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FULL LENGTH ARTICLE

Reactive oxygen species and ascorbate–glutathione interplay in signaling and stress responses in *Sesamum orientale* L. against *Alternaria sesami* (Kawamura) Mohanty and Behera

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Abstract *Sesamum orientale* wild and cultivar Thilarani exposed to *Alternaria sesami* infection triggered the signal cascade H_2O_2 content that was positively correlated with lipid peroxidation. The data were also supported by H_2O_2 localization as observed by scanning electron microscopy. Parallely, infection altered chloroplasts marginally and mitochondria effectively in susceptible cultivar than wild sesame. Deformities in the structure of these organelles were accompanied by changes in antioxidant machinery. H_2O_2 can be effectively detoxified via the ascorbate–glutathione cycle. Increases in ascorbate peroxidase, and glutathione reductase activities concomitant with ascorbate (AsA) and glutathione interplay, as well as AsA regeneration ability, function to keep the balance of cellular H_2O_2 under pathogenicity. Dehydroascorbate reductase and monodehydroascorbate reductase are responsible for AsA regeneration. Oxidative damage in Thilarani cultivar compared to wild sesame is attributed by a lower induction of the ascorbate–glutathione cycle as an antioxidant defense system and was not sufficient to protect mitochondria but prevent ultrastructural damage of chloroplasts. Overall, the availability of antioxidants and the induction of antioxidant enzyme activities for detoxifying reactive oxygen species (ROS) are regulated effectively in wild sesame against *A. sesami* induced oxidative stress. The experiments using ROS scavengers demonstrate that the antioxidant defense system is modulated by $O_2^{\cdot-}$ or H_2O_2 signals.

1. Introduction

Production of reactive oxygen species (ROS) is linked to signaling in both developmental and stress responses. The level of ROS is controlled by both production and removal through various scavengers including ascorbic acid and glutathione.

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Ascorbic acid and glutathione are central components in regulating the redox balance of the plant cell. For successful survival, plants have to adapt and acclimatize to the surrounding environment. ROS are used by plants as signaling molecules in a variety of processes ranging from defense against pathogens to developmental process (Love et al., 2005; Torres et al., 2006; Torres, 2010; Jannat et al., 2011). It has become increasingly clear that signaling pathways in plants are not organized into linear pathways; instead, as a web of interactions. Not even individual ROS give uniform responses; instead, separate molecules (hydrogen peroxide, superoxide, and singlet oxygen), acting at different subcellular locations give rise to unique changes in gene expression (Wrzaczek et al., 2010). ROS production and scavenging are intimately linked, and the balance between them will determine defense signaling output as well as damage and cell-death responses (Apel and Hirt, 2004; Cheng and Song, 2006). An active phase of enzymatic ROS scavengers (including catalase, superoxide dismutase, and ascorbate peroxidase) and low-molecular-weight scavengers (ascorbate, glutathione, and α-tocopherol) protect plants from excessive ROS production (Ahmad et al., 2008; Gill and Tuteja, 2010; Sharma et al., 2012). Ascorbate and glutathione are connected through the ascorbic acid–glutathione cycle (Bhattacharjee, 2012) and are essential for plants; *Arabidopsis thaliana* mutants with very low concentrations of either compound have severe developmental defects (Dowdle et al., 2007). In addition to ROS scavenging and redox chemistry, ascorbic acid also has a role in regulating the cell cycle and is a substrate or cofactor of many enzymes (Brosché and Kangasjarvi, 2012). Although the implication of the oxidative stress in plant–pathogen interactions is well documented (Mendoza, 2011), to date very little information is available on the involvement of the AsA-GSH cycle in plant defense against biotic stress. This picture is further complicated by the fact that the ROS-mediated plant response is variable and depends on the pathogen life style (biotrophy versus necrotrophy), the type of plant–pathogen interaction (compatibility versus incompatibility) and the stage of plant development (Huckelhoven and Kogel, 2003). Thus, the tightly regulated balance between ROS production and removal at both the cellular and subcellular levels seems to be of primary importance for fulfilling the multiple functions of ROS and controlling redox homeostasis. At this juncture, the objective of the present study is to understand the regulation of the antioxidant defense system and ultra structural changes in *Sesamum orientale* wild and cultivar – Thilarani – in response to *Alternaria sesami* infection.

