Antioxidant enzymes as biochemical markers for sharka resistance in apricot

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Abstract

The activity of antioxidant enzymes in different apricot cultivars, resistant or susceptible to PPV, was analyzed during the years 2002 and 2003. Resistant cultivars showed higher activities of antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) than susceptible cultivars. Five superoxide dismutase (SOD) isozymes (CuZn-SODs) were detected in the apricot cultivars. However, no correlation was observed between this isozyme pattern and the resistance to PPV. On the other hand, PPV-resistant apricot cultivars showed a greater capability for elimination of H_2O_2 and recycling of ascorbate-glutathione cycle (ASC), and they have at least two of these enzymatic activities (CAT, APX and DHAR) over the average. This fact could contribute, among other factors, to their resistance to PPV. In contrast, this response was not observed in the susceptible cultivars. All these data suggest that the activity level of antioxidant enzymes CAT, APX and DHAR could be used as biochemical markers of PPV resistance in apricot and open a way for the further studies necessary for a better understanding of the role of antioxidant enzymes in the PPV resistance in *Prunus*.

Additional key words: Prunus armeniaca, Plum pox virus, susceptibility

Abbreviations: AOS - activated oxygen species; APX - ascorbate peroxidase; ASC-GSH cycle - ascorbate-glutathione cycle; CAT – catalase; CuZn-SOD – copper,zinc-containing superoxide dismutase; DHAR – dehydroascorbate reductase; Fe-SOD - iron-containing superoxide dismutase; GR - glutathione reductase; GPX - glutathione peroxidase; H_2O_2 - hydrogen peroxide; MDHAR - monodehydroascorbate reductase; Mn-SOD - manganese-containing superoxide dismutase; $O_2^{\cdot -}$ - superoxide radicals; $^{\cdot}OH$ - hydroxyl radicals; POX – peroxidase; PPV – Plum pox virus; SEO – Stark Early Orange; SOD -superoxide dismutase; WClMV - White clover mosaic potexvirus.

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Introduction

Sharka, a disease caused by Plum pox virus (PPV), is a serious limiting factor for temperate fruit production in affected areas. PPV affects most *Prunus* species, resulting in severe economic losses in apricot, plum, prune and peach (Németh 1994). Described for the first time in Bulgaria in 1917, sharka has spread throughout Europe, North Africa, India and Chile, and, more recently, to North America (Kölber 2001).

This viral disease is characterized by its difficult control, due to its easy transmission by aphids. Short-term control methods in the field include removal of diseased plants and planting of certified material. However, the release and cultivation of new resistant cultivars seems to be the definitive solution. The development of new resistant apricot genotypes and the search for new sources of resistance to sharka are two of the most important objectives in apricot breeding programmes (Audergon *et al.* 1994, Martínez-Gómez and Dicenta 2000). Evaluation of PPV resistance in controlled conditions is a time-consuming and expensive process (Martínez-Gómez and Dicenta 1999). Therefore, the search for biochemical and molecular markers associated with resistance would be of a great interest.

Plants possess several mechanisms that detoxify superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), called antioxidant systems. The primary components of these antioxidant systems include non-enzymatic antioxidants (carotenoids, ascorbate, glutathione and tocopherols) and enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), peroxidases (POX, EC 1.11.1.7), and the enzymes involved in the ascorbate-glutathione cycle (ASC-GSH cycle), ascorbate peroxidase (APX, EC 1.11.1.1), dehydroascorbate reductase (DHAR, EC 1.8.5.1), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2) (Hernández *et al.* 2000, Jiménez *et al.* 1997).

Activated oxygen species (AOS), including H_2O_2, $O_2^{\cdot -}$ and hydroxyl radicals ($^{\cdot}OH$), are involved in plant responses to pathogen infections (Baker and Orlandi 1995). Hydrogen peroxide has been implicated in direct killing of pathogens and host cells. In addition, AOS may function as part of a signal transduction pathway leading to the induction of PR protein synthesis and systemic resistance in infected and non-infected parts of plants (Mehdy *et al.* 1996). Hernández *et al.* (2001a) suggested an important role for H_2O_2 in the response of apricot cultivars to PPV.

