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Variability in Superoxide Dismutase Isoforms in Tall and Dwarf Cultivars of Coconut (*Cocos nucifera* L.) Leaves

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Superoxide dismutases (SODs) are ubiquitous metallo-enzymes that constitute the first line of defense against reactive oxygen species (ROS). They constitute one of the major enzymatic components of detoxi?cation of superoxide radicals generated in biological system by catalyzing its dismutation to H_2O_2 and finally to H_2O and O_2. Most plant species contain numerous SOD isoforms differing in their active site metal ions. In the present investigation, we have attempted to identify variability in superoxide dismutase (EC 1.15.1.1) isoform pattern of 13 coconut genotypes comprising six talls, five dwarfs along with two reciprocal hybrids of WCT (Tall) with COD (Dwarf). Among the genotypes studied, a significant variation was observed in SOD enzyme activity as well as in SOD isoforms pattern. A total of eight to fourteen SOD isoforms were detected in different coconut cultivars. The variation was observed only in Mn-SOD isoforms while Fe-SOD (two) and Cu/Zn-SOD (five) isoforms were similar in all the analyzed cultivars. Mn-SOD isoforms varied from one to five in numbers. Among the tall cultivars, WCT, FMST and WCT X COD showed highest number (five) of Mn-SOD isoforms as well as highest enzymatic activity followed by LCT while TPT, PHOT and ADOT showed only single isoform for Mn-SOD. All dwarfs studied were found to have similar SOD isozyme profile for all SODs i.e. one Mn-SOD, five Cu/Zn-SOD and two Fe-SOD isoforms. It was also observed that Mn-SOD does not follow the Mendelian pattern of inheritance i.e. reciprocal crosses showed Mn-SOD isoform pattern similar to their mother palm. SOD activity and isoform pattern can be utilized as a biochemical marker for varietal identification and for abiotic stress/drought tolerance breeding.

Key words: Coconut, ROS, SODs, isoforms, reciprocal crosses

All aerobic organisms utilize the oxidation potential of atmospheric oxygen to drive chemical reactions (1). As a consequence of aerobic metabolism as well as abiotic stresses, there is continual production of reactive oxygen species (ROS) that cause oxidative stress by disrupting metabolism, especially by the degradation of complex biomolecules, including nucleic acids, proteins, and membrane lipids (2). ROS are highly reactive and when the scavenging capacity of plant is lower than the ROS production, they can seriously disrupt normal metabolism through oxidative damage of lipid, protein and nucleic acids (3-5). Plant cells contain a variety of enzymes and antioxidants that continually detoxify ROS (6). Antioxidant enzymes are the most important components in the scavenging system of ROS. In this antioxidant enzyme system, superoxide dismutases (SOD: EC 1.15.1.1) are ubiquitous metalloenzymes (7, 8) that constitute the first line of defense against reactive oxygen species and one of the most effective components of the antioxidant defense system in plant cells against ROS toxicity. It catalyzes the dismutation of superoxide radicals to H_2O_2 (2). By removing $O2^{\bullet-}$, SODs decrease the risk of $OH^{\bullet}$ formation via the metal catalyzed Haber-Weiss-type reaction because this reaction has a 10,000-fold faster rate than the spontaneous dismutation (9). This enzyme is unique in that its activity determines the concentrations of $O_2^{\bullet-}$ and H_2O_2, the two Haber-Weiss reaction substrates, and it is therefore likely to be central in the antioxidant defense mechanism (10, 11). The SOD system in higher plants exists in multiple isoforms in different subcellular compartments that are developmentally regulated and highly reactive in response to exogenous stimuli. These are classi?ed according to the active site metal into three major groups (types): Fe-SOD (iron cofactor), Mn-SOD (manganese cofactor) and Cu/Zn-SOD (copper and zinc as cofactors; copper is the redox active catalytic metal) (12, 13). Different SODs are located in various cellular compartments viz. Fe-SODs are located in plastids, Mn-

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SODs in mitochondrial matrix, peroxisomes and in cell wall, while Cu/Zn-SODs occur in cytosol, peroxisomes, plastids, and possibly extracellular space (12, 14, 15, 16).

Kumar *et al.* (17) reported four to six SOD isoforms in different genotypes of citrus and Kumquat and correlated the higher number of isoforms with abiotic stress tolerance. Momcilovik *et al*. (18) identified one Mn-SOD, three Fe-SOD and five to six Cu/Zn-SOD isoforms in potato leaves. Kumar *et al.* (19) reported a total of 14 SOD isoforms including five each of Mn-SOD and Cu/Zn-SOD, two of Fe-SOD and two unknown higher molecular weight isoforms, in WCT cultivar of coconut (Fig. 1). A relationship between basal ROS remediation and resistance to biotic stress in plants has also been proposed (20). The significance of all the SODs has been confirmed in the direct or indirect efficient metabolism of different ROS and its reaction products in numerous studies (9, 12). The present investigation was planned to find out natural variation if any in superoxide dismutase isoforms in coconut genotypes which can be utilized for development of abiotic stress tolerant coconut hybrids which can make positive impact on sustainable coconut production under changing climatic scenario. With this objective, studies were carried out on SOD activity assay as well as in gel SOD isoenzyme pattern in eleven coconut cultivars and two reciprocal hybrids of WCT with COD.