2. Materials and methods

2.1. Plant materials

S. orientale wild and Thilarani the cultivar were raised from seeds in healthy conditions in a glasshouse. For *in vitro* fungal inoculation studies, mature plants were inoculated with 20 μL of *A. sesami* conidial suspension (1×10^3 conidia mL^{-1}) from pure culture or 20 μL of water (mock inoculation). The inoculated plants, along with their respective healthy controls, were then maintained at 30 °C in a temperature controlled glasshouse under a photoperiod of 12/12 h (light/dark) and 60% RH. After the development of symptoms in infected plants (after 10 and 14-d of inoculation in the wild and Thilarani plants, respectively) the experiment was terminated and the plants harvested for all analysis.

2.2. Transmission electron microscopy (TEM)

Small pieces (1 mm^2) of *S. orientale* control and infected leaves were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 6.8, for 2 h at 0–4 °C. After washing with the same buffer, small pieces of leaves were post fixed in 1% OsO_4 at 0–4 °C for 2 h. The material was dehydrated in ethanol, then propylene oxide and afterward embedded in Spurr-/Epon mixture. Ultrathin sections cut on a Ultratome were stained with uranyl acetate and lead citrate (Terai et al., 2000) and examined in a JEOL 1010 transmission electron microscope at 80 kV. The ultrastructure of chloroplasts was studied in mesophyll cells from the middle parts of the fourth leaves from the control and treated plants.

To localize *in situ* H_2O_2 in sesame leaves of wild-type and Thilarani plants, the cytochemical method based on the generation of cerium perhydroxides developed by Lherminier et al. (2009) was used.

2.3. Estimation of lipid peroxidation, peroxide, and H_2O_2 level

Leaves of approximately 0.1 g were ground in liquid nitrogen and then 1 mL of 5% (w/v) trichloroacetic acid (TCA) was added. After centrifugation at 12,000*g* for 10 min at 4 °C, the supernatant was collected as a TCA extract for the determination of lipid peroxidation, peroxide, and H_2O_2.

The levels of lipid peroxidation were determined from the thiobarbituric acid reacting substance (TBARS) contents resulting from the thiobarbituric acid (TBA) reaction as described by Devasagayam et al. (2003). The TBARS contents were calculated based on A_{532}–A_{600} with the extinction coefficient of 155 mM^{-1} cm^{-1}.

H_2O_2 content was determined based on the decomposition of H_2O_2 by peroxidase as described by Okuda et al. (1991).

Peroxide contents were determined by the absorbance at A_{480} and extinction coefficient of 13.93 μM^{-1} cm^{-1} according to Sagisaka (1976).

2.4. Determination of ascorbate and glutathione contents

0.1 g was ground in liquid nitrogen and then 1 mL of 5% (w/v) trichloroacetic acid (TCA) was added. After centrifugation at 12,000*g* for 10 min at 4 °C, the supernatant was collected as a TCA extract for the determination of water-soluble antioxidant contents.

The measurement of total ascorbate and reduced ascorbate (AsA) contents was modified from the method of Hodges Charles et al. (2001). Total ascorbate contents were determined in a 1 mL mixture containing 200 μL TCA extract, 50 mM potassium phosphate buffer (pH 7.4), 3 mM EDTA, and 1 mM dithiothreitol (DTT). The mixture was incubated at 25 °C for 10 min, 100 μL of N-ethylmaleimide was added, and then 400 μL of 0.61 M TCA, 400 μL of 0.8 M orthophosphoric acid, and 400 μL of α,α′-bipyridyl were added. Finally, 200 μL of $FeCl_3$ was added and the mixture was incubated in a 55 °C water bath for 10 min and the absorbance was detected at A_{525}. AsA contents were determined by adding distilled

water instead of DTT and N-ethylmaleimide and then followed the same method as above. Total AsA and AsA contents were estimated from the standard curve of 0–40 nmol L-AsA determined by the above methods. DHA contents were calculated by the subtraction of AsA from total AsA.

Total glutathione contents were determined by the absorbance at 570 nm according to the method of Mytilineou et al. (2002). The contents of glutathione (reduced form) were estimated from the standard curve of 0–20 nmol glutathione. After the removal of glutathione by 2-vinylpyridine derivative, glutathione disulfide contents were determined, and the glutathione contents were calculated by the sub traction of glutathione disulfide contents from total glutathione contents.

