Interaction of plant cell signaling molecules, salicylic acid and jasmonic acid, with the mitochondria of *Helicoverpa armigera*

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Abstract The cotton bollworm, *Helicoverpa armigera* is a polyphagous pest in Asia, Africa, and the Mediterranean Europe. Salicylic acid (SA) and jasmonic acid (JA) are the cell signaling molecules produced in response to insect attack in plants. The effect of these signaling molecules was investigated on the oxidative phosphorylation and oxidative stress of *H. armigera*. SA significantly inhibited the state III and state IV respiration, respiratory control index (RCI), respiratory complexes I and II, induced mitochondrial swelling, and cytochrome c release in vitro. Under in vivo conditions, SA induced state IV respiration as well as oxidative stress in time- and dose-dependent manner, and also inhibited the larval growth. In contrast, JA did not affect the mitochondrial respiration and oxidative stress. SA affected the growth and development of *H. armigera*, in addition to its function as signaling molecules involved in both local defense reactions at feeding sites and the induction of systemic acquired resistance in plants.

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Introduction

Helicoverpa armigera (Hubner) (Noctuidae: Lepidoptera), commonly called cotton bollworm or legume pod borer, is one of the major constraints to crop production in Asia, Africa, Australia and the Mediterranean Europe (Sharma 2005). It is a polyphagous pest and has been reported to attack more than 200 different plant species including cotton, grain legumes, cereals, vegetables, fruits and forest trees. Control of *H. armigera* is heavily based on insecticides, and as a result, it has developed high levels of resistance to commonly used insecticides (Srinivas et al. 2004). Lepidopteran larvae in general are 'eating machines', and grow much faster than the young mammals and birds and have highly active metabolic processes (Chamberlin 2004). This phenomenal growth occurs due to large midgut epithelium, which helps in efficient digestion and absorption of the nutrients from the consumed food. Because of its reliance on aerobic metabolism, it also requires a highly active mitochondrial system for oxidative phosphorylation to meet the energy demands of the insect undergoing metamorphosis. This metabolic system conceptually divides oxidative phosphorylation into three reaction blocks, the substrate oxidation system, the phosphorylation system, and the proton leak (Chamberlin 2006).

Plants are continuously exposed to the challenge of a variety of herbivores. However, to defend themselves against the herbivores, plants have evolved constitutive and inducible defense mechanisms. Salicylic acid (SA) and jasmonic acid (JA) function as signaling molecules in

plants, mediating induced plant responses against herbivores and pathogen infection, leading to the activation of genes involved in host plant resistance to insects (Arimura et al. 2005). Both, SA and JA are needed for the plants' induced defense response, and both cause systemic acquired resistance (SAR) to pathogens and mechanical injury in plants (Cohen and Flescher 2009). SA is involved in regulating plant metabolism, and local and endemic disease resistance in plants in response to pathogen infection (Alverez 2000). More recently, elevated levels of SA have been found to be associated with tobacco mosaic infection and herbivore attack in tobacco (Malamy et al. 1990) and tomato (Peng et al. 2004) plants, respectively. SA induces plant defense responses by altering a mitochondrial enzyme, alternative oxidase, which mediates the oxidation of the ubiquinol pool and reduction of oxygen to water, without the synthesis of ATP. This altered activity of alternative oxidase affects the reactive oxygen species (ROS) levels in mitochondria, inducing defense responses in plants (Norman et al. 2004). JA causes the induction of proteinase inhibitors (Farmer and Ryan 1990) and also raises the ROS level in plants, which confers resistance to insect pests. JA is produced quickly following insect feeding (Bi et al. 1997). Based on the accumulated evidences, it has been proposed that plant signaling molecules are strong candidates for regulation of programmed cell death during pathogen infection (Zhang and Xing 2008). Topical application of SA and JA induces the production of pathogenesis related proteins, and subsequent resistance to phytopathogens (Bi et al. 1997). Induction of resistance through elevation of oxidative stress in plants against herbivores by exogenous application of SA, acetylsalicylic acid, methyl salicylate, jasmonate, jasmonate methyl esters and other chemicals have been well documented (Metraux et al. 2002; Syeed et al. 2011).

SA and its analogues have shown adverse effects on oxidative phosphorylation and swelling in rat liver mitochondria (Battaglia et al. 2005). It also induces the mitochondrial permeability transition (MPT) in the presence of calcium in isolated liver mitochondria (Biban et al. 1995). Brain and liver mitochondria exhibit morphological alterations such as matrix swelling, decreased matrix density, and loss of cristae in response to SA (Partin et al. 1971). Recent studies have shown that jasmonates can also suppress proliferation of human cancer cells, and one of the probable reasons may be mitochondrial dysfunction, consisting of mitochondrial membrane permeability transition, dissipation of inner membrane potential, osmotic swelling of the matrix and release of cytochrome c (Rotem et al. 2005). Since the level of these signaling molecules increases during insect and pathogen attack, as well as exogenous application, induces defense mechanism in plants. The present studies were therefore undertaken to determine whether SA and JA could have a direct effect on mitochondrial

oxidative phosphorylation and the respiratory chain complexes of *H. armigera*. Further, we investigated the effect of these signal molecules on the oxidative stress in *H. armigera* which could affect the larval growth and development.