Materials and Methods

The leaf samples from two years old seedlings were collected from 13 coconut genotypes/accessions comprising six tall (West Coast Tall - WCT, Tiptur Tall - TPT, Federated Malay States Tall - FMST, Laccadive Ordinary Tall - LCT, Philippines Ordinary Tall - PHOT and Andaman Ordinary Tall -ADOT), five dwarfs (Chowghat Orange Dwarf - COD, Chowghat Green Dwarf - CGD, Malayan Yellow Dwarf – MYD, Malayan Orange Dwarf – MOD, and GBGD – Gangabondam Green Dwarf) and two reciprocal crosses of WCT with COD (WCT x COD and COD x WCT).

Isolation of leaf proteins: Fresh leaves (2.5 g) were cut in to small pieces and homogenized in 25 ml of chilled 0.1 M Sodium Phosphate buffer pH 7.5 containing 2% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) and 200 µl â-mercaptoethanol and transferred into pre-chilled centrifuge tubes. Protein extracts were centrifuged at 12,000 rpm for 15 min at 4 ^{0}C in refrigerated centrifuge (Hareus, Germany). The supernatant was decanted and re-centrifuged at the same conditions. The extracted protein was partially purified from the supernatant by ammonium sulfate precipitation (final concentration 85%), and subsequent dialysis. The clear pale yellow dialysate after centrifugation was used for SOD enzymatic activity assay and for SOD isozymes identification. An aliquot of the enzyme extract was used to determine its SOD enzymatic activity by measuring its ability to initiate photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich (21) and protein content by the method of Lowry *et al.* (22).

Electrophoresis and SOD isoforms visualization: SOD isoforms were separated on non-denaturing polyacrylamide gels (12% resolving gel; 4% stacking gel) at 160 mA for 4-5 h at 4^{0}C according to the method of Hames and Rickwood (23). In each lane, 150 µg of total protein was loaded. After electrophoresis, the gels were incubated in dark for 20 minutes in a solution containing 100 ml 0.05M Tris-HCl (pH-8.2), 2.45 mM NBT, 28 mM ribo?avin and 10 mM EDTA, followed by illumination of the gels until the white bright bands became apparent as described by Beauchamp and Fridovich (21). The SOD isoforms were identified (Fig. 1) according to the method already standardized in coconut by Kumar *et al.* (19).

Results and Discussion

Significant variation was observed in SOD specific activity as well as in isozyme pattern in different coconut genotypes. The SOD specific activity varied from 4.02 to 6.39 (Table 1) and SOD isoforms visualized in native gel ranged from 8 to 14 in number (Fig. 2 and 3). Overall, WCT had highest specific activity (6.39) as well as maximum number of SOD isoforms i.e. 14 isoforms including five each of Mn-SOD and Cu/Zn-SOD, two Fe-SOD and two unknown isoforms of higher molecular

weight which were missing in all remaining cultivars, followed by WCT x COD and FMST, which showed 12 SOD isoforms, similar to WCT except for the absence of two higher molecular weight SOD isoforms.

Laccadive Tall contained 10 No. of SOD isoforms with three Mn-SODs, five Cu/Zn-SODs and two Fe-SOD isoforms while all remaining accessions showed only eight SOD isoforms comprising of one Mn-SOD, five Cu/Zn-SODs and two Fe-SODs. The variability in SOD isoforms was observed only in Mn-SOD isoforms pattern, whereas the pattern of Cu/Zn-SOD and Fe-SOD isoforms was similar in all the genotypes investigated in the present study. Among tall cultivars as well as among all investigated genotypes WCT and FMST had highest numbers of Mn-SOD isoforms (five each) followed by LCT which had three Mn-SOD isoforms, while all remaining genotypes showed only single Mn-SOD isoform. All dwarfs showed a similar SOD isozyme pattern with one Mn-SOD, five of Cu/Zn-SOD and two of Fe-SOD isoforms. The SOD isoform pattern of reciprocal hybrids revealed that the inheritance of Mn-SOD is not following the Mendelian pattern of inheritance i.e. progenies showed similar Mn-SOD isoform pattern as of it female parent. Therefore, these results support the earlier reports that Mn-SOD is present in mitochondria and does not follow the Mendelian pattern of inheritance as in many other plant species like maize (24), tobacco (25), mung beans (26) and watermelon (27). It has been reported that Mn-SOD is a mitochondrial enzyme. Many studies indicated that higher level of indigenous SOD or over expression of SOD enzyme activity is correlated with abiotic stress tolerance in many plant species. For instance, Sunkar *et al.* (28) reported that the posttranscriptional induction of two Cu/Zn-SOD genes in *Arabidopsis* was critical for oxidative stress tolerance. Over expression of a Cu/Zn-SOD (acytosolic SOD from pea) in transgenic tobacco plants could able to enhance O_3 tolerance (29). Kumar *et al.* (17) studied the antioxidant isozyme variability in different genotypes of citrus and kumquat and concluded that kumquat had higher SOD and catalase activities as well as higher number of their isoforms and consequently had greater potential to remove reactive oxygen species.