It has been proposed that a decline in AOS-scavenging capacity may be required before a rapid increase in virus replication can take place. In *Phaseolus vulgaris* L. plants treated with the cytokinin dihydrozeatin, salicylic acid or jasmonic acid showed elevated CAT, GR and peroxidase activities. These treatments, when applied before inoculation with WClMV (White clover mosaic potexvirus), inhibited virus replication and symptom development (Clarke *et al.* 2002). It is possible that, in some plant-virus interactions, the maintenance of enzymes involved in antioxidant metabolism prevents the establishment of the virus and the progression of the disease (Clarke *et al.* 2002, Deak *et al.* 1999). It is also possible that a decline in antioxidant enzymes and an increase in AOS could be necessary for the establishment of infection and the replication and spread of some plant viruses. All these data suggest that an enhanced capability for elimination of AOS could play a role in avoiding or hindering the replication of plant virus. Recently, it has been suggested the involvement of the glucose-6-phosphate dehydrogenase, the key enzyme of the oxidative pentose phosphate pathway, not only in the biosynthesis of nucleotide precursors but also in others processes, possibly connected with defense mechanisms of plant host against virus infection (Sindelárova and Sindelar 2003/4).

In this work, we examined antioxidant enzyme activity in different PPV-resistant and PPV-susceptible non-infected apricot cultivars, to evaluate if any differences exist in

the constitutive levels of these antioxidant enzymes, and to correlate enzymatic levels with the PPV-susceptibility/resistance character in apricot.

Material and Methods

Plant material

Plant material assayed included three traditional Spanish cultivars (Canino, Currot, and Real Fino) and a new selection from the CEBAS-CSIC (Z50308), all these cultivars described as susceptible to PPV. On the other hand, four representative North American apricot cultivars [Goldrich, Orange Red, Stark Early Orange (SEO), and Stella] described as PPV-resistant were also included in the study (Martínez-Gómez and Dicenta 2000) (Table 1). Apricot trees were grown in an experimental orchard in Cieza (South-East Spain). Fully expanded green young leaves were randomly sampled in the sun-faced parts of the trees and they were taken at the same level. Activities of antioxidant enzymes were evaluated during years 2002 and 2003. Five samples from each apricot cultivar were analyzed during the two year of the study.

Leaf enzyme extraction

All operations were performed at 0 – 4 ºC. Leaf material (2g fresh mass), from the different apricot cultivars, was homogenized with 4 cm^3 of an ice-cold medium containing 50 mM K-phosphate (pH 7), 0.1 mM EDTA, 5 mM cysteine, 10 % (m/v) insoluble PVPP (polyvynylpolypyrrolidone), 0.1 mM PMSF (phenylmethanesylfonyl fluoride) and 0.2 % (v/v) Triton X-100. For the APX activity assay, 20 mM Na-ascorbate was added to the extraction medium. The extracts were filtered through two layers of nylon cloth and

centrifuged at 8000 g for 20 min, at 4 ºC. The supernatant fractions were then filtered on Sephadex G-50 M PD10 columns (Amersham Pharmacia Biotech. Wien, Austria) equilibrated with the extraction medium.

Enzymatic determinations and assays

APX, DHAR, MDHAR and GR activities were assayed as described in Hernández *et al.* (2000). Enzyme activities were corrected for non-enzymatic rates and for interfering oxidation. For APX, the oxidation rate of ascorbate was estimated between 1.0 and 60 s after starting the reaction with the addition of H_2O_2. Correction was made for the low non-enzymatic oxidation of ascorbate by H_2O_2. To determine MDHAR activity, the monodehydroascorbate was generated by the ascorbate/ascorbate oxidase system. The rate of monodehydroascorbate-independent NADH oxidation (without ascorbate and ascorbate oxidase) was subtracted from the initial monodehydroascorbate-dependent NADH oxidation rate (with ascorbate and ascorbate oxidase). For DHAR activity, the reaction rate was corrected for the non-enzymatic reduction of DHA by GSH. A 2% contribution to the absorbance by GSSG was also taken into account. Values of GR activity were corrected for the small, non-enzymatic oxidation of NADPH by GSSG (Jiménez et al., 1997). Total SOD activity was determined according to McCord and Fridovich (1969). Proteins were estimated according to Bradford (1976). SOD isozymes were separated by PAGE on 10% gels using a Bio Rad Mini Protean II dual slab cell, and they were localised by the photochemical method (Weissiger and Fridovich 1973). Assignment of the different SOD isoforms (CuZn-SOD, Mn-SOD and Fe-SOD) was performed by selective inhibition with KCN or H_2O_2 (Hernández *et al.* 2001b). Cu,Zn-SODs are inhibited by CN^- and H_2O_2, Fe-SODs are resistant to CN^- but inactivated by H_2O_2, and Mn-SODs are resistant to both inhibitors (Bridges and Salin 1981).