Materials and methods

Chemicals

NADH, bovine serum albumin (BSA), ADP, jasmonic acid, salicylic acid and cyclosporine A were purchased from Sigma Aldrich (Mumbai, India). Sucrose, pyruvate, and malate were purchased from Qualigens (Mumbai, India), while the other chemicals used were of analytical grade.

Insects

Larvae of *H. armigera* were obtained from the insect rearing laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. The larvae were reared on a chickpea based semi-synthetic diet under laboratory conditions at $27 \pm 1°C$, $65 \pm 5\%$ RH, and 12 h photoperiod (Armes et al. 1992).

Isolation of mitochondria

The fourth- and fifth-instar larvae were starved for 3 h, their midgut content was removed, washed in cold distill water, and then homogenized in dounce homogenizer under cold conditions in 0.25 M sucrose solution containing 0.1% defatted bovine serum albumin (BSA). The homogenate was filtered through a moist muslin cloth, the filtrate centrifuged at $800 \times g$ for 10 min at 4°C, and the residue was re-suspended in extraction buffer, centrifuged at $800 \times g$ for 5 min. The supernatants from both the centrifugations were combined and centrifuged at $8,000 \times g$ for 10 min. The mitochondrial pellet was re-suspended in the reaction mixture, and used immediately for measuring oxygen consumption.

Mitochondrial respiration

Polarography determination of oxidative phosphorylation was made in oxygraph (Hansatech Instruments Limited, Bachofer, Reutlingen 72734, Germany) fitted with a Clark type oxygen electrode. The reaction system contained 5 mM HEPES buffer, pH 7.2, 50 mM sucrose, 120 mM KCl, 5.55 mM $MgCl_2$ and freshly isolated mitochondria in a total reaction volume of 1.5 ml. After the addition of substrate [NADH (1 mM) or succinate (10 mM) or pyruvate plus malate (5 mM each)], the rate of state III respiration was measured by the addition of 0.1 mM ADP and state IV

respiration was measured after the exhaustion of ADP. Protein concentration was determined by Lowry's method (1951) using BSA as standard.

Enzyme assays

Complex I activity was measured using ferricyanide as electron acceptor. The reaction system contained 250 mM sucrose, 50 mM potassium phosphate buffer, pH 7.2, 1 mM KCN, 5 mM $MgCl_2$, 1 mM potassium ferricyanide and 200 mg mitochondrial protein in a total volume of 1 ml. The reaction was started with 0.4 mM NADH and the rate of disappearance of either NADH (340 nm) or potassium ferricyanide (420 nm) was followed spectrophotometrically (Powel and Jackson 2003). Complex II activity was measured using phenazinemethosulphate (PMS) as electron acceptor. The reaction mixture was same as used for the assay of complex-I except that ferricyanide was substituted with PMS (1 mM) and 70 μM 2,6-dichlorophenol indophenol (DCPIP). The rate of reduction of DCPIP was followed at 600 nm ($\mathcal{C}_{\mu M}$ 16.2) (Powel and Jackson 2003). Complex IV was measured in a 2 ml reaction mixture containing 60 μM reduced cytochrome c in 50 mM phosphate buffer, pH 7.2. The reaction was initiated by adding mitochondrial protein, and oxidation of cytochrome c was measured at 550 nm (Theron et al. 2000). F_0F_1 ATPase activity was determined by quantifying the release of inorganic phosphate from ATP in 50 mM Tris–HCl, pH 7.4. The released phosphate was measured calorimetrically at 660 nm (Baginski et al. 1967).

Mitochondrial swelling

The mitochondrial swelling in isolated mitochondria was determined as a function of time by monitoring the absorbance changes at 540 nm in the standard reaction mixture used for polarographic studies in presence of 0.1 mM Ca^{2+}. Succinate was used as the oxidizable substrate (Battaglia et al. 2005).

Detection of cytochrome c release

Mitochondria (1 mg protein) were incubated in 50 mM phosphate buffer, pH 7.2, containing varying concentrations of SA and JA at different time intervals in the presence of 0.1 mM Ca^{2+}. The reaction mixtures were centrifuged at 12,000×g for 10 min at 4°C to obtain mitochondrial pellets. The supernatants were further spun at 12,000×g for 1 h to eliminate mitochondrial fragments. The supernatants were collected and the released cytochrome c was estimated according to Chamberlin (2004).

In vivo effect of SA and JA on the mitochondrial respiration and respiratory enzyme complexes

Fourth-instar larvae were fed on artificial diet containing 0.5 mM SA and 0.5 mM JA separately. After 24 h, mitochondria were isolated from SA- and JA-fed larvae, and the isolated mitochondria evaluated for mitochondrial respiration and enzyme activities as described above.

Lipid peroxidation

Fourth-instar larvae were fed with different concentrations of SA and JA (0–2 mM), and lipid peroxidation was measured by quantifying malondialdehyde (MDA) level in larval homogenates on the basis of reaction with thiobarbituric acid to form a pink colored complex. MDA produced was measured at 532 nm, and the nonspecific absorbance was subtracted by measuring the absorbance at 600 nm. Lipid peroxidation was calculated using 1.56×10^5 as the extinction coefficient and expressed as μmol of MDA/mg of protein extract (Poovala et al. 1999).

