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NUTRITIONAL EFFECT AND EXPRESSION OF SODs: INDUCTION AND GENE EXPRESSION; DIAGNOSTICS; PROSPECTIVE PROTECTION AGAINST OXYGEN TOXICITY

LUIS A. DEL RÍO, FRANCISCA SEVILLA*, LUISA M. SANDALIO and JOSÉ M. PALMA

*Unidad de Bioquímica Vegetal, Estación Experimental del Zaidín, CSIC, Apdo. 419, E-18080 Granada, Spain; *Unidad de Alimentación y Desarrollo de la planta, CEBAS, CSIC, Apdo. 195, E-30080 Murcia, Spain*

The effect of micronutrient stress (either deficiency or toxicity) on the expression of different superoxide dismutase isoenzymes in plants is reviewed. The induction of Fe-SOD and Mn-SOD by different metals and the potential use of the metalloenzyme system SOD for the appraisal of the micronutrient status of plants, is examined. At subcellular level, evidence for the participation of peroxisomal SOD in the molecular mechanism of plant tolerance to Cu is presented, and the activated oxygen-dependent toxicity of a xenobiotic (clofibrate) in plant peroxisomes is examined.

KEY WORDS: Superoxide dismutase, activated oxygen, plant nutrition, gene expression, metal stress, peroxisomes.

EXPRESSION OF SODs BY NUTRITIONAL STRESS

The presence of metals at the active sites of superoxide dismutases makes that stress situations in certain micronutrients, either deficiency or toxicity, can determine the expression of SODs. The generally accepted essential trace elements for plants, also know as micronutrients, are Fe, Mn, Cu, Zn, B, Cl, and Mo.[1] The effect of metal deficiencies on the isozyme pattern of SOD has been studied in several plant species. In leaves of pea plants (*Pisum sativum* L.) grown under limiting Mn nutrient levels a statistically significant inhibition of isozyme Mn-SOD was found, which was simultaneous with an increase in the level of the CuZn-SODs present in this plant species.[2] So, a restriction in the nutrient concentration of Mn can inhibit the synthesis of isozyme Mn-SOD but, apparently there exists a compensatory mechanism involving the induction of CuZn-SODs, in order to keep an adequate level of SOD for the cell protection against indirect deleterious effects of superoxide radicals. In a parallel work, equivalent results were obtained with the SODs of the fungus *Dactylium dendroides* when it was grown under Cu-limiting conditions.[3] In this case, there was a decrease in the activity of CuZn-SOD and a compensatory increase in Mn-SOD, in such a way that the total SOD activity of the cell remained constant. These results also suggested that the biosynthesis of the CuZn- and Mn-containing enzymes was coor-

Correspondence: Prof. Luis A. del Río, Unidad de Bioquímica Vegetal. Estación Experimental de Zaidín. (CSIC), Apdo. 419, E-18080 Granada, Spain.

dinated, and that the decrease in CuZn activity at low Cu concentration was a result of decreased synthesis of that protein rather than the production of an inactive apoprotein.[4] More recently, the response to Cu in *S. cerevisiae* has been reexamined with the conclusion that the induction of CuZn-SOD by copper is related to a sequence of events requiring both Cu and O_2.[5] A counterbalancing effect of the activity of SOD isozymes under Mn deficiency has also been demonstrated lately in soybean plants.[6]

The effect of Cu deficiency on SOD has been studied by Barón and Sandmann in *Pisum sativum* L. plants.[7] Copper deficiency in pea plants is difficult to obtain due to the small amounts of copper required for normal plant development (1 μM), but using two subsequent generations grown under Cu-deficient conditions, these authors obtained a more strongly expressed Cu deficiency. Under these conditions, there was a significant depression of CuZn-SODs which was parallel to an increase in the activity of Mn-SOD. Moreover, under copper deficiency a weak band of CN-resistant and H_2O_2-sensitive SOD, that is a Fe-SOD, was detected.[7] This Fe-containing SOD is not found in pea plants under normal nutrient conditions, and these results suggest that this isozyme could be induced as a result of Cu-restriction.