Data analysis

An analysis of variance (ANOVA) of the antioxidant enzyme activities was performed using Duncan´s multiple range test, to identify significant differences among means. Band scoring and separation of the activities of SOD isozymes were performed using GeneTools gene analysis software (SYNGENE, Beacon House, Cambridge, UK).

Results

CAT, POX, MDHAR and DHAR activities differed from year to year (Table 2). However, only CAT, MDHAR and DHAR activities were significantly higher in the year 2002 than in 2003, whereas POX activity did not show significant change (Table 3). Antioxidant enzyme activities showed differences when the ANOVA was applied to cultivars, except for SOD activity. However, there was no significant Year * Variety interaction (Table 2).

No clear correlation could be observed in the POX, MDHAR, DHAR, GR and SOD activities when the different apricot cultivars were analyzed regarding their level of susceptibility/resistance. However, and with a few exceptions, a clear tendency can be observed in the CAT, APX and DHAR activities. In this case, it seems that resistant apricot cultivars showed higher constitutive activity for these antioxidant enzymes than the susceptible cultivars (Table 3).

According to the Duncan's test, resistant cultivars showed higher activities for CAT, APX and DHAR than the susceptible cultivars (Table 3). However, it is important to note that, in the case of CAT, the higher activities corresponded to the susceptible cultivar Real Fino and all the resistant cultivars (Table 2). For APX, with the exception of the cultivar Currot, the higher activities corresponded to the resistant cultivars. Finally, for DHAR, the resistant cultivars Stella, Goldrich and SEO showed the highest activity values. However, only the resistant apricot cultivars have at least two of these enzymatic

activities over the total average showed in Table 3, and Stella has the three antioxidant enzymes over this total average.

Only CuZn-SODs isozymes were detected after 10% PAGE, according to their sensitivity to H_2O_2 and KCN. The apricot cultivars analyzed can be placed in three groups according to the number of their SOD isozyme: Group 1) Stella, Orange Red and Real Fino; Group 2) Goldrich, SEO, and Canino; and Group 3) Currot and Z50308. The first group seems to have three CuZn-SODs, named 1, 2 and 3, respectively, in order of increasing mobility, and with Rf values of 0.400, 0.466 and 0.533, respectively. In contrast, group 2 seems to have five CuZn-SODs, named 1 to 5 in order of increasing electrophoretic mobility. CuZnSODs 1, 2 and 3 had similar Rf values to cultivars from group 1, and CuZn-SODs 4 and 5 had Rf values of 0.560 and 0.600, respectively. However, group 3 also has three CuZn-SOD isoforms, named CuZn-SOD 1, 2' and 5. Whereas CuZnSODs 1 and 5 had Rf values similar to those of groups 1 and 2, CuZn-SOD 2' had a higher electrophoretic mobility (0.493) than CuZn-SOD 2 from the other groups, and, accordingly, we named it CuZn-SOD 2' (Table 4, Fig. 1).

No correlations can be observed between isozyme pattern and resistance or susceptibility to PPV. Similarly, the analysis of the different CuZn-SODs isozymes did not reveal any important differences in behavior between resistant and susceptible cultivars. The susceptible cultivar Z50308 had the highest values for CuZn-SODs 1 and 2', whereas the resistant Orange Red and the susceptible Real Fino and Canino exhibited the highest values for CuZn-SOD 2. The susceptible cultivar Canino also showed the highest activity for CuZn-SOD 4, whereas the resistant Stella and Orange Red cultivars and the susceptible Z50308 showed the higher values for CuZn-SOD 3. Finally, no significant differences were observed in CuZn-SOD 5 (Table 4).