Lactate dehydrogenase leakage

Fourth-instar larvae were fed with different concentrations of SA and JA (0–2 mM), and lactate dehydrogenase (LDH) activity was determined in larval homogenates by measuring the decrease in NADH content at 340 nm by using a UV

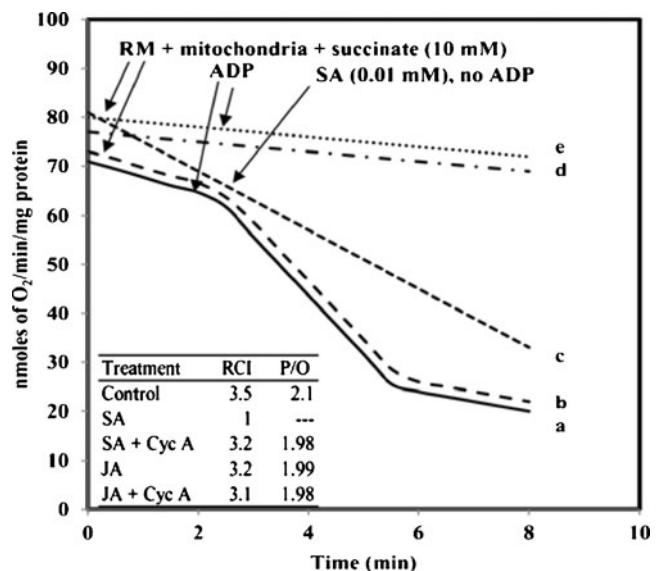| Treatment	RCI	P/O
Control	3.5	2.1
SA	1	---
SA + Cyc A	3.2	1.98
JA	3.2	1.99
JA + Cyc A	3.1	1.98

Fig. 1 Oxidation of succinate in isolated mitochondria. Oxygen consumption was measured using an oxygen electrode for the oxidation of succinate in isolated mitochondria in absence of the signaling molecules (control) (a), in presence of 2 mM JA (b), in presence of 0.01 mM SA (state IV respiration) (c), in presence of 2 mM SA (state III respiration) (d), and in presence of 1 mM KCN (e). In the corner, a table is shown indicating the RCI and P/O ratios

Table 1 In vitro effect of SA on oxidation of succinate, NADH, and pyruvate plus malate in mitochondria of *H. armigera*

SA (mM)	Succinate oxidation			NADH oxidation			Pyruvate and malate oxidation		
	nmoles of O_2 consumed / min/mg of protein			nmoles of O_2 consumed / min/mg of protein			nmoles of O_2 consumed / min/mg of protein		
	State III	State IV	RCI	State III	State IV	RCI	State III	State IV	RCI
0	57.23±2.89	19.22±0.61	2.98±0.12	77.96±1.32	27.49±0.31	2.83±0.04	63.33±0.94	20.58±1.28	3.07±0.14
0.1	53.72±1.70[a]	22.09±0.46	2.43±0.05[a]	73.61±0.86[a]	29.33±0.73	2.51±0.07	61.43±0.63[a]	22.3±0.76	2.77±0.14[a]
0.5	51.26±1.04[a]	24.82±0.80	2.07±0.03[a]	71.08±0.72[a]	31.85±0.63	2.23±0.04	58.89±0.44[a]	25.9±0.76	2.28±0.07[a]
1.0	51.18±0.93[a]	19.14±0.68	2.68±0.14[a]	59.03±0.76[a]	27.24±1.09	2.16±0.05	40.7±0.51[a]	18.83±0.89[a]	2.16±0.08[a]
1.5	42.64±0.76[a]	18.06±0.32	2.36±0.01[a]	33.05±0.84[a]	23.15±0.61[a]	1.42±0.007[a]	27.5±1.77[a]	17.64±0.17[a]	1.55±0.19[a]
2.0	28.12±1.63[a]	13.81±0.98[a]	2.03±0.28[a]	27.86±1.34[a]	19.10±0.66[a]	1.41±0.07[a]	23.79±1.51[a]	16.55±1.63[a]	1.44±0.10[a]

Values are mean ± SD (*n*=3)

[a] Significantly different at *p*<0.05 as compared to the control (no SA)

spectrophotometer (Hitachi, U-2900). The enzyme activity was expressed as mmoles/min/mg protein (Poovala et al. 1999).

Measurement of H_2O_2 content

Fourth-instar larvae were fed with different concentrations of SA and JA (0–2 mM), and H_2O_2 content was estimated in larval homogenates according to Noreen and Ashraf (2009), and expressed as μmoles of H_2O_2/mg protein.

Bioassay of SA and JA

SA and JA were incorporated into the artificial diet at different concentrations (0 to 2 mM). There were three replications for each treatment in completely randomized design, and there were 10 larvae in each replication. The larval weights were recorded 5 days after initiating the experiment.

Statistical analysis

Data were subjected to one way analysis of variance (ANOVA) to judge the significance of difference between the treatments by using F-test, while the significance of differences between the treatment means was judged by least significant difference (LSD) at *p*<0.05.