All these compensatory effects of SOD isozymes produced by Cu and Mn deficiencies indicate that the biosynthesis of SOD isozymes is interdependent and coordinated, and imply that the concentrations of the metal ions which are the prosthetic groups of the different types of SOD determine the balance between these isoenzymes.

On the other hand, excess metal concentrations can also induce SOD isozymes. The effect of excess nutrient levels of Zn and Mn on the activity of the leaf metalloenzymes catalase and SODs in pea plants, was studied. Moderately high nutrient levels of Zn (16 μM) and, to a lesser extent of Mn (90 μM), inhibited the growth of plants and produced an increase in the activities of Mn-SOD and catalase,[8] and the higher activity of Mn-SOD was found to be due to a new Mn-SOD isozyme. The relationship between catalase and total Mn-SOD activity in leaves from plants grown at high Zn and Mn nutrients levels, suggested the existence of a functional link between both metalloenzymes in the plant cell to remove the possible toxic effects of H_2O_2 and O_2^-. These results agreed with previous experiments where catalase and isozyme Mn-SOD were found to display similar activity patterns in leaves of different ages during development of *Pisum sativum* L. plants.[9] The induction of the Mn-SOD isozyme could take place as a result of interactions occurring between micronutrients within the plant which can produce synergistic and antagonistic effects in the plant nutrient status. If, as a consequence of metal interactions, the availability to the plant cell of either Mn, Cu, or Zn is altered and the synthesis of a determined SOD isozyme is limited, this situation could trigger a cellular response by inducing another SOD isozyme, whose prosthetic metal is available, in order to maintain an adequate level of SOD activity in the plant cell against indirect damage by O_2^- radicals. Alternatively, the production of O_2^--derived toxic species by an increase in the intracellular level of metals, could also induce the biosynthesis of Mn-SOD.

Salt stress by NaCl has been described to produce changes in the pattern of the SOD isozymes. Kayupova and Klyshev have found that under salinity there is an intensified formation of activated oxygen species in root cells, mainly $^1O_2$ and $^{\cdot}OH$, which could have an important role in the mechanism of salt injury.[10,11] In rice, the inhibition of seed germination by salinity was found to be correlated with a reduction in peroxidase and SOD activity, with a CuZn-SOD isozyme being more strongly suppressed than the other enzymes.[12] The effect of high NaCl concentrationss on the

activity of the metalloenzymes SOD, catalase, and cytochrome *c* oxidase was studied recently in leaves from bean plants (*Phaseolus vulgaris* L.)[13]. At 15 days growth, the level of Mn-SOD decreased slightly with salinity but this was compensated by a significant increase in the activity of isozyme CuZn-SOD I. However, this compensatory effect did not take place at 30 days growth, and activities Mn-SOD and CuZn-SOD II both diminished as well as catalase, whereas there was a rise in cytochrome *c* oxidase and fumarase activities. These results suggest that in leaves from plants under salt stress, an enhanced production of O_2^- and H_2O_2 could take place concomitant with a decrease in the enzymatic defenses against these forms of activated oxygen.

Nevertheless, salinity effects on SODs are difficult to interpret in higher plants and there always remains the possibility of indirect effects not specifically related to salt stress. No doubt, on this matter like in all the other nutritional experiments just mentioned, information is lacking on the genetic control of SOD isozyme production under different nutritional situations.

INDUCTION OF SODs AND DIAGNOSTIC VALUE

Iron-containing SODs were first thought to be exclusively restricted to procaryotic organisms and some eucaryotic algae. The distribution of SODs in 43 families of vascular plants was studied by Bridges and Salin,[14] and they found that Fe-SOD was present only in the families Gingkoaceae, Nymphaceae, and Cruciferae (Table I). Later on, the occurrence of Fe-SOD was also demonstrated in the plant families Rutaceae, Solanaceae, Leguminosae, Caryophyllaceae, and Rubiaceae (Table II). Interestingly, in leaves of *Coffea arabica* L. Fe-SOD was the most abundant isozyme and represented about 50% of the total SOD activity.[21] Therefore, it seems that Fe-SOD has a wider distribution in the plant kingdom than was previously thought.

