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Assesment of antioxidant and phenol related enzyme assays in Karanja (*Derris indica*)

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Abstract
The study was undertaken to identify the responses of five antioxidant enzymes viz., GPOD (guaiacol peroxidase), APX (ascorbate peroxidase), CAT (catalase), SOD (superoxide dismutase), GR (glutathione reductase) and one phenol related metabolism enzyme, PPO (polyphenol oxidase) in thirty accessions of Karanja. Highest GR (248.99 µM/min/g protein) and CAT (6.22µM/min/g protein) activity was found in NAUK-10 while highest GPOD, SOD, APX and PPO activity was found in NAUK-2 (165.16 µM/min/g protein), NAUK-13 (84.01 U/g protein), NAUK-24 (255.17 µM/min/g protein) and NAUK-11 (19.06 µM/min/g protein) respectively.

Keywords: Karanja, enzymes, antioxidant

Introduction
Karanja (*Derris indica* L., 2n=22) is an out-breeding drought resistant, semi-deciduous oilseed tree belonged to the family Fabaceae and subfamily Papilionaceae (Meera *et al.* 2003) [16]. It has recently gained a great commercial importance owing to their high oil content, (30-40%) which is explored as an alternate source of fuel and energy (Nagaraj and Mukta, 2004) [19]. The oil from karanja seeds (honge oil) used as lubricant for thousands of years (Kesari *et al.*, 2010) [12]. The juices from the different parts of the plant with oil are antiseptic and offer resistant to pests.

The plant phenotype was influenced by the level of different biomolecules like enzymes, proteins and various metabolites. In present study, we have studied the activity of reactive oxygen species (ROS) scavenging enzymes and phenol related metabolism enzyme. Reactive oxygen species (ROS) such as O_2 and H_2O_2 are produced during photosynthesis, photorespiration, respiration, flowering and other reactions of cellular metabolism (Winston 1990) [27]. Excessive release of ROS damages lipids, proteins and DNA which leads to oxidative stress, loss of cell function, and programmed cell death. ROS are also actively released as a response against bacterial and parasitic pathogens in different insect species. To regulate oxidative stress, the eukaryotic cell produces different ROS scavenging enzymes such as guaiacol peroxidase (GPOD), ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione oxidase and GR (glutathione reductase) which reduces H_2O_2 to H_2O. These enzymes provide cells with highly efficient machinery for detoxifying O_2^- and H_2O_2.

SOD (E.C. 1.15.1.1) is a family of metallo-enzymes that convert O_2^- to H_2O_2, protect the cell from damage caused by O_2^- and found virtually in all aerobic organisms (Fridovich, 1978). The balance between SOD and the different H_2O_2-scavenging enzymes in cells is considered to be a crucial point determining the steady state level of O_2^- and H_2O_2 (Asada, 1987). CAT (EC 1.11.1.6.) is located mainly in peroxisomes (80%), cytosol (20%) and also in mitochondria. In plants, catalase is the main enzymatic H_2O_2 scavenger of photosynthetic cells which converts H_2O_2 into H_2O and O_2 (Scandalios, 1987) [24]. GR (EC 1.6.4.2.) is a crucial enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form (GSH) by oxidation of NADPH. The increase in the activity of this enzyme in stress situations (herbicidal action, pollutants, low temperature, high light intensity and water stress) has been identified (Rennenberg H., 1982) [22]. GR is localized mainly in the chloroplast stroma but is also found in the mitochondria and cytosol (Edwards *et al.*, 1990) [8]. Peroxidase (EC 1.11.1.7) is a multipurpose enzyme that catalyses the condensation of phenol into lignin and plays a specific role in the hypersensitive containment of the pathogen (Dignum *et al.*, 2001). APX (EC 1.11.1.11) is localized in the chloroplast, peroxisomes and mitochondria, responsible for H_2O_2 removal.

