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A New Candidate of Catalase Appears during the Germination after Priming in Naturally Aged Neem (*Azadirachta indica*) Seeds

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Abstract

Loss of Neem (*Azadirachta indica*) seeds viability remarkably occurs with natural storages and related to decreased seed germination. We investigated that osmopriming induces hydrogen peroxide during germination that could be signaling molecule for germination improvement. A new catalase isozyme appeared during post-priming germination. The results show a high coordination between hydrogen peroxide and catalase and H_2O_2 may play a role in regulation of new catalase isozyme expression during post-priming germination of aged neem seeds.

Keywords: Seed priming; Germination; Catalase; Hydrogen peroxide; Isozyme

Introduction

Ageing accompanies diverse deleterious changes in cells that accumulate over the time that eventually leads to death. Seeds too lose viability with the progress of time. Seed viability is a complicated trait under the control of genetic, developmental, and environmental factors [1,2]. Often these factors interact with each other and produce deleterious effects during the process of seed maturation and harvest. Seed moisture and storage temperature play a significant role and confer varying length of seed viability. Concisely, we come across wide variability in seed viability, within and among seed lots and species that attributed to the interactions of above mentioned factors.

In recent years, increasing evidencehave been accumulated suggesting a functional significance for reactive oxygen species in seed aging and germination [3,4]. Reactive Oxygen Species (ROS) are metabolites of molecular oxygen (O_2) that have higher reactivity than O_2. ROS can include unstable oxygen radicals, such as superoxide radical ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), and non-radical molecules, like hydrogen peroxide (H_2O_2). Thus, all organisms living in an aerobic environment are exposed to ROS on a continual basis. Up to 2% of the oxygen utilized is converted into these deleterious agents and related reactive ROS, mostly, but not exclusively, within mitochondria. An enzymatic dismutation step must firstly take place to produce more stable H_2O_2 derivative from the $O_2^{\bullet-}$. H_2O_2 often travel across the cell membrane and is required for a viable long-range cell-to-cell signal [5]. Additional reactive $OH^{\bullet}$ can be formed from $O_2^{\bullet-}$ and H_2O_2 through Fe-catalyzed Haber-Weiss reaction that may cause lipid peroxidation [6]. The concentration of ROS is tightly controlled by ROS-scavenging pathways that metabolize ROS. However, an imbalance in generation and metabolism of ROS leads to a variety of physiological challenges by disrupting redox homeostasis of the cell, which is collectively known as "oxidative stress". Plant possesses an efficient antioxidative defense that protects the cell from oxidative damage. To minimize and/or to protect against the toxic effects of these damaging ROS, cells have evolved highly regulated enzymatic and non-enzymatic mechanisms to keep a balance between ROS production and destruction in order to maintain cellular redox homeostasis. These protective systems are composed of low molecular and enzymatic scavengers, such as superoxide dismutase (SOD), catalase (CAT) and enzymes of the Halliwell Asada pathway: ascorbate peroxidase (APX), dehydro ascorbate reductase (DHAR) and glutathione reductase (GR), connected with antioxidant compounds: ascorbate and glutathione [7]. SOD is a key enzyme in the regulation of the amount of superoxide radicals and peroxides. The removal of H_2O_2 through CAT as well as a series of reactions is known as an ascorbate glutathione cycle in which ascorbate and glutathione participate in a cyclic transfer of reducing equivalents resulting in the reduction of H_2O_2 to H_2O using electrons derived form nicotinamide adenine dinucleotide phosphate (NADPH) [8]. The cooperative activities of these enzymes and high levels of the low molecular antioxidants may increase the resistance to oxidative injury and minimize cell damage.

Additionally, increased level of ROS, including H_2O_2 [9,10], $O_2^{\bullet-}$ [11,12], and hydroxyl radicals [10], as well as reactive nitrogen species have been reported to play positive role during seed germination in plant species of varied taxonomic backgrounds. Seed priming affects germination rate, seed vigor, and seedling development. It has been shown that seed priming with water or solutions of various substances positively affects traits of seeds and seedlings [13,14]. Many research groups evaluated the ROS and detoxifying enzyme activity during seed priming and germination [15,16]. Bailly et al. proposed that an oxidative window for germination restricts the occurrence of cellular germination related events to a defined range of ROS level [17].