In Fe-deficient lemon leaves the induction of SODs by Fe(II) was studied by Sevilla *et al.* By isoelectric focusing of crude extracts from lemon leaves (*Citrus limonum* R.) the occurrence of 9 SODs was demonstrated.[22] Excised Fe-deficient leaves were vacuum-infiltrated with 5 mM $FeSO_4$, preincubated in the dark for 24 h, and then illuminated under controlled conditions of aeration, temperature and humidity during 24–48 h. Iron deficiency produced a considerable depression in the activity of Fe-SODs and Mn-SODs but vacuum-infiltration with Fe(II) solutions brought about the induction of both Fe-SOD and Mn-SOD isozymes.[22]

TABLE I
Presence of Fe-SOD in higher plants

Plant family	Plant species	No. of isozymes	Ref.
Cruciferae	*Brassica campestris*[a]	1	14
	Brassica rapa	1	
	Brassica oleracea	1	
	Raphanus sativus	1	
	Rorippa sessiliflora	1	
Nymphaceae	*Nymphaeae odorata*	1	14
	Nuphar luteum[a]	4	
Ginkgoaceae	*Ginkgo biloba*[a]	2	14

[a]Plant species where Fe-SOD has been purified and characterized

TABLE II
Presence of Fe-SOD in higher plants

Plant family	Plant species	No. of isozymes	Ref.
Rutaceae	*Citrus limonum*[a]	3	15,16
	Citrus sinensis	2	
	Citrus paradisi	2	
	Citrus aurantium	1	
	Citrus reticulata	1	
Solanaceae	*L. esculentum*[a]	1	17
Leguminosae	*V. unguiculata*	1	18
	P. vulgaris	1	19
Caryophyllaceae	*D. caryophyllus*	2	20
Rubiaceae	*Coffea arabica*	1	21

[a]Plant species where Fe-SOD has been purified and characterized

In bacteria and some eucaryotic organisms the induction of SOD isozymes in response to pO_2 or to enhanced rates of intracellular O_2^- production has been reported.[23,24] The effect of Fe(II) on *Citrus limonum* L. Fe-SODs agrees with results described in *E. coli* by Pugh and Fridovich.[25] If this mechanism also operates in lemon leaves, it seems likely that the supply of Fe(II) to Fe-deprived lemon leaves would favour the conversion of apo-SOD to active Fe-SOD. However, the possible involvement of O_2^- radicals in the induction of lemon Fe-SODs as a protection of chloroplasts against the restored production of this activated oxygen species by photosystem I,[26] cannot be discarded. On the other hand, the induction of Mn-SOD isozymes observed after vacuum-infiltration with Fe(II) could be due to intracellular production of O_2^- radicals or other activated oxygen species, as a result of the joined effect of light intensity and oxygenation.[26] Under these conditions, more Mn(III) would be available and, accordingly, more Mn-SOD would be produced, since Mn(III) competes more favourably with Fe(II) for the apo-SOD.[25]

A metal-independent induction of the Mn-containing SODs was observed when Fe-deficient leaves, previously vacuum-infiltrated with water, were subjected to illumination under continuous aeration.[22] The specific activity of Mn-SODs underwent about a three-fold increase after 48 h illumination, whereas Fe-SOD isozymes showed only minor changes under similar conditions. The induction of mitochondrial and/or peroxisomal Mn-SOD activity by the action of light under oxygenation implies an induction mechanism mediated by activated oxygen species generated by photooxidative processes.[26] But the question of whether the induction of Mn-SODs is produced through a mechanism involving autogenous repression by the apo-enzyme[25] or by means of a synthesis *de novo* of the protein[23] is something that remains to be seen.