Results

The effect of SA and JA on mitochondrial respiration in vitro

Figure 1 shows the effect of SA and JA on the oxidation of succinate in intact mitochondria, with RCI and P/O ratios. SA inhibited the ADP-stimulated respiration (state III) in a dose-dependent manner. At 2 mM, SA inhibited state III respiration by 64%, 62%, and 58% for the oxidation of NADH, succinate, and pyruvate plus malate, respectively. The oxygen consumption was stimulated at lower concentrations (<1 mM) but declined at higher concentrations (above 1 mM). Similarly at 2 mM, it inhibited state IV respiration by 30%, 28%, and 20%, when NADH, succinate, and pyruvate plus malate were the substrates, respectively. Respiratory control index (RCI) decreased with an increase

Table 2 In vitro effect of JA on oxidation of succinate, NADH, and pyruvate plus malate in mitochondria of *H. armigera*

JA (mM)	Succinate oxidation			NADH oxidation			Pyruvate plus malate oxidation		
	nmoles of O2 consumed/min/mg protein			nmoles of O2 consumed/min/mg protein			nmoles of O2 consumed/min/mg protein		
	State III	State IV	RCI	State III	State IV	RCI	State III	State IV	RCI
0	109.6±3.90	34.44±1.14	3.18±0.17	80.23±4.56	26.67±4.57	3.01±0.48	88.48±8.53	28.23±4.06	3.13±0.36
0.1	108.42±5.14	34.41±1.60	3.15±0.18	78.65±6.04	26.55±4.16	2.96±0.65	85.44±6.53	28.05±1.13	3.05±0.11
0.5	105.76±9.89	33.85±8.49	3.12±1.31	77.54±7.07	26.54±5.74	2.92±0.59	84.88±5.85	27.79±0.78	3.05±0.27
1.0	104.42±2.26	33.77±8.53	3.09±0.32	77.87±9.06	25.89±3.18	3.01±0.49	84.51±8.04	27.65±0.53	3.06±0.35
1.5	99.36±3.40	32.23±0.57	3.08±0.14	76.89±4.72	25.77±5.07	2.98±0.31	84.48±4.06	27.59±2.01	3.06±0.22
2.0	95.02±0.47	31.58±1.50	3.01±0.16	75.5±7.14	25.56±2.78	2.95±0.07	82.25±5.24	26.89±1.69	3.06±0.40

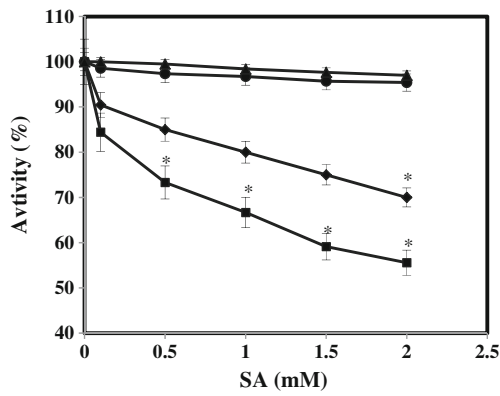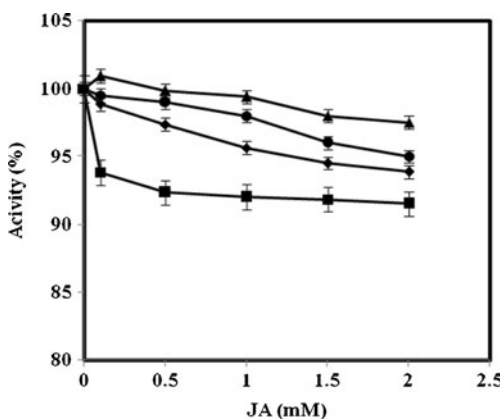Values are representation of mean ± SD (*n*=3)

Fig. 2 Effect of SA on NADH dehydrogenase (*Black Diamond*), succinate dehydrogenase (*Black Square*), cytochrome oxidase (*Black Circle*) and F_0F_1 ATPase (*Black Triangle*) of isolated mitochondria. Enzyme activities were assessed as described in text. The data represents the mean ± S.D. ($n=3$). (Significantly different from control at *$p<0.05$)

in SA concentration. The RCI was decreased by 50%, 31%, and 53% by 2 mM SA, when NADH, succinate, and pyruvate plus malate were the substrates, respectively (Table 1). However, JA did not show any effect on the oxidative phosphorylation and RCI of the isolated mitochondria with any of the substrates (Table 2).