Previously research found that Neem (*Azadirachta indica*) seeds lose their viability during natural ageing [18,19]. The seed priming improved germination to a limited extent [20]. The aims of this work are to examine H_2O_2 metabolism during ageing and its post-priming germination.

Materials and Methods

Seed collection and extraction

Mature, ripe yellow fruits of *Azadirachta indica* (Neem) were collected manually from the trees growing on the campus of Pt. Ravishankar Shukla University, Raipur, (21° 14'14" N latitude and

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81°38'55" E longitude), and brought to the laboratory immediately. The initial water content of seeds was determined using randomly chosen mechanically depulped seeds. Seeds with endocarp were extracted from the fruit tissue by soaking them in 20% H_2SO_4 (Merck, India) for 30 min following depulping by rubbing over a wire mesh and washing repeatedly under tap water. Seeds with hard endocarp were dried to their initial water content under shade for storage [21]. Seeds were harvested from hard endocarp by braking gently to analyze biochemical variables after every 15 days following storage.

Sample collection and priming treatment

The seeds were collected at different percentage of germination and denoted as $G_{100\%}$, $G_{90\%}$, $G_{70\%}$, $G_{50\%}$, $G_{30\%}$ and $G_{0\%}$. Seeds with 100% germination referred as a control. The osmopriming treatments of naturally aged seeds were carried out in 12-cm-diameter petri dish with single layers of filter paper moistened with 5 ml of -0.78 MPa PEG solutions for germination improvement as optimized for neem seed priming [20]. Ten seeds of each differentially aged ($G_{90\%}$ to $G_{0\%}$) in five replicates were placed for treatment. After one day priming at 30°C, the seeds were washed in sterile water for twice, blotted on filter paper and immediately used for germination tests and other analysis. After each stage of ageing and treatment, following sets: dried (NA-WI), primed (Primed-NA-WI), germinated (NA-RE) and post-priming germinated (Primed-NA-RE) seeds were immediately frozen in liquid nitrogen to stop the chemical reaction, and grounded to a fine powder with a pestle and mortar in LN_2. The powder was stored at -80°C in humidity-proof vials until use.

Hydrogen peroxide estimation

Batches of five seeds at differentially aged and treated were pre-incubated for 30 min in 3 ml of K-phosphate buffer (20 mM, pH 6.0) to remove pre-formed H_2O_2 and were then incubated in 3 ml of the same buffer containing 5 µm scopoletin and 3 µg ml^{-1} horseradish peroxidase (Boehringer Mannheim) in darkness at 25°C on a shaker. The estimation of H_2O_2 was performed according to Schopfer et al. by an increase in fluorescence (excitation: 346 nm, emission: 455 nm) in the incubation medium and was measured using reagent blanks as a reference [10]. Results corresponded to the mean ± SE of four replicates and are expressed in fluorescence, 455 nm (relative unit) per seed.

Catalase (CAT) enzyme analysis and assay

Stored LN_2 crushed differentially aged and treated seed tissue samples were weighed (Fifty mg embryonic axes), grounded again in liquid nitrogen and homogenized in cold borate buffer (0.2 M, pH 7.4) containing 4% poly-vinylpyrolidone (PVP-40 Himedia), followed by centrifugation at 17,890 g for 20 min. The supernatants were stored at -20°C for later determination of enzyme activity spectrophotometrically.

Catalase (EC 1.11.1.6) activity was estimated measuring the decomposition of H_2O_2 at 240 nm as per the method of Aebi [22]. Enzyme activity was performed by taking, in a quartz cuvette, 2.74 ml of potassium phosphate buffer (37.5 mM, pH 6.8), to which 60 µl enzyme extract was added and the absorbance was zeroed at 240 nm. Catalase activity was triggered by adding 200 µl of H_2O_2 (60 mM). The change in absorbance was recorded for 6 min at 15 sec intervals. The blank was without enzyme extract. Catalase activity was expressed in $A_{240}min^{-1}mg^{-1}$ protein.