APX utilizes Ascorbic acid (AsA) as its specific electron donor to reduce H_2O_2 to water with concomitant generation of monodehydroascorbate (MDAsA). Polyphenol oxidase (E.C. 1.14.18.1) catalyses the oxidation of polyphenols, the hydroxylation of monophenols and lignification of plant cells (Rivero *et al.*, 2001) [23]. Recent studies indicated that phenol-oxidizing enzymes may participate as a response to the defense reaction and hypersensitivity for inducing resistance of plants to biotic and abiotic stress. (Jouili and El FE, 2003) [11].

Materials and Methods

Planting Material

Karanja germplasms were collected from different regions of Gujarat and established at germplasm bank of Navsari Agricultural Universty, Navsari. Leaf samples of progeny of candidate plus trees (CPT's) of Karanja were selected for experimental purpose.

Enzyme Extraction

For antioxidant enzyme assays, frozen leaves were ground to a fine powder with liquid nitrogen and were extracted with ice-cold 0.05 M sodium phosphate buffer (pH 7.4) containing 1mM Ethylene diamine tetra acetic acid (EDTA) and 1% (W/V) polyvinylpyrolidone (PVP). The homogenate was centrifuged at 10000 g for 20 min, at 4°C, and the supernatant was used for enzyme activity and protein determinations. For determination of PPO activity, leaves were grinded in 0.1 M sodium phosphate buffer (pH 6.0). The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used for enzyme assays. Protein concentration was determined by Lowry's method. Preparations for enzyme extraction and enzyme assay were carried out at 4 °C.

Protein determination

The concentration of protein was determined by the method of Lowry *et al*., (1951) [13] using BSA as a standard.

Enzymes Assay

The activities of antioxidant enzymes namely, APX, GPOD, CAT, GR, SOD and PPO were determined spectrophotometrically.

Antioxidant and phenol related enzyme assay

Super oxide dismutase activity was measured based on inhibition of photochemical reduction of nitroblue tetrazoilum (NBT). The 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8) 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 0.1 ml enzyme extract. Riboflavin was added last (Van Rossun *et al*., 1997) [25]. The test tubes were shaken and placed 30 cm below light source consisting of four 15-w fluorescent lamps. The reaction was allowed to run for 10 minutes and stopped by switching off the light. The photoreduction in NBT was measured as increase in absorbance at 560 nm. Blanks and controls were run in the same way but lacking illumination and enzyme, respectively. In the presence of $\bullet O^{2-}$, NBT is reduced to blue formazan, a bluish purple compound. Enzyme unit of SOD was calculated according to formula given by Constantine and Stanley (1977) [5].

Guaiacol peroxidase activity was determined in the homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (ε = 26.6 mM-1 cm-1) in a reaction mixture containing 50 mM sodium phosphate buffer pH 7.0, 0.1 mM EDTA, 0.05 ml enzyme extract, 10 mM guaiacol and 10 mM H_2O_2 (Costa *et al*., 2002) [6].

Ascorbate peroxidase activity was measured immediately in fresh extract and was assayed as described by Nakano and Asada (1981) [20]. 3 ml reaction mixture contained 50 mM sodium phosphate buffer pH 7.0, 0.1 mM H_2O_2, 0.5 mM ascorbic acid, 0.1 mM EDTA and 0.1 ml enzyme extract. The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}).

Total catalase activity was determined in the homogenates by measuring the decrease in absorption at 240 nm as H_2O_2 (ε = 39.4 mM^{-1} cm^{-1}) was consumed according the method of Aebi (1984) [2] and enzyme activity was expressed as µmol H_2O_2 oxidized min^{-1} g^{-1} protein. The 3 ml mixture containing 50 mM sodium phosphate buffer (pH 7.0), 10mM H_2O_2 and 50 µl enzyme extract.

Polyphenol oxidase activity was measured in fresh extract and was assayed as described by Malik and Singh, 1980. The reaction mixture contained 2.9 ml of catechol (0.01 M catechol in 10 mM phosphate buffer pH 6.0) and reaction was initiated by the addition of 0.1 ml of enzyme extract. The change in the colour due to the oxidized catechol was read at 490 nm for one minute at an interval of 15 second. Molar extinction coefficient for catechol is 25.5 mM^{-1} cm^{-1}.

