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H_2O_2 as a better index of seed quality and mechanism of cucumber (*Cucumis sativus*) seed deterioration

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ABSTRACT

The present investigation was conducted at research farm of ICAR-Indian Agricultural Research Institute, New Delhi for 2016–17 and 2017–18 to study the seed deterioration in cucumber (*Cucumis sativus* L.) cv PusaBarkha by mimicking seed ageing with accelerated ageing. The fresh seeds were subjected to accelerated ageing at 40±1˚C and 100% RH for a duration of 2, 4, 6 days along with control (0 days) in desiccators. With the progression of ageing, a gradual decline in the seed quality parameters, viz. seed germination and seed vigour indices was observed. The H_2O_2 content was within the threshold level from 0 to 4 days of ageing but beyond this, it damaged the cell membrane in seeds. Similarly, antioxidant activity (SOD, CAT, POX and higher GSH/GSSG ratio) increased initially and maintained the redox state by quenching the H_2O_2 effectively. Whereas, the content of H_2O_2 reached above the oxidative window as the activity of enzymes also decreased beyond 4 DAA. The study suggested that the H_2O_2 within oxidative window could be quenched efficiently; beyond this it is toxic and affects longevity as enzymes get inactivated with ageing in storage.

Keywords: Accelerated ageing, Antioxidants, Cucumber, Glutathione, H_2O_2, Seed deterioration, Seed germination, Seed vigour

Cucumber (*Cucumis sativus* L), member of *Cucurbitaceae* family and indigenous to India is the most important vegetable worldwide having short-life cycle (i.e. three months from seed to seed). Cucumber is a summer cultivated crop. It has the highest export potential but many problems are encountered during its cultivation starting with dormancy, lower seed quality and non-availability of sufficient quantity. The seed is the prime factor which determines the quantitative and qualitative characteristics of the crop. Loss in seed viability directly affects the production, seed sale and ultimately huge losses to the agricultural sector every year. Seed health significantly decreases with increasing moisture and temperature (Chhabara *et al.* 2019).

The process of seed ageing primarily depends upon physiological conditions, genetic potential and storage condition. In general, seed deterioration can be defined as the "deteriorative changes occurring with the time that increase the seed's exposure to external challenges and decrease the ability of the seed to survive". In plants, ageing causes several biochemical changes which help to protect the plants. Depending upon the intensity of stress, plant induces protection mechanism. Numerous oxidants are produced during stress such as ROS, free radicals which causes numerous physiological alterations including impairment of protein synthesis, lipid peroxidation, DNA damage, and membrane disruption which became a major cause of seed deterioration. Eventually, ageing leads to seed deterioration, loss of vigour and increase sensitivity to stresses upon germination (Walters *et al.* 2010, Nigam *et al.* 2019). Rapid germination and emergence are essential for successful crop establishment, for which proper seed storage and identification of the causes and related indicators could play an important role. Therefore, the present investigation was envisaged to understand the mechanism of seed deterioration in cucumber seeds and to find out the protection mechanism of plant against the ROS.

MATERIALS AND METHODS

The freshly harvested seeds of cucumber cv Pusa Barkha were procured from Division of Vegetable Science, ICAR-IARI, New Delhi (2016–17 and 2017–18). Seeds were surface sterilized with 1% NaOCl, dried to its original moisture and used for further studies (2017 and 2018). Seeds

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were subjected to accelerated ageing (100% RH & 40±1˚C) for 0 to 6 days to understand the mechanism of seed ageing.

To adjudge the seed quality, viz. seed germination percentage, ISTA rules (Anon 2019) were followed with a modification of using 25 seeds, each in three replicates. The seed germination was recorded on 4th day (first count) and 8th day (final count) from seed planting and, the seed vigour index, antioxidant activity, catalase activity, peroxidase activity, superoxide dismutase, glutathione and hydrogen peroxide were calculated after 6 days of accelerated ageing (DAA).

Seed vigour index: The seed vigour indices were determined as (Abdul-Baki and Anderson 1973).

Seed vigour index (SVI-I) = Germination % × Seedling lenath (cm)

Seed vigour index (SVI-II) = Germination % × Seedling dry weight (g)

Antioxidant activity: Antioxidant activities were determined by following the methodology of Dhindsa *et al.* (1981) and Hasan *et al.* (2018).

Catalase activity: Catalase enzyme activities were determined by following the methodology of Aebi *et al.* (1984).

Peroxidase activity: Peroxidase activities were determined by following the method of Oswald *et al.* (1992).