2.5. Determination of antioxidant enzyme activity

Lyophilized leaves of approximately 1.0 g were first homogenized in liquid nitrogen and 0.6 mL of 0.1 M sodium phosphate buffer (pH 6.8) containing 1% (w/v) polyvinyl polypyrrolidone (PVPP) and 0.25% (v/v) Triton X-100 was then added. After centrifugation at 12,000*g* for 10 min at 4 °C, the supernatant was used for enzyme activity assay of SOD, CAT, POX, GR, MDHAR, and DHAR. For the determination of APX, 1.0 g of lyophilized leaves was homogenized in liquid nitrogen and 0.6 ml of extraction buffer (0.1 M sodium phosphate buffer (pH 6.8) containing 1% (w/v) PVPP, 0.25% (v/v) Triton X-100, and 0.5 mM L-ascorbate) was added. After centrifugation at 12,000*g* for 10 min at 4 °C, the supernatant was used for the APX assay. The soluble protein contents were determined by the Coomassie blue dye binding method (Bradford, 1976) with bovine serum albumin as the standard curve.

The CAT activity was measured at A_{420} for H_2O_2 decomposition rate using the extinction coefficient of 40 mM^{-1} cm^{-1} according to Lijun et al. (2005). Guaiacol POX activity was determined by the formation rate of tetraguaiacol detected at A_{470} with the extinction coefficient of 26.6 mM^{-1} cm^{-1} according to Lijun et al. (2005). SOD activity was determined by the inhibition of photo chemical inhibition of nitro blue tetrazolium according to Lijun et al. (2005). APX activity was determined at A_{290} for DHA according to the extinction coefficient of 2.8 mM^{-1} cm^{-1} (Chen and Wang, 2002). GR activity was monitored by A_{340} for β-NADPH oxidization as GSSG reduction according to Chen and Wang (2002).

Both MDHAR and DHAR activities were determined according to Song et al. (2005). DHAR activity was measured by monitoring the absorbance at 265 nm for 3 min in the reaction mixture that consisted of enzyme extract, 50 mM Na-phosphate buffer (pH 7.0), 0.3 mM glutathione, 0.06 mM Na_2-EDTA and 0.2 mM DHA. MDHAR activity was measured by the monitoring the absorbance at 340 nm for 3 min in 50 mM Na-phosphate buffer (pH 7.6), 0.1 mM NADPH and 0.1 unit AsA oxidase (Sigma, MO, USA) and 2.5 mM AsA. Non-enzymatic reduction of DHA or MDHA in phosphate buffer was measured in a separate cuvette at the same time.

2.6. Statistical analysis

The data were analyzed by SAS (SAS version 8.1, NC, USA). The present results were the mean of three replicates with a pot as a replicate. The effects of infection on TBARS contents, peroxide contents, H_2O_2 contents, water-soluble antioxidant contents, and enzyme activities, and the effects of ROS scavengers were analyzed by 1-way analysis of variance (ANOVA). The difference among means was analyzed by Duncan's new multiple range test followed by significant ANOVA at $P < 0.05$.

3. Results

3.1. Ascorbate and glutathione contents

Ascorbate and glutathione contents were analyzed to test whether the water-soluble antioxidants are involved in the defense system against *A. sesami* induced oxidative stress in *S. orientale*. The contents of total AsA, AsA, and DHA and the ratios of AsA/DHA were significantly influenced by pathogenicity (ANOVA, $P < 0.05$) (Table 2) i.e., increased remarkably in wild sesame compared to cultivar Thilarani and the respective controls (Table 2). To analyze the regeneration rate of AsA, the ratio of AsA/DHA was calculated. Interestingly, AsA/DHA increased with the maximum followed by a drop.

Total glutathione, glutathione, and glutathione disulfide contents and glutathione/glutathione disulfide ratios were also affected (ANOVA, $P < 0.05$) (Table 3). The contents of total glutathione, glutathione, and glutathione disulfide were increased followed by a marginal decrease. Wild sesame responded more significantly than the cultivar Thilarani. Glutathione/glutathione disulfide ratio as an indicator of glutathione regeneration rate decreased with pathogenicity because the magnitude of glutathione disulfide increase was higher than that of glutathione increments (Table 3). *A. sesami* infection increased the contents of TBARS and H_2O_2 significantly in the cultivar Thilarani compared to its control and wild sesame (Table 1) i.e., TBARS increased in the infected leaves to 3.5 folds and similarly H_2O_2 content to 2.5 folds.

Table 1 H_2O_2, peroxide and TBARS contents in infected (I) and control (C) Wild & Thilarani leaves of sesame from 10 to 24 days.