Nutritional studies of SOD in higher plants suggest that the biosynthesis of SOD isozymes is interdependent and is probably coordinately controlled. The detection of a Fe-SOD isozyme in Cu-depleted leaves of pea plants and the induction of Fe-SODs by Fe(II) in Fe-deficient lemon leaves, implies that the presence of Fe-SODs in these higher plants could be due to the expression of silent genes coding for Fe-SODs as a result of environmental pressures, a hypothesis that has been previously formulated to explain the distribution of iron-containing SODs in different plant families.[14]

In the last few years attention has started to be payed to the molecular genetics of plant superoxide dismutases. As Danièle Touati has pointed out, a full understanding of mechanisms involved in the expression and regulation of genes requires their

TABLE III
Diagnosis of metal deficiencies using SODs

Metal deficiency	SOD isozyme	Plant species	Ref.
Mn	Mn-SOD	peas	2
		soybean	6
		lemon	15
Cu	CuZn-SOD	peas	7
		clover	37
Zn	CuZn-SOD	duckweed	38
Fe	Fe-SOD	lemon	15,22

isolation and characterization.[27] In this sense, cDNA has been recently isolated and the nucleotide sequences determined for cytosolic CuZn-SOD from maize[28] and tomato[29] and for chloroplastic CuZn-SOD from petunia,[30] tomato[29] and peas.[31] For Mn-SOD, cDNa has been isolated and the nucleotide sequences determined for the mitochondrial enzyme from maize (SOD 3).[32] The expression of genetically distinct SODs in maize seedlings during development has been studied by Baum and Scandalios.[33] They found that the four SOD isozymes of maize are encoded by four nuclear independent structural genes (Sod 1, Sod 2, Sod 3, and Sod 4). In maize, two of the SODs are compartmentalized in the cytoplasm (SOD-2 and SOD-4 CuZn-SOD); the other two are found in mature form in the chloroplast (SOD-1 CuZn-SOD) and in the mitochondria (SOD-3, Mn-SOD) after being processed from larger precursor polypeptides.[34] SOD-3 has been shown to be synthesized as a precursor (preSOD-3) and is translocated into isolated maize mitochondria.[34] The genetic control of the mitochondrial form of superoxide dismutase has also been studied in hexaploid wheat.[35]

However, in plants there is a lack of information on the genetic control of the expression of the SOD isozymes in response to diverse environmental effects. This knowledge could throw light on many experimental findings now available regarding the nutritional effect and expression of SODs. Studies similar to that carried out on the effect of the herbicide paraquat on the expression of the superoxide dismutase (Sod) genes in maize[36] would be very useful. But, in any case, as indicated by Touati, "few data are presently available on the molecular basis of mechanisms that regulate the expression of SOD".[27] In higher plants, this type of studies are difficult due to the complexity of the plant physiology, compared to other simpler biological systems, like *E. coli* or yeast, where the major part of research on molecular genetics of SOD has been conducted.

Nevertheless, the response of SOD isozymes to changing concentrations of micronutrients can be exploited for the diagnosis of metal deficiencies in plants. On the basis of nutritional experiments made under greenhouse conditions, it can be said that the metalloenzyme system SOD could be employed for estimating the metal nutrient status of plants, as far as Mn, Cu, Fe, and Zn is concerned. In Table III several cases are shown where leaf activities of SOD isozymes were found to be positively correlated with metal nutrient concentrations.

METAL TOLERANCE, SOD, AND PEROXISOMES

The effect of high nutrient levels of copper (240 μM) on the activity of different

metalloenzymes containing Cu, Mn, Fe, and Zn, distributed in chloroplasts, peroxisomes, and mitochondria was studied in leaves of two varieties of *Pisum sativum* L. with different sensitivity to copper (a sensitive and a relatively tolerant variety).[39] The metalloenzymes studied were cytochrome *c* oxidase, catalase, Mn-SOD, CuZn-SOD I and CuZn-SOD II. In leaves of pea plants the subcellular distribution of SODs has been previously studied and is known that Mn-SOD is mainly found in mitochondria and also in peroxisomes; isozyme CuZn-SOD II is located in chloroplasts; and CuZn-SOD I is distributed in the cytosol and in mitochondria.[40,41]

In plants grown in nutrient solutions containing 240 μM Cu^{2+} the activity of mitochondrial SOD isozymes (Mn-SOD and CuZn-SOD I) was very similar in Cu-tolerant and Cu-sensitive plants, whereas cytochrome *c* oxidase was lower in Cu-sensitive plants. Chloroplastid CuZn-SOD activity was the same in the two plant varieties. In contrast, the peroxisomal Mn-SOD activity was considerably higher in Cu-tolerant than in Cu-sensitive plants, and the activity of catalase was also increased in peroxisomes of Cu-tolerant plants. Naiki[42] working with a Cu-resistant strain of yeast grown in high Cu, also found that this metal increased the activity of Mn-SOD rather than CuZn-SOD.