Superoxide dismutase activity: Superoxide dismutase activities were determined by following the method of Beauchamp and Fridovich (1971).

Glutathione assay: Glutathione is an antioxidant and non-enzyme antioxidant enzyme. Glutathione was analyzed by the following method of Calvi *et al.* (2017).

Hydrogen peroxide assay: Hydrogen peroxide was determined by the following method of Mukherjee and Choudhury (1983).

RESULTS AND DISCUSSION

The seed germination percentage registered a gradual decrease with the increased seed ageing. Seed vigour indices (SVI-I and SVI-II) also steadily declined with longer duration of accelerated ageing. SVI-I (1714.96) and SVI-II (78) from untreated control (0-DAA) seeds, reduced to minimal values, *i.e.* SVI-I (510.91) and SVI-II (24), respectively, during 6-DAA (Table 1). The decline in seed quality parameters can be attributed to damage in membrane integrity, owing to lipid peroxidation during seed ageing process (Parrish and Leopold 1978). During accelerated ageing, seeds are exposed to longer duration of higher moisture and temperature which leads to accelerated seed deterioration. Similar findings were reported by Trivedi *et al.* (2018) in cumin seeds, Chandel *et al.* (2016) in soybean seeds, Kumar *et al.* (2019) in sesame seeds and Nigam *et al.* (2019) in tomato seeds.

The changes in antioxidants comprising enzymatic, catalase, peroxidase, superoxide dismutase; non-enzymatic glutathione and ROS, i.e. hydrogen peroxide in cucumber seeds during the accelerated ageing test were estimated and presented in Table 1. Catalase undertakes the activity of breaking down of H_2O_2 into water and oxygen. It increased up to 8-folds (14.77 to 120.17 µmol/g_{fw}/min) during 0 to 4-DAA followed by reduction (58.44 µmol/g_{fw}/min) in 6-DAA (Fig 1). Peroxidase also quenches the reactive oxygen species produced during accelerated ageing. It utilizes H_2O_2 to produce water with phenol or ascorbic acid as electron donors. Like catalase, the peroxidase activity also increased 6 to 7-folds (9.37–66.50 µmol/g_{fw}/min) during 0 to 4-DAA; followed by reduction (21.95 µmol/g_{fw}/min) in 6-DAA. Superoxidedismutase, another antioxidant, catalyzes the dismutation reaction and converts superoxide to hydrogen peroxide and oxygen. It initially increased half fold (59.47 to 86.65 unit of enzyme/g_{fw}/min) during 0 to 2-DAA, further decreased (63.6 and/or 61.18 unit of enzyme/g_{fw}/min) during 4 or 6-DAA, respectively. The results are in line with the findings of Chandel *et al.* (2016) in soybean seeds, Kavitha *et al.* (2017) in maize seeds and Nigam *et al.* (2019) in tomato seeds. The results thus showed significant increase in antioxidants enzymes activities from 0 to 4 DAA and then decrease in activities from 4–6 DAA which indicated protective mechanism of seeds against oxidative stress. The decreasing activities of these antioxidants from 4 to 6 DAA were related to progressive water content decline.

Glutathione is a major non-enzymatic antioxidant which

Table 1 Seed quality during accelerated ageing of cucumber seeds

DAA	Physiological parameters			Biochemical parameters						
	Germination (%)	SVI-I	SVI-II	CAT (µmol/g FW/min)	POX (µmol/g fw/min)	SOD (unit/g fw/min)	GSSG (nmol/ min/g fw)	GSH (nmol/ min/g fw)	GSH/GSSG (nmol/min/g fw)	H_2O_2 (µmol/g fw)
0 (control)	88.33	1725.07	78.39	14.77	9.31	59.47	10.61	1029.39	97.02	5.42
2	77.33	1425.11	56.73	66.48	33.69	86.65	18.95	953.42	50.30	11.77
4	69.33	1037.73	43.98	120.17	66.50	63.60	33.37	695.65	20.84	16.38
6	49.33	513.19	24.90	58.44	21.94	61.18	39.37	515.89	13.10	21.97
SE±m	1.42	33.41	2.81	1.56	0.001	0.15				0.03
CD (P=0.05)	4.71	110.66	9.33	5.18	0.002	N/A				0.11

SVI-I, Seed vigour index-I; SVI-II, Seed vigour index II, DAA, Days of accelerated ageing; CAT, Catalase; POX, Peroxidase; SOD, Superoxidedismutase; GSSG, Glutathione (oxidised); GSH, Glutathione (reduced); H2O2, Hydrogen peroxide.