		Wild						Cultivar					
Days		14	16	18	20	22	24	10	12	14	16	18	20
H_2O_2	C	6.6	7	7.2	7.3	7.5	7.5	7.8	7.8	7.8	7.9	8	8
(μmol/g)	I	7.7	10	12.5	13.9	14.4	15	10.4	12.6	15.2	19.8	20.9	20.9
Peroxide	C	0.3	0.4	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.6
(μmol/g)	I	0.6	0.8	0.9	1	1	1.2	0.9	1.4	1.6	1.9	2.0	2.0
TBARS	C	286	286	290	290	290	290	358	358	358	358	358	358
(μmol/g)	I	858	912	926	960	984	1011	974	999	1100	1212	1286	1372

3.2. TEM analysis

A. sesami infection affected the ultrastructure of chloroplasts but more severely the mitochondria (Fig. 1). Major structural changes observed are change in the shape, thylakoids are swollen with wave-like nature. Internal lamellar system is retained but separated from the chloroplast membrane (Fig. 2). Malformed elongated mitochondria with prominent vacuolation, altered cristae with electron transparent areas are noticed.

Transmission electron microscopy (TEM) study, using the cerium chloride technique which leads to the formation of perhydroxide precipitates in the presence of H_2O_2, was performed to localize H_2O_2. Cells exhibited a weak cerium staining in the control (uninfected) whereas, densely packed precipitates along the PM (Fig. 3) were noticed in the cultivar sesame. Meanwhile, the wild sesame shows mild precipitates along the PM. This is also consistent with the detection of H_2O_2 accumulation along the PM of cultured cells of *Rubia tinctorum* challenged with a fungal elicitor (Boka et al., 2007).

Table 2 Ascorbate (AsA), dehydroascorbate (DHA), total ascorbate contents and AsA/DHA ratio in infected (I) and control (C) Wild & Thilarani leaves of sesame from 10 to 24 days.

		Wild						Cultivar					
Days		14	16	18	20	22	24	10	12	14	16	18	20
AsA	C	48.9	53	60	64	68	68	35	38	38	40	40	40
(μmol/g)	I	70	88	105	110	115	120	37	39	41	44	46	48
DHA	C	21	23	24	28	28	28	16	15	16	17	18.9	18
(μmol/g)	I	30	35	40	50	54	55	25	28	30	33	34	38
Total AsA	C	69.9	76	84	92	96.2	96.2	56.1	53	54	58	58.9	59.8
(μmol/g)	I	100	123	145	160	169	175.6	62	67	71	74	78	80.2
AsA/DHA	C	2.32	2.3	2.5	2.28	2.42	2.42	2.18	2.53	2.4	2.22	2.35	2.2
ratio	I	2.33	2.51	2.62	2.20	2.12	2.18	1.48	1.39	1.36	1.35	1.33	1.22

Table 3 Reduced glutathione (GSH), oxidized glutathione (GSSG), total glutathione and GSH/GSSG ratio in infected (I) and control (C) Wild & Thilarani leaves of sesame from 10 to 24 days.

		Wild						Cultivar					
Days		14	16	18	20	22	24	10	12	14	16	18	20
GSH	C	8	8	9	9	10	10	7	7	7	7.5	7.5	8
(μmol/g)	I	9	9	10	10	10	9	7	7	7	7	6	5.7
GSSG	C	3	3	3	3.5	4	3.6	3	3.6	4	4.6	4.6	4.8
(μmol/g)	I	5	5.3	6	6.8	7	6.2	4	4.5	5	5	5	5
Total glutathione	C	11	11.6	12	12.5	13	13.6	10	10.6	11	11	11	11.2
(μmol/g)	I	14	15.2	16	16.8	16	15.8	11	11.5	12	12	10.6	9.9
GSH/GSSG	C	2.66	2.66	3.0	2.56	2.5	2.77	2.33	1.94	1.75	1.6	1.6	1.66
ratio	I	1.8	1.7	1.66	1.47	1.42	1.45	1.75	1.55	1.40	1.40	1.20	1.14

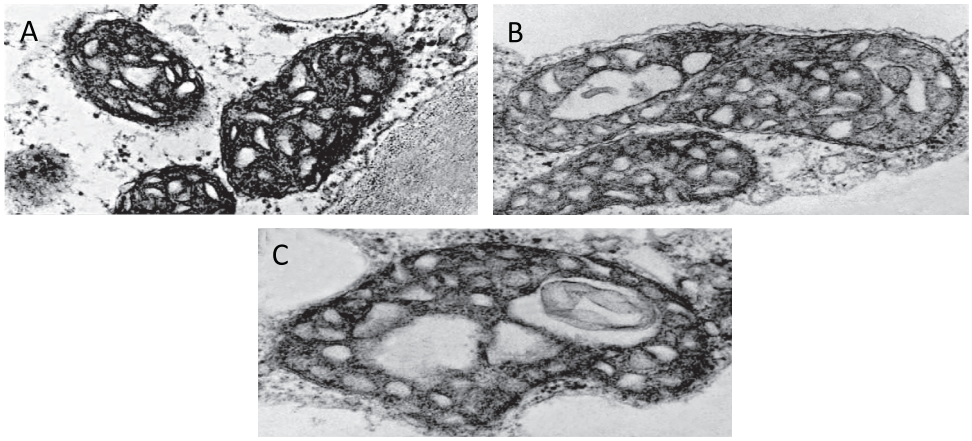

Figure 1 Ultrastructural changes in mitochondria due to *A. sesami* infection on sesame. A-Control, B-Wild infected and C-Cultivar infected.