Results and Discussion

The analysis of variance showed significant difference in the thirty germplasms of Karanja for superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase and polphenol oxidase. By correlation analysis, polyphenol oxidase showed significant positive association with catalase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase (Table 1). The peroxidases, guaiacol peroxidase and ascorbate peroxidase was significantly associated with each other. Super oxide dismutase is the first line of defense against toxic ROS. The SOD activity was about eight times higher in NAUK-13 compared to NAUK-29 (Table 2). Reduction in SOD activity possibly relates to the higher accumulation of ROS. Hence the capacity of NAUK-13 to degrade ROS is higher than that of genotype NAUK-29 and might be sufficient to cope with higher superoxide generation, in turn capable for drought stress (Abedi and Pakniyat 2010) [1]. The NAUK-11 showed highest GR activity (248.02 µM/min/g protein) among 30 genotypes (Table 2). GR supplied GSH to the ascorbate-glutathione cycle for scavenging active oxygen species (Foyer and Halliwell, 1976) [9]. GSH have protective roles including maintenance of thiol groups like Cys, homocysteine, and sulfhydryl proteins to the reduced form (Rennenberg, 1982) [22]. These roles of GSH could be important for the tolerance of plants to photo-oxidative stress. Catalase activity was recorded maximum in NAUK-11 (13.09 µM/min/g protein) which was ten fold higher than NAUK-15 which was recorded the minimum value for this parameter. As by the result, catalase is the most effective antioxidant enzyme in NAUK-11 preventing oxidative damage (Mittler, 2002) [17]. The increase in the activity of catalase indicates its active involvement in the decomposition of hydrogen peroxide. Thus catalase detoxifies inorganic peroxide and prevents from the harmful effect of peroxides. The changes in catalase activity may depend on the species, the development and metabolic state of the plant as well as on the duration and intensity of the stress (Chaparzadeh *et al.*, 2004). An increase in catalase activity has been reported as an early response to different stresses

and may provide cells with resistance against formation of H_2O_2 (Willekens *et al.*, 1997) [26]. Highest APXs activity was observed in genotype NAUK-24 (255.17 µM/min/g protein) while genotype NAUK-8 had lowest APXs activity of about 15.80 µM/min/g protein (Table 2). Thus genotype NAUK-24 may scavenge excess H_2O_2 (which is formed in plant cells) under normal and stress conditions. The ascorbate system is not only important as a detoxification mechanism, but it also has an obligatory role in the regulation of electron flow *in vivo* (Asada 1992) [3]. GPOD activity in Karanja accessions ranged from 9.71 µM/min/g protein (NAUK-21) to 165.16 µM/min/g protein (NAUK-2). In Olive cultivar Oblica, higher GPOD activity related with its thicker lignified cell walls in vascular tissue and more suberin in cuticular layer of epidermis (Pfeiffer *et al.*, 2010) [21]. Studies have suggested that peroxidase plays a role in lignification, cross-linking of cell wall structural proteins and defense against pathogen (Kawano, 2003). Thus genotype NAUK-2 may have higher rate of lignifications in cell wall and defense against pathogens compared to all other genotypes and also may be capable for drought stress (Abedi and Pakniyat 2010) [1]. Highest PPO activity was noticed in NAUK-11 (19.06 µM/min/g protein) and lowest in NAUK-19 (2.22µM/min/g protein) shown in Table 2. The positive correlation between levels of PPO and the resistance to pathogens and herbivores is frequently observed in plants (Mayer, 2006) [15]. Thus NAUK-11 may show more disease and pest resistance characteristics among thirty genotypes. Highest and lowest PPO activity influences on lignin of plant cells, interferences in browning of wounding tissue and constitutes defensive barriers against diseases (Mohamadi and Kazemi, 2002) [18]. The changes in PPO activities is an indicator when important endogenous changes occur and the enzyme play a possible protective role with other components in the defensive mechanisms against water soluble frost stresses and unfavourable environmental conditions.

