Research Article

Responses of Antioxidative Defense System and Composition of Photosynthetic Pigments in *Brassica juncea* L. upon Imidacloprid Treatments

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Abstract | The effect of the insecticide Imidacloprid on photosynthetic pigments and antioxidative enzymes in *Brassica juncea* L. cv. alankar was investigated. Forty-day-old pot plants were exposed to different concentrations of the insecticide, ranging from 0 to 40 grams active ingredient per hectare (g.a.i ha^{-1}) through foliar spray. Analyses were done at days 3, 7, and 15 after treatment. Lipid peroxidation rates and contents of proline, ascorbate (ASC), glutathione (GLU), antioxidative enzymes; superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were assessed. Imidacloprid enhanced malondialdehyde (MDA) and proline contents over their respective controls at all doses and growth stages observed, attaining maximum with 40 grams Imidacloprid active ingredient per $hectare^{-1}$ at day 15 after treatment. The antioxidative enzyme activity of SOD, APX and GR showed increasing trend in a dose-dependent manner, attaining maximum at 40 g.a.i ha^{-1} of Imidacloprid treatment at day 7 after treatment (DAT) followed by 3 and 15 DAT, respectively. On the contrary, the CAT activity was increased at lower concentrations of Imidacloprid but showed a dose dependence decline at higher concentrations of the insecticide at all the observed growth stages, with maximum decrease under 40 g.a.i ha^{-1} at 15 DAT followed by 3 and 7 DAT respectively. Among the non-enzymatic antioxidants, a dose dependent decline was observed in Ascorbate (ASC), dehydroascorbate (DHA) and reduced glutathione (GSH) levels. The photosynthetic pigments showed slight increases over their respective controls at lower concentrations of the insecticide at all the growth stages, but at higher concentrations (30 and 40 g.a.i ha^{-1}), both the pigments were decreased considerably at all the observed growth stages. These results suggested that the lower concentrations of Imidacloprid applied on the mustard might have no severe effect on the mustard plant because of the regulation of the anti-oxidative enzyme system and MDA, but provoked oxidative stress when applied at high dosages. Therefore, pesticide application at high concentrations can be considered as a kind of stress, similarly to other stresses such as salt, drought and low or high temperatures.

Received | September 04, 2015; **Accepted** | October 24, 2015; **Published** | January 07, 2015
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DOI | http://dx.doi.org/10.17582/journal.absjournal/2015/1.1.3.15
Citation | Dar, M. I., F. A. Khan and F. Rehman. 2015. Responses of antioxidative defense system and composition of photosynthetic pigments in *Brassica juncea* L. upon imidacloprid treatments. *Abiotic and Biotic Stress Journal*, 1(1): 3-15.

Introduction

Pesticides were introduced to agriculture to fulfill the increased food needs of the growing world population. The use of synthetic insecticides has made the production of higher yields of crop possible in areas where insects would have otherwise damaged the crop (Gonias et al., 2008). On the other hand, uptake of many pesticides in plants also causes chemical shock, which results in interference of growth and metabolism by triggering secondary responses such as oxidative damage by producing highly reactive oxygen species (ROS) (Halliwell, 1987). Cellular systems scavenge these active oxygen species by invoking an

increase in antioxidative machinery, composed of enzymes and non-enzymatic components. The enzymatic components include ROS scavengers like superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) (Khan and Kour, 2007; Ganguly et al., 2010). Such enzyme-based anti-oxidative system has been one of the important strategies for plants to respond to environmental stresses. In most cases, toxic organic compounds can give rise to the increased activities of antioxidant enzymes such as SOD, POD or ascorbate peroxidase (APX), which reflect not only the degree of toxicity but the ability to tolerate the stress as well (Mishra et al., 2009). Additionally, non-enzymatic defense systems (e.g., glutathione (GLU), tocopherols, ascorbate, proline and carotenoids) protect cells from damage by scavenging ROS (Bashir et al., 2007a; Jan et al., 2012a).

Brassica juncea L. is the major oil seed crop cultivated in the entire Indian sub-continent, but infestation of this crop with insect pests is a major problem in the region (Rehman et al., 2013). Farmers deal with the issue of insect pests by spraying various insecticides and pesticides. However, some insect pests develop resistance to the conventionally used insecticides. Imidacloprid [1-(6-chloropyridin-3-yl)-N-nitroimidazolidin-2-ylideneamine], a nicotinoid insecticide, has been found very effective against these resistant pests (Conway et al., 2003) and therefore is used widely in areas where mustard crop is attacked by insects.