Enzyme assays

Mitochondrial respiratory enzyme complexes were measured in the presence of different concentrations of SA and JA (0 to 2 mM). Among the enzyme complexes measured, SA inhibited the respiratory complexes I and II in a dose-dependent manner. At 2 mM concentration, it inhibited 30% and 40% of the enzyme activities, respectively. No significant effect of SA was observed on the activities of complex

Fig. 3 Effect of JA on NADH dehydrogenase (*Black Diamond*), succinate dehydrogenase (*Black Square*), cytochrome oxidase (*Black Circle*) and F_0F_1 ATPase (*Black Triangle*) of isolated mitochondria. Enzyme activities were assessed as described in text. The data represents the mean ± S.D. ($n=3$). (Significantly different from control at *$p<0.05$)
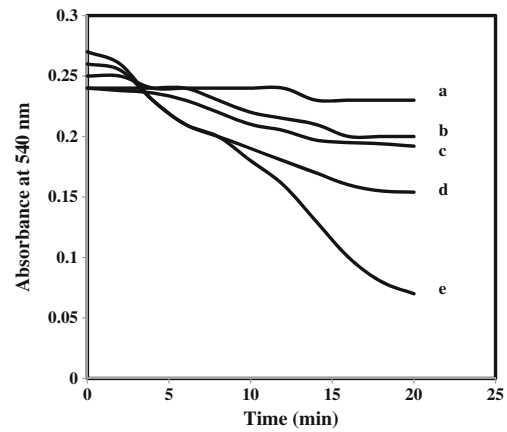

Fig. 4 Effect of SA and JA on the rate of swelling of mitochondria energized with succinate. Decrease in absorbance was measured at 540 nm in control mitochondria (**a**), in presence of 0.5 mM JA (**b**), in presence of 0.5 mM SA plus 50 μM cyclosporine A (**c**), in presence of Ca^{2+} (**d**), and in presence of 0.5 mM SA (**e**). The traces are the representative of three individual experiments

IV and F_0F_1 ATPase (Fig. 2). In contrast, JA had no effect on any of the respiratory enzyme complexes, even at higher concentrations (Fig. 3).

Mitochondrial swelling

Mitochondria incubated with 0.5 mM SA in standard reaction mixture in the presence of calcium exhibited extensive swelling, as evidenced by a strong decrease in the apparent absorbance of the mitochondrial suspension. However, pretreatment with cyclosporine A (50 μM), an inhibitor of membrane permeability transition (MPT) pore, greatly inhibited the decrease in absorbance, indicating that SA-
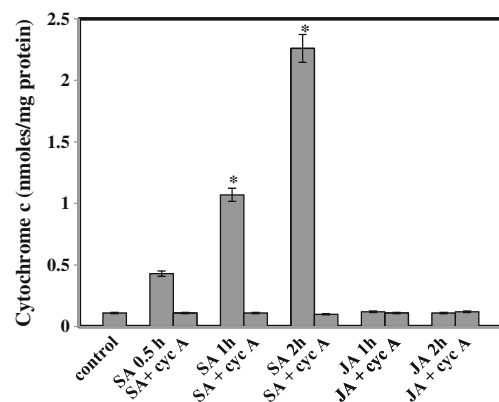

Fig. 5 Time-dependent response bars for the effect of SA and JA on the release of cytochrome c. Intact mitochondria (about 1 mg protein) was incubated with 1 mM of SA and JA in presence of 0.1 mM of Ca^{2+}, in presence or absence of cyclosporine A, and cytochrome c was estimated at different time intervals as described in the text. The data represents the mean ± S.D. ($n=3$). (Significantly different from control at *$p<0.01$)
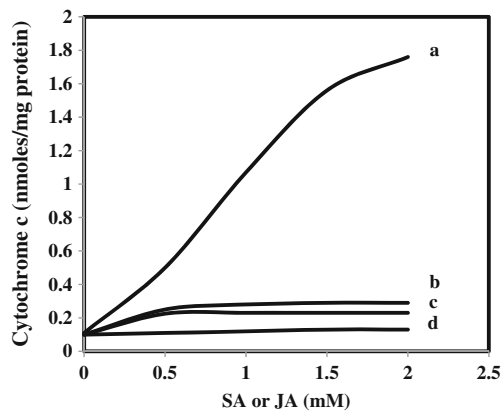
Fig. 6 Dose-dependent response curves for the effect of SA and JA on cytochrome c release. Cytochrome c release was estimated by incubating isolated mitochondria (1 mg protein) in presence of SA (**a**), in the presence of SA and 50 μM cyclosporine A (**b**), in presence of varying concentrations of JA (**c**), and in presence of JA and cyclosporine A (**d**). The traces are the representative of three individual experiments
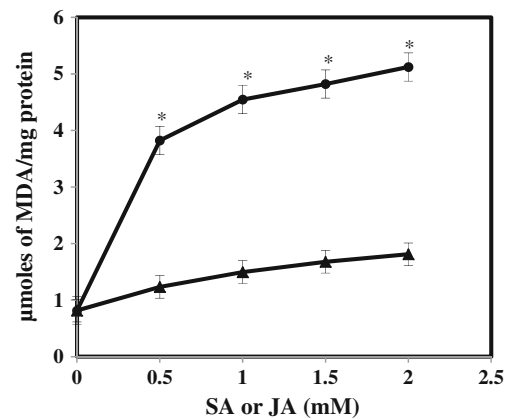

Fig. 7 Dose-dependent response for lipid peroxidation in the larvae fed on an artificial diet containing varying concentrations of SA (*Black Circle*) and JA (*Black Triangle*).The data represents the mean ± S.D. ($n=3$). (Significantly different from control at $*p<0.01$)

induced mitochondrial swelling depends on the opening of the MPT. However, JA did not induce mitochondrial swelling (Fig. 4).