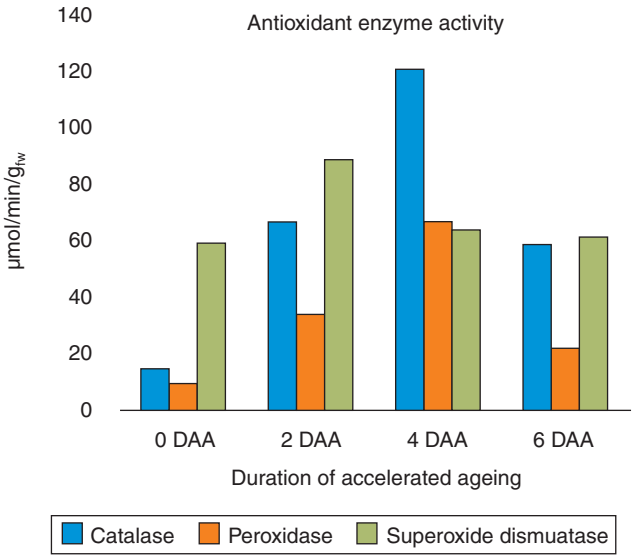

Fig 1 Changes in catalase, peroxidase and superoxidedismutase from cucumber seeds during accelerated ageing. Where Values are the mean of three replicates; 0 DAA: Control (without accelerated ageing), 2 DAA: 2-days accelerated ageing, 4 DAA: 4-days accelerated ageing, 6 DAA: 6-days accelerated ageing.

is involved in protective mechanism during stress, and helps to improve the longevity of seeds. It was estimated in two forms, i.e. reduced form (GSH) and oxidised (GSSG) form (Table 1). GSH activity registered highest value (1029.39 nmol/min/g_{FW}) in 0-DAA which was reduced to 695.65 and/or 515.89 nmol/min/g_{FW} during 4 and/or 6-DAA, respectively. In contrast, GSSG activity was very low (10.61 and 18.95 nmol/min/g_{FW}) during 0 and 2 DAA; and increased slightly (39.37 nmol/min/g_{FW}) on 6-DAA. The ratio of GSH/GSSG was maximum (97.02 nmol/min/g_{FW}) on 0-DAA. Therefore, among the studied various enzymes (strong or weak), positive or negative correlations, in general, had depicted an increasing trend from 0 to 4 DAA, which declined with further accelerated ageing (6-DAA). Glutathione reduced form (GSH), a major non-protein thiol group, the most abundant in plant species, plays a crucial role for maintaining redox status of the cell and seed storage and also transports reduced sulphur, necessary for protein synthesis. The primary oxidation product of GSH is its disulfide, GSSG, which is produced *in-vivo* mainly via thiol-disulfide exchange with proteins; so also by a variety of oxidants. GSSG can be reduced back to GSH by the action of glutathione reductase in the presence of NADPH. In non-stressed cells, glutathione is mainly present as GSH but during higher RH and temperature, i.e. during accelerated ageing, the glutathione redox status may shift to a more oxidized form due to an increased GSH oxidation and/or a decreased GSSG. The findings of the present study were supported by De *et al.* (1994) in tomato, Hsu and Sung (1997) in triploid watermelon and Xin *et al.* (2014) in soybean.

H_2O_2 is a signalling molecule from within an oxidative window (during germination) increased to beyond oxidative window (during accelerated ageing), and acts as reactive oxygen species responsible for damaging the cell membrane. The concentration of H_2O_2 had increased up to four-folds (5.42–21.97 µmol/g_{fw}) during 0 to 6-DAA seeds, respectively (Table 1). ROS such as superoxide radicals ($O^{2.-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH^-$) are produced in mitochondria, peroxisomes and the apoplastic space of germinating seeds as a result of aerobic metabolism. Accumulation of the ROS is detrimental to seed viability since it imposes oxidative stress during the seed desiccation or ageing. ROS acts as signaling molecules that break dormancy and facilitate seed germination. H_2O_2 has been reported to promote germination by acting as a secondary messenger in the seed germination process (Barba-Espin *et al.* 2011). It has been demonstrated that hydration of seeds causes a release of free radicals from the trapped state. The level of H_2O_2 in the plants is maintained by several enzymes such as superoxide dismutase, peroxidase, oxalate oxidase, xanthine oxidase, membrane linked nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and amine oxidase among which cell wall peroxidase plays a major role in the generation of H_2O_2. The role of peroxidase can be antagonistic depending on the stage of the plant and the site of its production. The changes in the activities of catalase and peroxidase were observed in seeds on different days of seeds ageing, i.e. on 4-DAA, the peroxidase activity was maximal (over control) which was reduced on 6-DAA (Fig 2). During the oxidative cycle of peroxidase, O^{2-} is converted to H_2O_2 by extracting electron from an electron donor, i.e. NAD reduces O_2 to O^{2-} that can be converted to H_2O_2 by oxidizing extra NADH to NAD as reported in *Arabidopsis* (Liszkay *et al.* 2003). The catalase undertakes the activity of breaking down the H_2O_2 into oxygen and water molecule. Reduced catalase activity was observed for control seeds which could explain the increased levels of H_2O_2 in 4-DAA seeds. The critical levels of H_2O_2 that promote germination in cucumber seeds are thus maintained by a concerted synthesis and breakdown action of peroxidase and catalase, respectively. Superoxide dismutase, the unstable and harmful reactive forms of oxygen, stops the