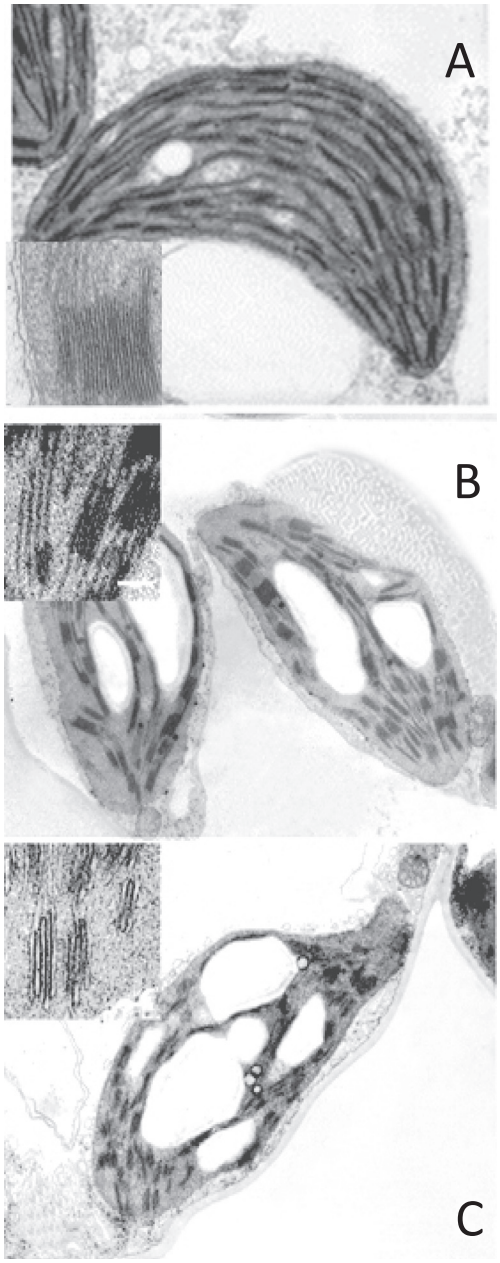

Figure 2 Ultra structural changes in chloroplast due to *A. sesami* infection on sesame. A-Control, B-Wild infected and C-Cultivar infected.

3.3. Enzyme activities

A. sesami significantly affected SOD activity (ANOVA, $P < 0.05$) that increased in wild sesame up to 18th day after infection meanwhile 16th day after infection in Thilarani cultivar and subsequently decreased in due to course of time (Table 4). Similarly, both CAT and POX activities increased initially and then decreased but higher than the control ($P < 0.05$) (Table 4).

The activities of major enzymes in the ascorbate–glutathione cycle were also affected ($P < 0.05$) upon infection. APX, MDHAR and GR activities increased and then decreased gradually, but the activities were still higher than the control. Compared with the response of MDHAR, DHAR responded differently (Table 5). Lipid peroxidation in terms of TBARS and H_2O_2 level strongly corroborates with the antioxidant machinery in the wild and cultivar sesame (Table 1).

4. Discussion

This study was aimed for a better understanding of the regulation of the antioxidant defense system in *S. orientale* in response to *A. sesami* infection. Using total peroxide and lipid peroxidation as indicators, the tolerance limit has been identified. Increased TBARS and total peroxide contents in cultivar – Thilarani suggest the occurrence of oxidative damage. However, the oxidative damage was mild in wild sesame as revealed by lipid peroxidation level and total peroxide content. Meanwhile, in the cultivar Thilarani defense against pathogen and repairing systems are operating at a low pace suggesting its susceptible nature. A survey of the pathogen tolerance of crops from tropical habitats showed that pathogenicity caused less pronounced impact in wild species when compared with cultivar species (Jensen et al., 2008). Similarly, *Citrus* cultivars responded differently to the antioxidant enzymes and metabolites of the ascorbate glutathione pathway to oxidative stress caused by biotic stress (Peroni et al., 2007). The present study suggests that the antioxidant defense system has evolved in wild land races to protect them from oxidative stress induced by pathogens.