CAT Isozyme

For CAT isozyme assays, stored LN_2 crushed differentially aged and treated seed axes samples were homogenized in ice chilled Zivy's buffer (containing 30 mM Tris-base pH 8.5, 0.25 mM ascorbic acid, 1mM EDTA, 5 mM $MgCl_2$, 1 mM DTT and 0.1 mM PMSF) and centrifuged at 10,000 rpm for 10 min at 4°C [23].

CAT Isozymes were separated on 7% non-denaturing poly acrylamide gel electrophoresis and 30 µg protein per well was loaded. Gels were run at 20 mA constant current till the dye front reaches the bottom of the gel. Gels were stained by the method followed by Woodbury et al. [24]. After completion of gel running, gelwas soaked in DDW for 15 min and Incubated in 0.03% H_2O_2 for 10 min and stained with a solution of 1% ferric chloride and 1% Potassium ferricyanide. Gel turns Prussian blue except the position exhibiting CAT activity.

The protein content of the extract was determined by Bradford method using bovine serum albumin as standard [25].

Statistical analysis

Data were analyzed by one-way ANOVA in combination with Duncan's multiple-range tests at 5% level of significance ($p \leq 0.05$) for post-hoc comparisons of means. Statistical tests were carried out using SPSS (version 16) for Microsoft Windows (SPSS, INC., Chicago, IL).

Results and Discussion

Seed ageing/ loss of viability addresses stochastic passive degenerative processes that may be initiated by external factors but are endogenously influenced by an uncontrollable oxidative stress. ROS accumulation induces oxidative stress when the imbalance between ROS production and antioxidant defense against these ROS takes place. The findings of the present study show that hydrogen peroxide (H_2O_2) increased with its priming (primed-WI), germination (RE) and post-priming germination (primed-RE) compared with dried/ without imbibed (WI) by about 16.68, 28.85 and 39.24% at each level of ageing (Table 1). Many earlier studies have highlighted the increase in oxidative stress during seed ageing [26-28]. The increased H_2O_2 level in seed in the current experiment observed after priming (Primed-WI), germination (RE) and post-priming germination (primed-RE) compared to dried/ without imbibed (WI), suggest that free-radical mediated processes were initiated by re-aeration of hypoxic tissues and/or higher respiration. Ageing induced a decrease in CAT activity in differentially aged seeds (Table 2). Catalase activity increased with priming, germination as well as post-priming germination in embryonic axes at each level of ageing, but such improved activity turned down with ageing. Interestingly, CAT activity was enhanced on average 35.39% after their priming (Primed-WI), 34.55% during germination (RE) and 64.82% post-priming germination (primed-RE) as compared to dried/without imbibed (WI) embryo axes during natural ageing. Gels stained for CAT activity revealed only one CAT isoform in naturally aged embryonic axes; however, new isoform appeared during post-priming germination. The band intensity gradually decreased with different level of ageing, whereas, the intensity increased after its priming as well as radicle emergence at each level of ageing (Figure 1).

Conclusion

Hydrogen peroxide enhanced during radicle emergence (RE), priming (primed-WI) and priming subsequent radicle emergence (primed-RE) compared with the dried/ without imbibed (WI) aged seeds. It is suggested that free-radical mediated processes were initiated by re-aeration of hypoxic tissues and/or higher respiration. Catalase enzyme significantly decreased with ageing CAT activity was enhanced with osmopriming and/or radicle emergence. The occurrence of extra