Discussion

The PPV-resistant apricot cultivars analyzed had a higher capability for elimination of H_2O_2 and recycling of ASC, and they have at least two of these enzymatic activities (CAT, APX and DHAR) over the total average. These facts could contribute to their resistance. However, this response was not showed by the susceptible cultivars.

In previous work carried out with two apricot cultivars, grown in controlled conditions in an insect-proof greenhouse and inoculated by chip grafting, we showed different behavior of SODs (H_2O_2-generating enzymes) and APX (an H_2O_2-removing enzyme) in both cultivars. In the resistant cultivar studied (Goldrich), PPV infection produced an induction of SOD activity, whereas no change in APX was observed. However, in the susceptible cultivar (Real Fino), inoculation with PPV brought about a decrease in SOD and a rise in APX activity. These results suggested an important role for H_2O_2 in the response to PPV in the resistant cultivar (Hernández *et al.* 2001a). Under control conditions, the susceptible cultivar had higher SOD, APX and DHAR activities than the resistant cultivar, which had higher MDHAR activity values (Hernández *et al.* 2001a). However, under non-inoculated field conditions, our present results showed that resistant cultivars had higher APX, CAT and DHAR values than susceptible cultivars.

After 10% PAGE of crude leaf extracts from the different apricot cultivars, only CuZn-SOD isozymes were detected. In contrast, in other woody plants, such as poplar and lemon trees, the presence of Mn-SOD, CuZn-SOD, and Fe-SOD was described (Almansa *et al.* 2002, Bernardi *et al*. 2004). The presence of only CuZn-SOD isozymes was observed previously in greenhouse-grown apricot plants (Hernández *et al.* 2001a). The presence of only CuZn-SOD isozymes is surprising, because these isozymes are inhibited by H_2O_2, which is produced in different cell compartments (Halliwell and Gutteridge 1989), such as mitochondria and peroxisomes, where the presence of the H_2O_2-resistant Mn-SOD has been described (Del Rio *et al.* 1985, Sandalio and Del Rio 1987). In

glyoxisomes of watermelon, as well as in wheat germ and pea leaves, a partly H_2O_2-sensitive CuZn-SOD isozyme has been described (Del Rio *et al.* 1985, Sandalio and Del Rio 1987, Beauchamp and Fridovich 1973). However, no correlation was observed between SOD isozyme pattern and the resistance and/or susceptibility to PPV. The fact that Currot and Z50308 had the same SOD isozyme pattern is not surprising, because the hybrid Z50308 proceeds from the cross Currot x SEO, from the apricot breeding programme of CEBAS-CSIC.

In conclusion, all these data suggest that activity level of some antioxidant enzymes as CAT, APX and DHAR could be used as biochemical markers of PPV resistance in apricots. We propose that if an apricot cultivar has at least two of these enzymatic activities over the total average, it could be considered as a putative PPV-resistant. However, some exceptions were found in these results, and these data must be confirmed by the analysis of more resistant and susceptible apricot cultivars. These results open a new way for the further studies necessary for a better understanding of the possible use of antioxidant enzyme activities as biochemical markers for PPV resistance.

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Table 1. Pedigree, origin and main agronomic characteristics (skin and flesh colors, and PPV resistance) of the apricot cultivars assayed.

Cultivar	Pedigree	Origin	Skin color	Flesh color	PPV resistance[1]
Goldrich	Sunglo x Perfection	USA	Orange	Orange	Resistant
Orange Red	Lasg. Mas x NAJ2	USA	Red	Orange	Resistant
SEO	Unknown	USA	Yellow	Light Orange	Resistant
Stella	Unknown	USA	Dark Orange	Orange	Resistant
Canino	Unknown	Spain	Light Orange	Orange	Susceptible
Currot	Unknown	Spain	Cream	White-Orange	Susceptible
Real Fino	Unknown	Spain	Cream	White - Yellow	Susceptible
Z503/08	Currot x SEO	Spain	Red-Orange	Light Orange	Susceptible

[1]PPV resistance level determined by Martínez-Gómez and Dicenta (2000).