Cytochrome c release

SA induced the efflux of cytochrome c in a time- and dose-dependent manner when incubated with isolated mitochondria in the presence of Ca^{2+}. When mitochondria (about 1 mg protein) was incubated with SA (2 mM), 1.76 nmol of cytochrome c released into the supernatant and this efflux was inhibited by cyclosporine A. JA did not induce the release of cytochrome c when incubated with isolated mitochondria even at high concentrations (Figs. 5 and 6).

In vivo effect of SA and JA on mitochondrial respiration and respiratory enzyme complexes

Mitochondria isolated from the SA-fed larvae exhibited normal state III respiration. However, state IV respiration was significantly increased (28.34 nmoles of O_2/min/mg protein) as compared with the control larvae (21.24 nmoles

of O_2/min/mg protein), indicating the uncoupling behavior of the molecule under in vivo conditions (Table 3). RCI was also significantly affected in SA-fed larvae. Mitochondria prepared from the control and SA-fed larvae showed an RCI of 3.07 and 2.11 respectively (Table 3). None of the enzyme complexes were affected in SA-fed larvae, but a drop of about 50% activity was observed for F_0F_1 ATPase as compared with the control larvae. In contrast, mitochondria isolated from JA-fed larvae showed normal respiration, and no respiratory enzyme complex was affected (Table 3).

Lipid peroxidation, LDH leak and H_2O_2 content

Lipid peroxidation, LDH leak and H_2O_2 content were significantly higher in SA-fed larvae as compared with the control larvae. There was a proportional increase in these components in a time- and dose-dependent manner. The JA-fed larvae did not show any significant differences in these components as compared with the control larvae (Figs. 7, 8, 9, 10, 11 and 12).

Bioassay of SA and JA

SA inhibited the larval growth in a dose-dependent manner. At 2 mM, the larval weight was decreased by 66%.

Table 3 In vivo effect of SA and JA on oxidation of succinate and respiratory enzyme complexes in mitochondria of *H. armigera*

	Control	SA-fed larvae	JA-fed larvae
State III respiration (nmoles/min/mg protein)	65.23±2.88	59.89±1.23	59.34±0.236
State IV respiration (nmoles/min/mg protein)	21.24±2.475	28.34±1.11[a]	20.67±1.136
RCI	3.07±1.163	2.11±1.108[a]	2.87±0.207
Complex I (μmoles/min/mg protein)	59.75±6.47	51.37±0.68	56.28±3.46
Complex II (μmoles/min/mg protein)	3.16±0.41	2.83±0.55	2.91±0.51
Complex IV (μmoles/min/mg protein)	4.64±0.69	4.56±0.71	4.43±0.95
Complex V (nmoles/min/mg protein)	5.73±1.08	3.04±0.43[a]	6.11±0.125

Values are mean ± SD ($n=3$)
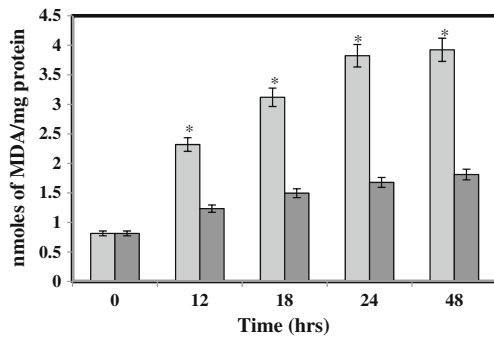[a]Significantly differ from the control larvae at $p<0.05$

Fig. 8 Time-dependent response for lipid peroxidation in the larvae fed on an artificial diet containing 0.5 mM SA (*light gray color*) and 0.5 mM JA (*dark gray color*).The data represents the mean ± S.D. (*n*=3). (Significantly different from control at *$p<0.01$)
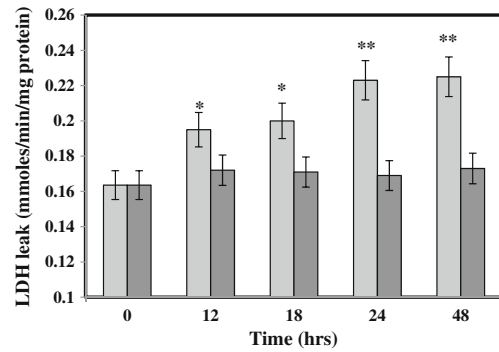

Fig. 10 Time-dependent response for LDH leak in the larvae fed on an artificial diet containing 0.5 mM SA (*light gray color*), and 0.5 mM JA (*dark gray color*). The data represents the mean ± S.D. (*n*=3). (Significantly different from control at *$p<0.05$ and **$p<0.01$)

However, there was no significant effect on the weights of larvae fed on JA amended diets (Fig. 13).