	Without Imbibed		Radicle Emergence	
	Aged	**Primed**	**Aged**	**Primed**
Natural Ageing				
$G_{100\%}$	80.13 ± 1.23	-§	95.61 ± 2.21	-§
$G_{90\%}$	82.50 ± 2.14	95.44 ± 2.77	113.03 ± 3.38	127.43 ± 2.69
$G_{70\%}$	90.03 ± 3.56	110.05 ± 3.33	131.26 ± 3.31	149.90 ± 5.41
$G_{50\%}$	102.56 ± 5.34	135.23 ± 3.32	156.33 ± 6.07	184.78 ± 4.80
$G_{30\%}$	132.60 ± 2.73	167.20 ± 5.49	171.34 ± 5.02	211.42 ± 4.16
$G_{0\%}$	185.02 ± 6.82	198.60 ± 3.61	-	-
F-value (1)	**97.368**	**121.115**	**53.372**	**71.798**
Df	**5, 18**	**4, 15**	**4, 15**	**3, 12**
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**
§ Control ($G_{100\%}$) excluded in osmopriming treatment				

Table 1: Hydrogen peroxide (H_2O_2) released by aged and primed seeds at two different stages, without imbibed (WI) and radicle emergence (RE) during different levels of natural ageing. Values were represented in relative fluorescence mean at 455nm ± SE and data were expressed in per seed basis.

	Without Imbibed		Radicle Emergence	
	Aged	**Primed**	**Aged**	**Primed**
Axes				
$G_{100\%}$	1.25 ± 0.03	-§	1.36 ± 0.02	-§
$G_{90\%}$	0.72 ± 0.04	1.21 ± 0.03	1.23 ± 0.01	2.23 ± 0.05
$G_{70\%}$	0.66 ± 0.05	1.08 ± 0.08	1.09 ± 0.01	2.17 ± 0.09
$G_{50\%}$	0.57 ± 0.03	0.99 ± 0.02	0.96 ± 0.04	1.54 ± 0.05
$G_{30\%}$	0.52 ± 0.02	0.85 ± 0.02	0.93 ± 0.02	1.28 ± 0.07
$G_{0\%}$	0.37 ± 0.01	0.44 ± 0.02	-	-
F-value (1)	**97.887**	**49.832**	**51.554**	**49.256**
Df	**5, 18**	**4, 15**	**4, 15**	**3, 12**
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**
§ Control ($G_{100\%}$) excluded in osmopriming treatment				

Table 2: Catalase (CAT) activity in axes tissue of aged and primed seeds at two different stages, without imbibed (WI) and radicle emergence (RE) during different levels of natural ageing. Values were presented in activity mean ± SE and expressed in A_{240} min^{-1} mg^{-1} protein.

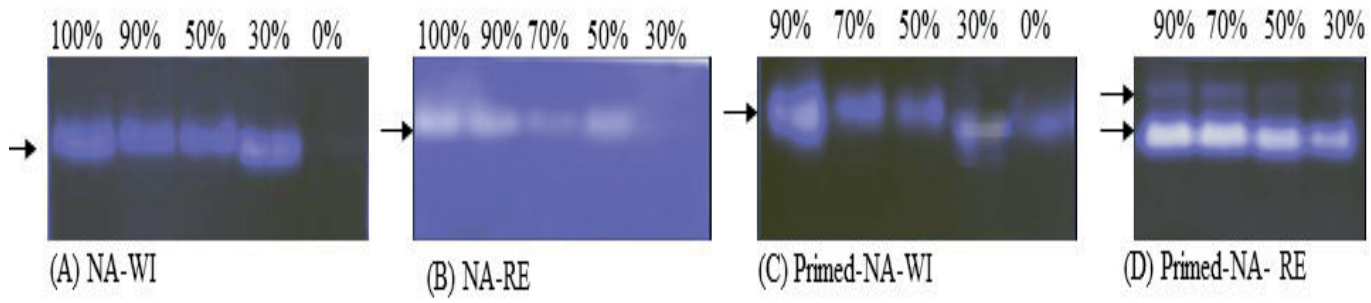

Figure 1: Native gel stain for embryonic-axes CAT of aged and primed seeds during without imbibed (WI) and radicle emergence (RE) at different level of natural ageing. Arrows indicate number of CAT isoform appeared on native gel.

CAT isoform during germination in osmoprimed seeds confirmed that it was produced by a separate CAT gene and probably expresses during germination.

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