Table 2. ANOVA (*P* values x 100) of the levels of antioxidant enzymes catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and superoxide dismutase (SOD), in different apricot cultivars, carried out during the years 2002-2003.

	CAT	POX	APX	MDHAR	DHAR	GR	SOD
Year	0.05	0.01	16.74	0.01	0.13	14.58	27.30
Cultivar	0.02	0.02	0.69	1.57	0.99	0.78	8.83
Year * Cultivar	6.6	34.45	65.45	16.54	53.05	21.4	56.59

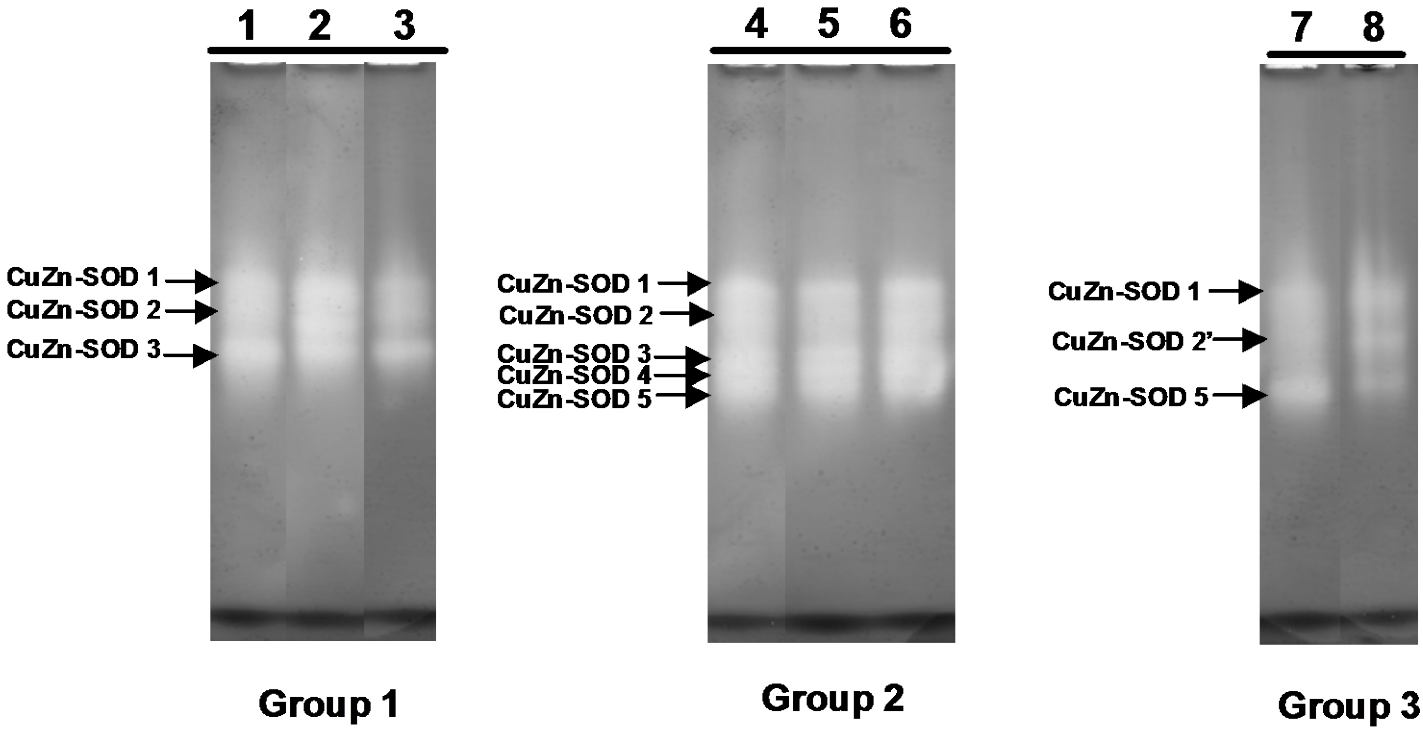
Table 3. Antioxidant enzyme activity variations with the year and the apricot cultivar. Catalase (CAT) and peroxidase (POX) are expresed as [µmol min^{-1} (prot) mg^{-1}], ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) are expressed as [nmol min^{-1} (prot) mg^{-1}], and superoxide dismutase (SOD) is expressed as [U mg^{-1} (prot)]. Different letters in the same column indicate significant differences according to Duncan's multiple range test ($P = 0.05$).

