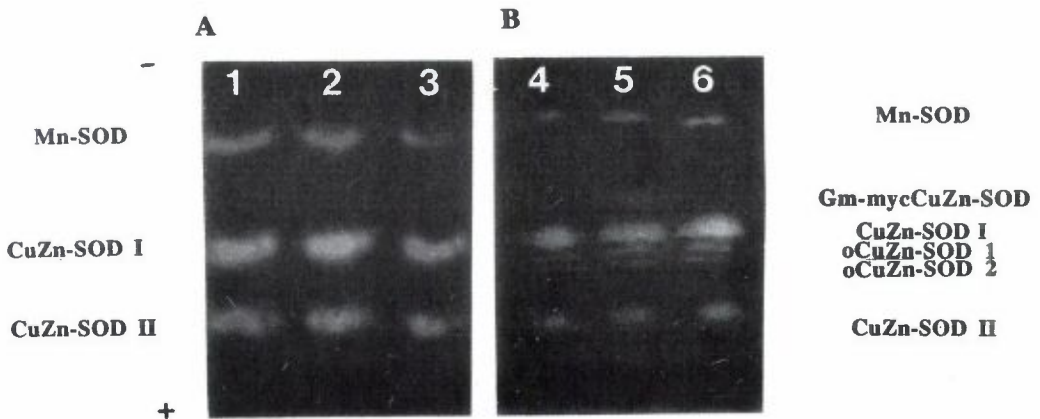

Figure 2.   Isozymes of SOD in crude extracts from uninoculated, *Glomus mosseae*-inoculated, and *Glomus intraradices*-inoculated onion plants at 15 (A) and 50 (B) days of growth. Proteins (35–40 mg lane^{-1}) were loaded onto gels and activity in gels was detected by the NBT photochemical reduction method. Lanes 1 and 4, uninoculated roots. Lanes 2 and 5, *G. mosseae*-inoculated roots. Lanes 3 and 6, *G. intrarradices*-inoculated roots.

4. Discussion

Several reports indicate that an enhanced production of O_2^- radicals, together with the synthesis of new SOD isozymes, could be a protective response of the plant cell induced in response to the entrance of a foreign organism. The expression of inducible SODs has been related to the resistance or susceptibility to rust in coffee plants (Daza et al., 1993). Also, changes in the isoenzymatic SOD pattern has been detected in plants of *Phaseolus vulgaris* L. susceptible or resistant to the pathogen *Uromyces phaseoli* as well (Buonario et al., 1987). Becana et al. (1989) also found that SOD plays an important role in the legumine-*Rhizobium* symbiosis. Recently, changes in the isoenzymatic pattern of SOD in several plant-mycorrhizal fungal symbioses have been reported (Palma et al., 1993; Arines et al., 1994a), and the involvement of the superoxide dismutase enzymatic system in the mycorrhization process has been postulated (Arines et al., 1994b). However, there are still no conclusions about what might be the specific role of SOD in the mycorrhizal symbiosis.

In this work, two kinds of plants, as well as two mycorrhizal fungal species were used to study total and isoenzymatic superoxide dismutase. Both a leguminous (red clover) and a non-leguminous (onion) plant were selected, in order to test whether the obtained results might be due to the effect of nodulation by *Rhizobium*. Also, two fungal species (*G. mosseae* and *G. intraradices*), which showed similar root colonization and plant growth effect in the plant species indicated above (results not shown) were chosen to develop our study.

Total SOD activity in 50-day-old red clover was double than that of 15-day-old plants. A similar increase was also found in inoculated plants, but only after 80 d of plant growth (Table 2). A rise in the activity of some SOD isozymes in senescent tissues of several plant species has been reported (Pastori and del Río, 1994a; Longa et al., 1994). In studies carried out in pea leaves aged 50 d, an increase in SOD activity has also been detected, and the tissue showed clear symptoms of senescence (Pastori and del Río, 1994b). Red clover is phyllogenetically close to pea, and their respective SOD isoenzymatic patterns are similar (Palma et al., 1993). The increase in the SOD activity found in uninoculated red clover plants might be the result of senescence of the plant material. The delay in the increase in SOD activity in inoculated plants could be due to the presence of the symbiotic fungus which supports the plant with nutrients until later stages, thus delaying plant senescence.

The isoenzymatic SOD patterns of red clover and onion plants at 15 d of growth showed three isozymes (Figs. 1 and 2). One Mn-SOD and two CuZn-SODs (I and II). However, in the two plant species studied, 30-, 50-, and 80-day-old plants showed two new bands which were inhibited by KCN, and were

present in all nonAM, in *G. mosseae*-inoculated, and in *G. intraradices*-inoculated plants (CuZn-SOD 1 and CuZn-SOD 2) (Figs. 1 and 2). Therefore, their expression seems to be mycorrhizal-independent, and may be assigned to a plant origin. In onion plants, two new SOD isozymes also appeared after 30, 50, and 80 d of growth, but no increase in total SOD activity was found at those growth stages.

One additional SOD isozyme was detected in 30-, 50-, and 80-day-old *G. mosseae*-mycorrhizal plants which was absent in *G. intraradices*-inoculated plants (Figs. 1 and 2). This new isozyme, which was cyanide-sensitive, was named mycCuZn-SOD in both symbioses, and presumably is the same as the isozyme mycCuZn-SOD described earlier (Palma et al., 1993). This isozyme is not attributed to a nodule origin, since it was also expressed in onion roots, where no symbiosis with nodule-forming bacteria is established. Moreover, it is not expressed by the plant alone, since it was not detected in nonAM plants. Therefore mycCuZn-SOD seems to be synthesized by *G. mosseae*. In preliminary experiments on SOD activity in *G. mosseae* mycelium, no isozyme behaving as mycCuZn-SOD could be detected. This suggests that this isozyme might be expressed by the fungus after mycorrhization takes place, and this may help the symbiosis to cope against superoxide radicals (O_2^-) generated by an increase in local O_2 pressure, as a consequence of the entrance of the fungus into the plant structure. However, the possible reasons why only *G. mosseae* synthesizes the new SOD isozyme, whereas *G. intraradices* does not is at present being investigated.

On the other hand, it might also be hypothesized that mycCuZn-SOD in both red clover- and onion-*G. mosseae* symbioses is of plant origin, and their expression is elicited in both plant species by the entrance of a foreign organism. Such a mechanism has been postulated for both pathogenic and non-pathogenic infections of plants (Fink et al., 1991; Ivanova et al., 1991; Williamson and Scandalios, 1992; Wojtaszek, 1997). This hypothesis implies that the colonization by *G. mosseae* is different at cellular and molecular levels from that of *G. intraradices*, although plant symbioses with the two fungi do not show any visible differences. Further research has to be conducted in order to know whether *G. mosseae* and *G. intraradices* are metabolically distinct throughout the symbiotic stages in spite of their similar colonization patterns.

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