Discussion

Plants react to pathogen attack by activating their defense mechanism in which SA and JA play a key role in signal transduction pathways, thereby, resulting in induced resistance to herbivores. SA and JA are widely distributed in plants, and play an important role in systemic acquired resistance (Cohen and Flescher 2009). These plant signal molecules raise the endogenous level of reactive oxygen species by regulating oxidative enzymes in plants (Xu and Tian 2008). H_2O_2 and other reactive oxygen species, thus produced, serve as secondary messengers to induce the expression of plant defense related genes (Conrath et al. 1995). A number of reports have demonstrated the involvement of SA and JA in host plant resistance to herbivores (Metraux et al. 2002; Syeed et al. 2011). Exogenous application

of SA (0.8 mM) caused the induction of protease inhibitors in chickpea plants which inhibited the proteases in *H. armigera* (Raju et al. 2009). The present studies demonstrated that SA also exhibit direct effects on insect growth through inhibition of respiration, but JA did not exhibit such effects on insect growth.

SA uncoupled the respiration of isolated mitochondria at lower concentrations (<1 mM), but inhibited at higher concentrations (>1 mM). SA also affected the RCI, which decreased with an increase in SA concentration, suggesting that it caused dysfunction of insect mitochondria *in vitro*, and impaired the mitochondrial respiration. Similar findings have earlier been reported for rat liver (Battaglia et al. 2005; Doi and Horie 2010) and tobacco mitochondria (Norman et al. 2004). SA inhibited the activities of NADH dehydrogenase and succinate dehydrogenase in *H. armigera*. Aspirin, a derivative of SA, has been reported as a uncoupler of oxidative phosphorylation in Yeast (Sapienza et al. 2008), and an inhibitor of the electron transport chain at complex I
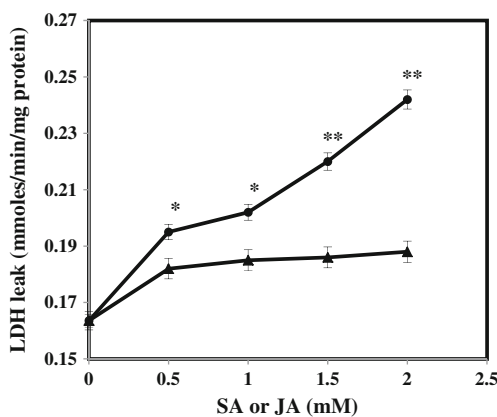

Fig. 9 Dose-dependent response for LDH leak in the larvae fed on artificial diet containing varying concentrations of SA (*Black Circle*) and JA (*Black Triangle*). The data represents the mean ± S.D. (*n*=3). (Significantly different from control at *$p<0.05$ and **$p<0.01$)
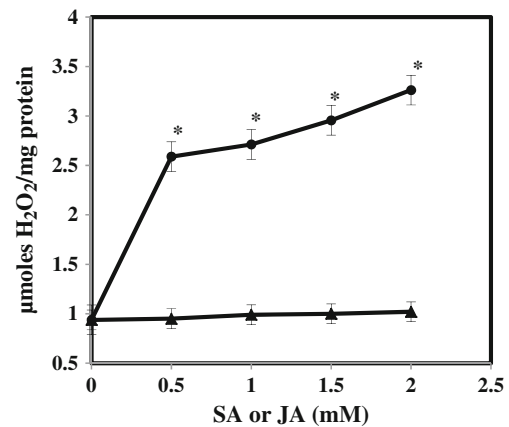

Fig. 11 Dose-dependent response for H_2O_2 production in the larvae fed on an artificial diet containing varying concentrations of SA (*Black Circle*) and JA (*Black Triangle*).The data represents the mean ± S.D. (*n*=3). (Significantly different from control at *$p<0.01$)
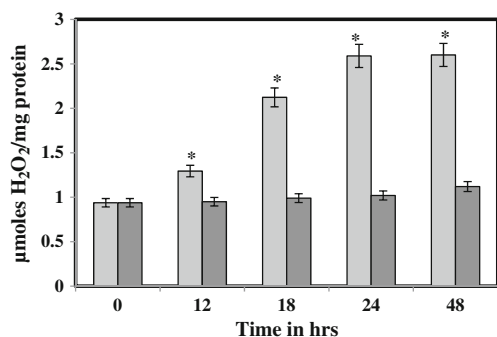
Fig. 12 Time-dependent response for H_2O_2 production in the larvae fed on an artificial diet containing 0.5 mM SA (*light gray color*), and 0.5 mM JA (*dark gray color*). The data represents the mean ± S.D. (*n*=3). (Significantly different from control at *$p<0.01$)