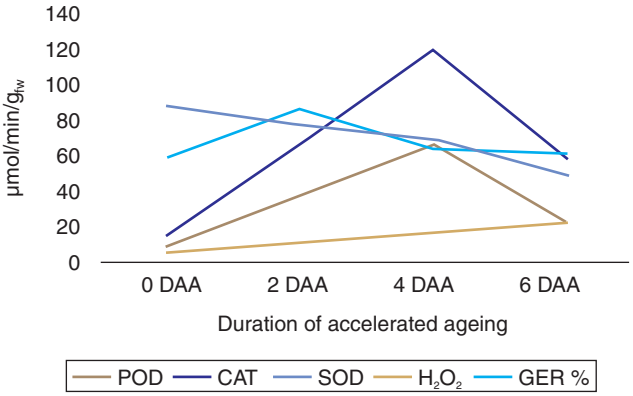

Fig 2 Changes in studied enzyme activities from cucumber seeds during accelerated ageing.

Table 2 Correlation studies between antioxidants, ROS and various seed quality parameters

	CAT	POD	SOD	H_2O_2	GSH	GSSG	GER	SVI	SVII
CAT	1								
POX	0.97*	1							
SOD	0.15	0.15	1						
H_2O_2	0.55	0.37	-0.12	1					
GSH	0.18	0.18	0.99**	-0.14	1				
GSSG	-0.32	-0.51	0.07	0.54	0.01	1			
GER	-0.37	-0.18	0.19	-0.97*	0.22	-0.67	1		
SVI	-0.41	-0.22	0.28	-0.97*	0.30	-0.61	0.99**	1	
SVII	-0.50	-0.32	0.12	-0.99**	0.15	-0.58	0.98**	0.98**	1

action of superoxide radical and dismutase it into H_2O_2 which is later acted upon by other enzymes like CAT and POX in a chain.

As the stress increased, the activity of SOD increased at an early stage and but stress for a prolonged period lead to decline in the activity because of exposure to a higher temperature and relative humidity. The extended ageing declined the activity of all the enzymes, viz. SOD, CAT and POX. The contents of GSH decreased with increased seed ageing (stress), and got converted to GSSG (oxidized form). The ratio of GSH/GSSG depicts the ability of the non-enzymatic antioxidant system to maintain the redox potential of the seed and improve longevity as reported in moong bean seeds (Shaheed and Abass 2014). Beyond the studied enzymes, reactive oxygen species play a major role in different aspects of seed physiology and may lead to oxidative damages and/or cellular damages - resulting in seed deterioration during seed ageing (stress). During stress, with increasing H_2O_2, the enzymes were also increased for the quenching of free radicals up to a certain limit, beyond which, *i.e.* oxidative window, it may lead to degeneration of cell and hampering of all biological processes, which ultimately reduces seed germination and vigour (Gupta *et al.* 2021a, b, c) as also reported in cucumber.

Correlation studies: Among antioxidants and/or ROS with seed quality parameters, correlations in general were either positive or negative. Seed germination, SV-I and SV-II showed positive correlations with SOD and GSH, whereas negative correlations with CAT, POX and GSSG. Seed germination recorded the strongest but negative correlation with H_2O_2 content (-0.979 at 5%), whereas negative but weakest correlation with POD (-0.18364 at 5%) (Table 2).

Since H_2O_2 was strongly correlated with all seed quality parameters, viz. germination and both vigour indices, thus H_2O_2 was identified as better indices for seed quality.

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