Induction of reactive oxygen species with infection reflects the H_2O_2 generation in the plants which may be considered as a signal cascade which in turn triggers the antioxidant system in the cells. Although the contents of $O_2^{\cdot-}$ have not been described in this paper, the induced H_2O_2 generation indirectly indicates that the first ROS species generated is $O_2^{\cdot-}$ which is the primary source of H_2O_2. Subsequently SOD-mediated dismutation of $O_2^{\cdot-}$ to H_2O_2 may contribute to H_2O_2 production in the plants under biotic stress. However, increase in SOD activity was followed by a decrease, reflecting that, in addition to SOD-mediated $O_2^{\cdot-}$ dismutation, H_2O_2 could be generated from other origins, for example, pH-dependent cell wall peroxidase, amine oxidase, and germin like oxalate oxidase (Karuppanapandian et al., 2011). Overall, the present study shows that $O_2^{\cdot-}$ and H_2O_2 are the ROS species generated in sesame due to pathogen invasion.

Malformations in chloroplast, mitochondria and other cellular organelles are observed in a variety of stress situations including metal toxicity, acidity, ion deficiency, oxygen stress and pathogen invasion (Breusegem et al., 2001). Similarly, Huang et al. (2008) observed ultrastructural and cytochemical changes in oil seed rape due to *Sclerotinia sclerotiorum* infection.

The over production of $O_2^{\cdot-}$ and H_2O_2 in Thilarani compared to wild sesame suggests the varied response of the species against *A. sesami*. The alleviation of oxidative damage induced H_2O_2 accumulation has an intimate relationship with stress induced oxidative damage. The magnitude of H_2O_2 level is also positively correlated with the extent of lipid peroxidation and total peroxide contents in both the species. A peak of SOD activity followed by a decrease reflects that SOD is responsible for $O_2^{\cdot-}$ detoxification, but the ability to detoxify $O_2^{\cdot-}$ decreases subsequently. SOD constitutes the first line of defense against ROS in plants (Morita et al., 2012), but the

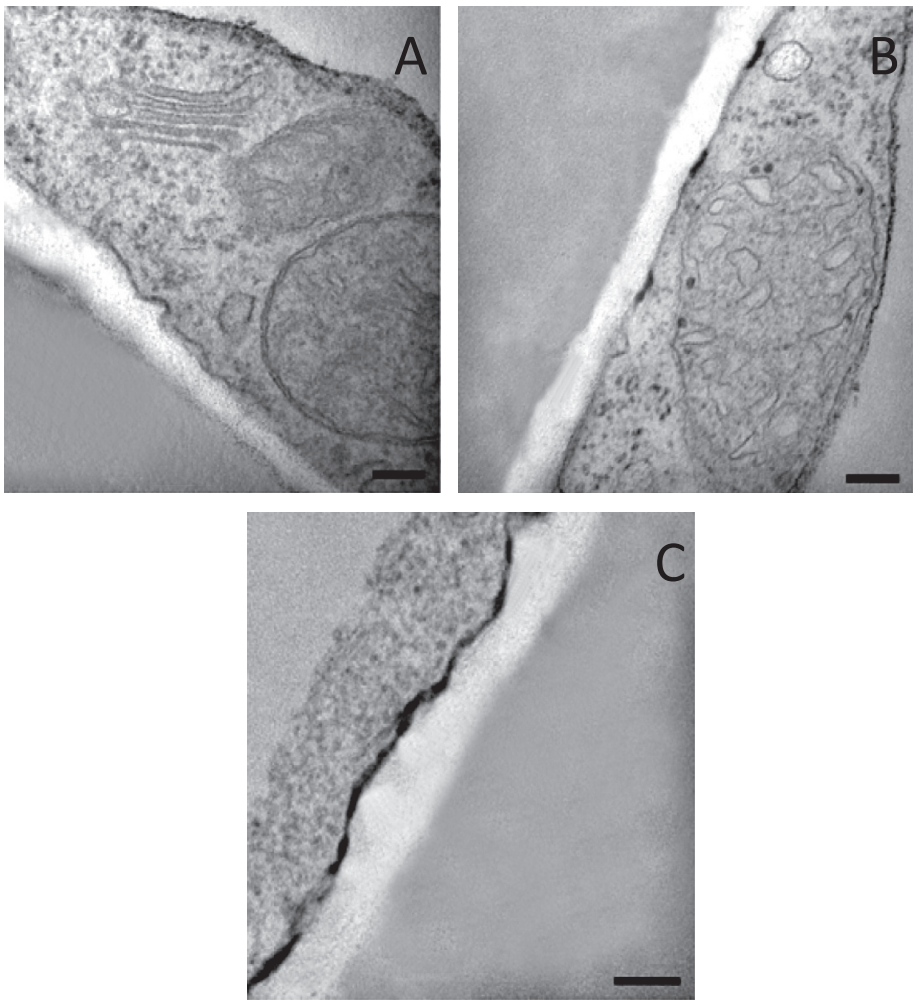

Figure 3 H_2O_2 localization by transmission electron microscopy A-Control, B-Wild infected and C-Cultivar infected.