The generation of superoxide free radicals by certain endogenous metabolites has been demonstrated in two types of plant peroxisomes (leaf peroxisomes and glyoxysomes).[43,44] Production of O_2^- in peroxisomal soluble fractions was xanthine- and hypoxanthine-dependent and was due to xanthine oxidase,[43] whereas in peroxisomal membranes NADH induced the generation of superoxide radicals.[43,44] These results suggest that O_2^- production could be a common metabolic property of plant peroxisomes and supports the existence of active oxygen-related roles for peroxisomes in cellular metabolism.

An increase in the peroxisomal concentration of copper, could under appropriate conditions, originate the production of the vastly reactive $^{\cdot}OH$ radicals by a metal-catalyzed Haber-Weiss or superoxide-driven Fenton reaction. Therefore, Cu-tolerant plants could have evolved a protection mechanism against the production in peroxisomes of O_2^--dependent toxic species by high levels of copper by inducing the peroxisomal Mn-SOD and catalase activities. In this way, O_2^- radicals and H_2O_2 could be effectively removed, and the eventual formation of $^{\cdot}OH$, highly toxic for biological membranes, avoided. So, these results indicate a role for peroxisomes in plant cellular metabolism related to copper toxicity, and suggest the involvement of active O_2 species, possibly generated in these oxidative organelles, in the mechanism of Cu lethality. Though further experiments are still necessary, superoxide dismutases appear to have a certain role in the molecular mechanism of plant tolerance to Cu in *Pisum sativum*.

ACTIVATED OXYGEN-DEPENDENT TOXICITY OF XENOBIOTICS AT SUBCELLULAR LEVEL

Certain hypolipidemic drugs can induce the proliferation of the peroxisomal population in some animal tissues, as well as the activity of certain enzymes of these organelles, particularly the H_2O_2-producing acyl-CoA oxidase.[45] Some of these peroxisomal proliferators, like clofibrate (ethyl-α-p-chlorophenoxyisobutirate) are proven carcinogenic agents in animals.[46] The possibility that sustained oxidative stress

resulting from the continued proliferation of peroxisomes might serve as an initiator and promoter in carcinogenesis, with the participation of highly reactive oxygen free radicals, has been suggested.[47,48] In experiments with peroxisomes isolated from plants treated with clofibrate, the activity of different peroxisomal enzymes (SOD, acyl-CoA oxidase, catalase, hydroxypyruvate reductase, and xanthine oxidase) was determined.[49] Results obtained showed that clofibrate stimulated the activity of acyl-CoA oxidase and xanthine oxidase, which are H_2O_2- and O_2^--producing enzymes, respectively. At the same time, catalase and Mn-SOD activities were both depressed.[49] In intact leaves incubated with clofibrate, the cytomorphology was studied by electron microscopy and the population of cell peroxisomes was counted after cytochemical staining of these organelles with diaminobenzidine (DAB). There was a considerable increase in the number of catalase-depleted peroxisomes and this demonstrated that clofibrate induced the proliferation of peroxisomes in plant leaves. The NADH-dependent production of superoxide radicals was studied in peroxisomal membranes and it was found that this was enhanced by incubation with clofibrate, as well as the lipid peroxidation rate.[49]

These results suggest that clofibrate, apart from proliferating the number of plant peroxisomes, also originates an overproduction of oxygen free radicals in these organelles (O_2^- and perhaps also $^{\cdot}OH$). The mechanism of toxicity of this xenobiotic, mediated by activated oxygen species (H_2O_2 and O_2^-) at the level of plant peroxisomes, perhaps could also be operative in peroxisomes from certain animals where clofibrate and other hypolipidemic drugs have been demonstrated to be carcinogenic.

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