and complex II in rat liver mitochondria (Somsundaram et al. 1997). According to Battaglia et al. (2005), the most probable site for binding the SA is Fe^{3+} of Fe-S clusters of mitochondrial complexes. SA also inhibited F_0F_1 ATPase slightly (<5%) at 2 mM, which is not significant, and these observations are similar to the results reported by Zhang and Ramirez (2000). SA induced mitochondrial swelling in the presence of Ca^{2+} ions, possibly due to the induction of membrane permeability transition (MPT), causing the accumulation of calcium ions in the matrix. SA-induced mitochondrial swelling was inhibited by cyclosporine A, a typical inhibitor of MPT, confirming the induction of MPT by SA. MPT is induced by the opening of a cyclosporine A sensitive pore in the mitochondrial inner membrane. With this, the electrochemical gradient collapses and the corresponding increase in the electron flux along the respiratory chain results in an increase in oxygen uptake and
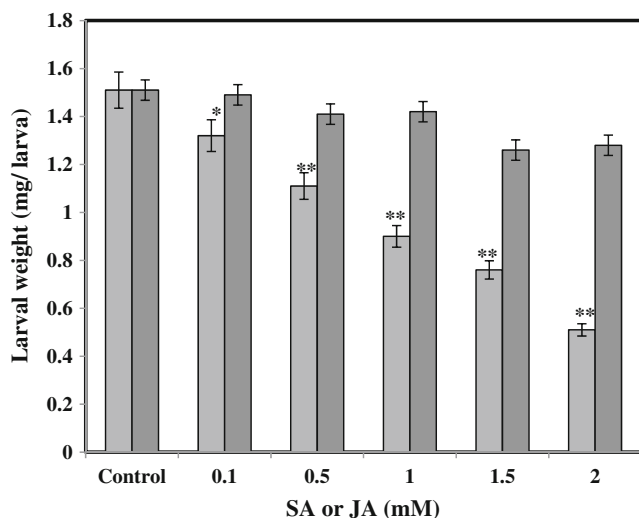

Fig. 13 Bioassay for SA and JA. Neonates were fed on an artificial diet containing varying concentrations of SA (*light gray color*), and JA (*dark gray color*). The data represents the mean ± S.D. (*n*=3) (Significantly different from control at *$p<0.05$, and **$p<0.01$)

consequently the production of reactive oxygen species, resulting in swelling and uncoupling. Enhancement of mitochondrial swelling by SA has been confirmed by spectrophotometrically and polarographically (Battaglia et al. 2005). SA induced the release of cytochrome c in the presence of calcium ions, but cyclosporine A inhibited the same effect. Release of cytochrome c by SA and aspirin has already been documented in rat liver mitochondria (Battaglia et al. 2005) and *Saccharomyces cerevisiae* cells (Sapienza et al. 2008), respectively. The impairment of mitochondrial functions observed with SA in vitro was mainly due to the induction of MPT and inhibition of the electron transport chain through complex I and II.

Mitochondria isolated from *H. armigera* larvae fed on the diet containing SA exhibited an uncoupling nature as evidenced by high state IV respiration, low RCI and a significant drop in the F_0F_1 ATPase activity. Inhibition of both ATP synthesis and respiratory oxygen uptake in tobacco cell cultures within minutes of incubation with SA has been reported (Xie and Chen 1999). Significant increase in lipid peroxidation, LDH leak and the H_2O_2 content were observed in SA-fed larvae. SA has been reported to generate H_2O_2 and other reactive oxygen species in isolated rat liver mitochondria (Battaglia et al. 2005) that resulted in induction of lipid peroxidation in cell membranes and LDH leak from mitochondria (Doi and Horie 2010). These results are similar to SA-induced oxidative stress observed in isolated rat liver hepatocytes (Doi and Horie 2010). Thus, the SA-induced lipid peroxidation accompanied by high levels of LDH activity and H_2O_2 content in SA-fed larvae supports the assumption that SA induces oxidative stress in cells. SA affected the larval growth, which can be explained in terms of increased oxidative stress, and impairment in the mitochondrial function as evidenced by the uncoupling effect on mitochondrial respiration, decrease in the respiratory control index (RCI) and inhibition of F_0F_1 ATPase activity under in vivo conditions. Since the mitochondrial respiratory chain produces the majority of ATP content of the cells, an impairment in the mitochondrial respiration could adversely affect the energetic state of the cell.

JA showed no effect on mitochondrial respiration, oxidative stress and the larval growth. It is known that JA specifically causes mitochondrial dysfunction in cancerous cells by disrupting membrane potential, enhancing swelling by inducing MPT, cytochrome c release, and induces the apoptotic pathway leading to cell death (Rotem et al. 2005). The composition and function of mitochondria of cancer cells differ from normal cells in having certain characteristics such as high membrane potential, possibly a module expression of the MPT component and enhanced rates of ATP generation through glycolysis rather than through oxidative phosphorylation (Rotem et al. 2005). Although both, SA and JA share the common function of anticancer activity by

disrupting the mitochondrial functions, JA is specific to cancer cell mitochondria, and differentiates between normal cells and cancer cell mitochondria. Hence, JA did not exhibit any effect on mitochondria of *H. armigera* and did not induce any oxidative stress in JA-fed larvae. Similar observations have earlier been reported by Rotem et al. (2005).

In summary, SA-induced mitochondrial dysfunction and oxidative stress in *H. armigera* under in vitro and in vivo conditions, suggests that SA affect the growth of Lepidopteran insects. Further, SA when applied exogenously could act as antifeedant and affect the growth and development of larvae by inducing mitochondrial dysfunction and oxidative stress in *H. armigera*. This capacity to induce deleterious effects on insect growth is expected to work in concert with its proven induced-resistance in plants, making SA an excellent candidate for the control of pests. Although JA did not affect the larval growth, it is known to induce resistance in plants against insects. Thus there is a need to develop strategies to exploit SA and JA for the control of Lepidopteron pests, and to reduce our dependence on chemical pesticides for crop production.

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