Table 4 Activity of antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) in infected (I) and control (C) Wild & Thilarani leaves of sesame from 10 to 24 days.

		Wild						Cultivar					
Days		14	16	18	20	22	24	10	12	14	16	18	20
SOD	C	5	5	5.5	5.5	5.8	6.2	2	2	2	2	2.5	2.5
(U/mg protein)	I	16.2	17	18.5	18	16.8	16	9.6	12.8	16.9	21.6	18	17
CAT	C	16.5	18	18.8	18	19	19.8	7	7	7	8	8	8.3
(U/mg protein)	I	36	41	42	39.5	37	36	11	13	15	14	12.4	10
POX	C	15.8	18	19	19	19	19	4.5	5	5	5	5	5.5
(U/mg protein)	I	24	28	29.8	28.6	26	26	9	9	9	9.4	8.8	8.4

responses of SOD to stress depend on genetics of the host and the pathogen. Studies have shown that ROS such as $O_2^{\cdot-}$ and H_2O_2 can also be produced extra cellularly in land plants (Kranner et al., 2010), bryophytes and lichens (Weissman et al., 2005; Cruz de Carvalho et al., 2012) through the reaction of cell wall-bound peroxidase and plasma membrane-bound NAD(P)H oxidase. Even if H_2O_2 was generated extra cellularly, it can be suggested that H_2O_2 could be diffused through the plasma lemma for releasing the toxic effects caused by harmful H_2O_2 accumulation under stress.

Present evidence shows that the availability of antioxidants and the activities of antioxidant enzymes in the ascorbate–glutathione cycle are primarily involved in H_2O_2 scavenging. Upon infection, a significant AsA regeneration, as indicated by a marked increase in AsA/DHA ratio, suggests that a rising AsA regeneration rate is crucial for the proper scavenging of accumulated ROS in the plants under pathogen infection. Compared with Thilarani, marked increases in the contents of AsA, DHA, and total AsA, as well as high ratios of AsA/DHA in response to *A. sesami* infection in wild sesame, indi-

Table 5 Activity of enzymes—ascorbate peroxidase (APX), monodehydro ascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) in infected (I) and control (C) Wild & Thilarani leaves of sesame from 10 to 24 days.

		Wild						Cultivar					
Days		14	16	18	20	22	24	10	12	14	16	18	20
APX	C	30.5	31	31	31	31	31	17	18.8	20.6	22	19	18.4
(μmol/min/mg protein)	I	65	74	77.5	72	69	67.5	44	50	52.7	48	40	40
MDHAR	C	19	19	19	19	19	19.4	15	15	15	15.7	15.8	15.8
(μmol/min/mg protein)	I	43	49	55	53	50	45.2	23	29	30	27	26	23
DHAR	C	13	13	13	13.2	13	13	7.9	8	8	8	8	8
(μmol/min/mg protein)	I	15	16.8	17	16	15	14.2	9.6	11	10	9	9	9
GR	C	7	7	7	7	7	7.3	4	4.6	4.6	4.7	4.9	4.9
(μmol/min/mg protein)	I	16	19	21	18.6	18	18	7.6	10	10.4	8	7.5	7

cate that an increasing AsA pool in combination with the rapid regeneration of DHA to AsA is attributable to oxidative defense in response to pathogenicity. The ability of AsA regeneration is inhibited in Thilaranias indicated by a significant drop in AsA/DHA ratios. Glutathione another component in defense system also responds to stress. Similar to ascorbate, the pools of glutathione and their regeneration rates are increased for detoxifying ROS under stress conditions. Although glutathione/GSSG ratios were decreased by infection, the data reveal that the GR activity of wild sesame was higher or remained unchanged compared with the control reflecting that glutathione regeneration was not inhibited; instead glutathione was consumed faster than its regeneration. In wheat, higher antioxidant levels are positively associated with their stress tolerance (Almeselmani et al., 2006). Instead of keeping a high quantity of antioxidants under normal condition, the antioxidant defense ability in wild sesame is achieved by increasing the amounts of AsA and glutathione as well as their regeneration rate under stress.