	CAT	POX	APX	MDHAR	DHAR	GR	SOD
Year 2002	16.74a	0.777a	173.1a	226.6a	182.9a	169.9a	51.0a
Year 2003	7.76b	1.182a	124.7a	102.7b	102.7b	197.5a	45.5a
Goldrich	6.49bc	0.84cd	177.3b	75.4c	153.5ab	218.2abc	44.24b
Orange Red	14.67ab	1.47a	179.5b	116.3bc	78.0b	165.8bcd	51.30ab
SEO	15.41ab	1.12bc	138.6b	158.0ab	185.8a	243.6ab	39.82b
Stella	17.60a	0.72d	343.1a	156.4ab	205.6a	118.4d	37.39b
Resistant cultivars	*13.71a*	*1.04a*	*209.6a*	*128.3a*	*158.2a*	*186.7a*	*43.20a*
Canino	4.31c	0.93cd	91.3b	167.5ab	142.7ab	270.2a	55.33ab
Currot	7.83bc	0.99bcd	170.3b	164.1ab	115.9ab	187.8bcd	39.14b
Real Fino	21.16a	1.06bc	107.5b	218.1a	116.7ab	146.2cd	43.61b
Z50308	2.11c	1.28ab	89.2b	104.6bc	55.2b	146.7cd	65.65a
Susceptible cultivars	*8.85b*	*1.06a*	*112.7b*	*161.9a*	*104.6b*	*187.7a*	*50.94a*

Table 4. Activities of superoxide dismutase (SOD) isozymes [U mg^{-1} (prot)] in leaves from healthy apricot cultivars resistant (Stella, Goldrich, Orange Red, SEO) and susceptible (Canino, Currot, Real Fino, Z50308) to PPV. Different letters in the same column indicate significant differences according to Duncan's multiple range test (P = 0.05).

	CuZn-SOD1	CuZn-SOD2	CuZn-SOD2'	CuZn-SOD3	CuZn-SOD4	CuZn-SOD5
Stella	10.37±1.76a	7.66±1.30a	No detected	19.35±3.30bc	No detected	No detected
Goldrich	11.57±2.10a	8.76±1.50a	No detected	8.14±1.46a	6.52±1.17a	9.24±1.60a
Orange Red	23.22±2.78b	12.7±1.50b	No detected	15.39±2.02b	No detected	No detected
SEO	9.52±1.41a	8.05±1.12a	No detected	6.84±0.95a	6.40±0.77a	8.99±1.17[a]
Canino	13.72±2.06a	11.25±1.68b	No detected	9.78±1.47[a]	9.58±1.15b	10.98±1.65[a]
Currot	14.01±2.66a	No detected	9.83±1.47a	No detected	No detected	15.25±2.75a
Real Fino	11.93±2.98a	11.63±2.33b	No detected	20.05±4.81c	No detected	No detected
Z50308	32.87±4.27c	No detected	21.99±2.41b	No detected	No detected	10.79±1.4a

Fig. 1. Superoxide dismutase (SOD) isozyme patterns observed in leaves of different apricot cultivars. 1.- Stella, 2.- Orange Red, 3.- Real Fino, 4.- Goldrich, 5.- SEO, 6.- Canino, 7.- Currot, 8.- Z50308.


1 2 3
CuZn-SOD 1
CuZn-SOD 2
CuZn-SOD 3
4 5 6
CuZn-SOD 1
CuZn-SOD 2
CuZn-SOD 3
CuZn-SOD 4
CuZn-SOD 5
7 8
CuZn-SOD 1
CuZn-SOD 2'
CuZn-SOD 5
Group 1
Group 2
Group 3