Table 1: Correlation values for enzyme assays among Karanja germplasms

	Superoxide dismutase	Glutathione reductase	Catalase	Ascorbate peroxidase	Guaiacol peroxidase	Polyphenol oxidase
Superoxide dismutase	1.000					
Glutathione reductase	-0.112^{NS}	1.000				
Catalase	0.104^{NS}	0.414*	1.000			
Ascorbate peroxidase	0.035^{NS}	0.341^{NS}	0.573**	1.000		
Guaiacol peroxidase	-0.194^{NS}	0.257^{NS}	0.180^{NS}	0.620**	1.000	
Polyphenol oxidase	0.104^{NS}	0.526**	0.571**	0.711**	0.432*	1.000

Table 2: Estimation of ROS scavenging and phenol related enzymes

Genotypes	Superoxide dismutase	Glutathione reductase	Catalase	Ascorbate peroxidase	Guaiacol peroxidase	Polyphenol oxidase
	U/g protein	µM/min/g protein	µM/min/g protein	µM/min/g protein	µM/min/g protein	µM/min/g protein
NAUK1	15.65	77.19	5.99	76.82	25.84	5.27
NAUK2	38.16	173.80	4.63	207.09	165.16	11.79
NAUK3	15.36	230.67	3.30	54.48	31.57	7.04
NAUK4	13.21	138.41	4.77	171.38	153.90	10.18
NAUK5	40.90	180.49	1.29	91.11	94.51	8.04
NAUK6	41.72	105.13	5.15	80.67	41.78	10.87
NAUK7	14.34	242.98	2.97	80.13	32.64	7.24
NAUK8	22.49	157.37	3.91	15.80	23.48	5.54
NAUK9	21.71	141.63	1.36	58.42	47.26	5.25
NAUK10	30.80	238.98	6.21	61.22	28.12	6.80
NAUK11	21.17	248.02	13.09	176.04	82.89	19.06
NAUK12	12.51	74.78	5.39	26.33	10.98	2.24
NAUK13	84.01	51.12	5.71	48.49	19.21	7.39
NAUK14	14.22	24.40	1.88	61.75	11.14	4.22
NAUK15	23.90	45.53	1.14	45.01	36.10	6.16
NAUK16	28.41	52.13	1.85	64.16	18.28	5.44
NAUK17	19.26	119.55	2.26	84.91	69.58	9.36
NAUK18	18.97	52.22	1.68	148.60	63.31	7.50
NAUK19	19.54	108.86	2.03	69.71	66.40	2.22
NAUK20	24.67	136.58	2.30	86.42	22.90	6.43
NAUK21	13.10	43.11	1.54	44.07	52.58	3.83
NAUK22	22.28	63.95	3.02	116.17	9.71	7.21
NAUK23	59.06	77.35	7.11	181.32	27.38	11.11
NAUK24	20.03	204.41	14.02	255.17	97.07	9.69
NAUK25	81.03	51.42	3.77	76.40	15.09	3.72
NAUK26	22.07	28.17	2.35	62.01	87.03	4.18
NAUK27	20.09	146.84	3.83	163.12	67.98	12.63
NAUK28	17.96	41.05	4.49	45.60	27.40	6.01

NAUK29	10.28	24.10	4.11	47.23	73.57	3.36
NAUK30	11.22	66.24	3.80	131.72	106.06	7.80
C.D.	6.07	6.02	0.50	12.67	2.71	0.44
SE(m)	2.14	2.12	0.18	4.47	0.96	0.16
SE(d)	3.03	3.00	0.25	6.32	1.35	0.22
C.V.	13.94	3.30	7.17	8.20	3.09	3.73
F value	74.83*	89.03*	42.07*	21.37*	59.90*	51.38*

The genotype NAUK-10 had highest GR and CAT activity while NAUK-2, NAUK-13, NAUK-24 and NAUK-11 expressed highest GPOD, SOD, APX and PPO activity respectively. Thus, by enzyme assays, we can select these genotypes for sreening against abiotic as well as biotic stresses in different environment for better performance.

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