Increased APX activity functions to keep the balance of cellular H_2O_2 components in response to fungal flux. Both CAT and POX are also responsible for the removal of excess H_2O_2, as indicated by increasing CAT and guaiacol POX activities. As the substrate of APX reaction, the availability of AsA is becoming essential under stress. Evidence shows that the recycling of AsA is mediated by MDHAR, DHAR and GR. DHAR is responsible for AsA regeneration while MDHAR operates at the latter stages. Glutathione is used as an electron donor for reducing DHA to generate AsA by DHAR. The decline of glutathione regeneration due to diminished GR activities explains that AsA was not regenerated via the DHAR route. Although AsA and glutathione pools are increasing, the extent of pathogen induced APX and GR activity increase was less.

H_2O_2 has been proven signal cascade for the induction of the antioxidant defense system in plants in response to biotic and abiotic stresses (Bhattacharjee, 2012). The present results support the induction of the antioxidant defense system in sesame mediated by $O_2^{\cdot-}$ and H_2O_2. The induction of APX gene expression by H_2O_2 has also been proved in germinating rice embryos (Yu-Chang Tsaia et al., 2005) and in *Arabidopsis* leaves (Brosché and Kangasjarvi, 2012).

Under normal conditions the levels of ROS in cellular compartments are determined by the interplay between the multiple ROS producing pathways and enzymes (e.g. respiration, NADPH oxidases, amine oxidases and cell wall-bound peroxidases) and the scavenging mechanisms which essentially constitute the basic ROS cycle (Bhattacharjee, 2012). Fluctuations in the antioxidative enzymes and metabolites observed in sesame could be because of the fine metabolic tuning performed by this cycle in terms of increasing active scavenging or suppressing metabolic activity responsible for ROS production. Further, recent evidence ascribes an alternative role of plant growth and cell cycle control to ascorbate and glutathione (Brosché and Kangasjarvi, 2012). More specifically, AsA stimulates cell cycle activity and DHA blocks the normal cell cycle progression. In this context the time course changes in these compounds under control condition could have been due to a cellular machinery regulating cell cycle.

Under stress ROS production is high resulting in oxidative damage. The combined action of SOD and CAT is critical in mitigating the effects of oxidative stress, since the former merely acts on the superoxide anion converting to another reactive intermediate (H_2O_2) and the latter acts on H_2O_2 converting to water and oxygen (Bhattacharjee, 2012). In the present study, it was seen that catalase did not participate in inactive H_2O_2 scavenging irrespective of species, although SOD was active in scavenging the superoxide produced by both the species. Differential CAT and SOD activity under abiotic stress induced oxidative damage has been reported in plants (Dickman and de Figueiredo, 2011).

APX was more efficient in destroying H_2O_2 than catalase in both the species. Unlike CAT which is present only in the peroxisome and has low substrate affinities since it requires simultaneous access of two molecules of H_2O_2, APX is present throughout the cell and has higher substrate affinity in the presence of AsA as a reductant (El-zbietaKuzniak and Sklodowska, 2005). Increase in APX activity was seen after the increase in SOD activity in the case of wild species whereas in Thilarani the increase in APX activity was before the increase in SOD activity. This suggests of a differential response to AsA signaling. It is possible that higher ROS generation in sesame acted as a signal transduction mechanism to increase DHAR/MDHAR synthesis resulting in higher AsA amounts. The high increase in AsA stress can be explained by the recycling function of the DHAR/MDHAR. DHA is rapidly and irreversibly hydrolyzed to 2,3-diketogulonic acid if not acted upon by DHAR. Moderate DHAR activity could

have generated more AsA from the DHA pool before hydrolysis. The increase in DHA coupled with MDHAR activity indicates that his metabolite was also formed by non-enzymatic disproportionation. Glutathione pool dynamics was similar although different in terms of amount of the metabolite present.

5. Conclusion

The H_2O_2 level as well as the availability of antioxidants and the activation of SOD, CAT, guaiacol POX, and reactive oxygen scavenging enzymes in the ascorbate–glutathione cycle suggest the varied defense response against oxidative stress occurring in wild and cultivar *S. orientale* upon *A. sesami* infection. Pathogen invasion disrupts the balance between the production and removal of H_2O_2 and subsequently accumulated H_2O_2 initiates the signaling responses leading to the induction of enzymatic antioxidant defense systems to overcome ROS production in *S. orientale*.

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