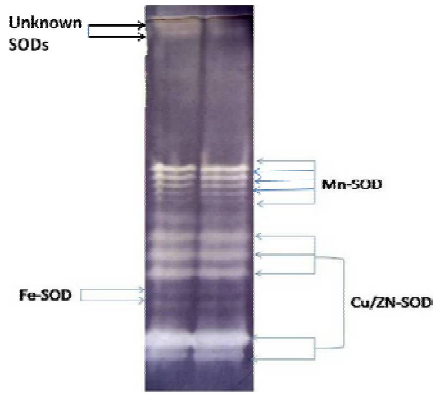

Fig. 1: Superoxide Dismutase isoforms identified in WCT accession of coconut leaves (19).

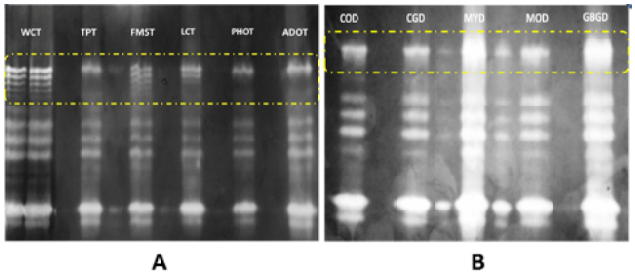

Fig. 2: Superoxide dismutase isoform variability in coconut genotypes (circled) A. Tall accessions B. Dwarf accessions

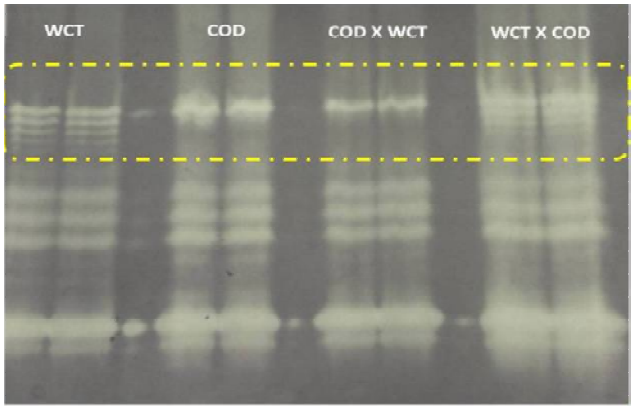

Fig. 3: Superoxide dismutase isoform pattern of WCT, COD and their reciprocal crosses

In citrus, callus, and cold-acclimated mandarin fruits, higher SOD activity conferred greater resistance to salt and chilling stress (30). Higher SOD activity was observed in frost resistant potato hybrids rather than in frost-sensitive *Solanum tuberosum* 'Matilda' (31). Xu et al. (32) reported that increased expression of native cytosolic Cu/Zn-SOD and ascorbate peroxidase improves the tolerance to chilling and oxidative stress tolerance in cassava.

Table 1: Superoxide dismutase (SOD) specific activity in leaves of 13 coconut genotypes

Coconut Genotypes	SOD Specific activity	
	Mean	S.E.
WCT	6.39	0.07
TPT	4.47	0.07
FMST	6.07	0.04
LCT	5.37	0.07
PHOT	4.36	0.07
ADOT	4.48	0.09
COD	4.84	0.04
CGD	4.13	0.05
MYD	4.54	0.04
MOD	4.02	0.02
GBGD	5.00	0.09
WCT X COD	6.12	0.07
COD X WCT	4.05	0.07
C.D. (p>0.001)	0.188**	

Rajgopal *et al.* (33) studied the drought tolerance level on the basis of some physiological parameters like stomatal conductance, leaf water potential and epicuticular wax content and scored them with 1 to 20 rank and WCT X WCT and FMST secured first and second ranks, respectively. Interestingly, in this study also, maximum number of SOD isoforms specially Mn-SOD were observed in these two genotypes, viz. WCT and FMST. They also concluded that in hybrids these parameters resemble their female parent. It has been reported in many studies that higher level of Mn-SOD is linked with abiotic stress tolerance and Melchiorre *et al.* (34) reported photooxidative stress tolerance, lower oxidative damage and higher H_2O_2 in *Triticum aestivum* plant transformed with Mn-SOD gene from *Nicotiana plumbaginifolia*. Wang et al. (35) also reported over expression of Mn-SOD gene in *Arabidopsis* leads to salt tolerance. Similarly, Rubico et al. (36) observed mild water stress tolerance and higher photosynthetic activity in *Medicago sativa* L. plants transformed with Mn-SOD and Fe-SOD from *Nicotiana plumbaginifolia* and *Arabidopsis thaliana*. SOD activity assay and SOD isoform pattern can be utilized as a biochemical marker for screening for abiotic stress tolerance in coconut germplasm as well as to differentiate between the tall and dwarf cultivars of coconut.

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