Although a large amount of work relating to the pesticide and insecticide induced effects on growth and photosynthetic pigment contents has been done, but to the best of our knowledge, there is little information available so far about the effect of Imidacloprid especially on ROS system in plants. Keeping in view the importance of oil seed crops, the present investigation was carried out to: (1) investigate whether Imidacloprid induces oxidative stress in plant cells, which might affect various enzymatic and non-enzymatic antioxidants involved in Ascorbate-Glutathione cycle in plants, and; (2) find out the optimum level of Imidacloprid for efficient growth and photosynthetic pigment contents in *B. juncea*.

Material and Methods

The present experiment was performed in a net house of the Department of Botany, Aligarh Muslim University, Aligarh, India, under natural conditions. The seeds of mustard (*B. juncea* L. Czern & Coss) cv. Alankar were surface-sterilized with 0.5% sodium hypochlorite for 20 min, rinsed and soaked overnight in double distilled water (DDW) for 12 h at 4°C for uniform germination. The seeds were then transferred to 23-cm-diameter earthen pots filled with 4 Kg of reconstituted soil (sand: clay: peat; 70: 20: 10, by dry weight). After two weeks of sowing, thinning was done and three healthy plants of uniform size were maintained in each pot. Plant pots were watered every alternate day and watering schedule was adjusted throughout the experimental duration in order to prevent leaching. Forty day-old plants were subjected to foliar application of five levels of commercial insecticide Imidacloprid 17.8% SL (trade name Ultimo™ 200 SL), ranging from 0 to 40 g.a.i ha^{-1} with a hand-held sprayer (adjustable nozzle). Doses were prepared by dissolving the required amount of Imidacloprid solution in double distilled water. Surfactant 'Tween-20' (0.5%) was mixed with each desired pesticide dose just before the spray to enhance spreading of spray droplets on the leaf surface and delays the drying of leaves. Control plants were sprayed with distilled water and 0.5% tween-20. The nozzle of the sprayer was adjusted in such a way that it pumped out 1 ml approximately in one sprinkle and each plant was sprinkled thrice. Accordingly, each plant received 3 ml of deionized water or insecticide solution. All the plants were healthy and disease free at the time of spraying. The maximum and minimum temperatures during the whole experimental season were 27°C and 13°C, respectively with relative humidity of 30%. Average sunshine recorded was 8.5 hours. There was no rainfall during the period of the present experiment.

Each experiment was repeated three times with four replicates in completely randomized blocks. Thus, each dose with four replicates contained 12 plants (3 × 4). Plant samples were collected 03 (43 days after sowing), 07 (47 days after sowing) and 15 (55 days after sowing) days after the treatment (DAT) to analyze the effect of different concentrations of insecticide on photosynthetic pigments, proline content, activities of enzymes ascorbate peroxidase (APX) (EC 1.11.1.11), superoxide dismutase (SOD) (EC 1.15.1.1), glutathione reductase (GR) (EC 1.6.4.2) and catalase (CAT) (EC 1.11.1.6) and activities of non-enzymatic antioxidants like ascorbate (ASC) and glutathione (GLU) content.

Measurement of proline and MDA content

The proline content in control and Imidacloprid

treated plants was estimated following the method of Bates et al. (1973). The absorbance of the chromophore extracted in toluene was taken at 520 nm. The concentration of proline was estimated using the standard curve prepared from L-proline (0–100 µg/ml). Proline concentration was expressed in µg g^{-1} of fresh weight of the sample.

Lipid peroxidation was estimated by measuring the formation of malondialdehyde (MDA), a breakdown product of lipid peroxidation, with 2-thiobarbituric acid (TBA) according to De Vos et al. (1989). The absorbance of resulting supernatant was taken at 532 and 600 nm. MDA content was determined by subtracting absorbance of supernatant at 600 nm from that of 532 nm and using an absorbance coefficient of 155 mM^{-1} cm^{-1} and was expressed as nmol g^{-1} fresh weight of sample.

Extraction and estimation of antioxidative enzymes

For enzyme extraction, leaf samples of *B. juncea* L. were homogenized in pre-cooled mortar and pestle in 5 ml extraction mixture (1 mM Ethylenediaminetetraacetic acid (EDTA), 0.05% Triton X - 100, 2% PVP, 1 mM ascorbate in 50mM phosphate buffer of pH 7). The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. Supernatants obtained were used for enzyme determinations.

Superoxide dismutase activity

SOD activity was assayed according to the method of Beauchamp and Fridovich (1971) in terms of the ability to inhibit the photochemical induction of nitroblue tetrazolium (NBT) to formazan at 560 nm. The amount of reduced NBT was calculated using the absorbance coefficient 100 mM^{-1} cm^{-1}. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT monitored at 560 nm by a spectrophotometer (T70 UV/VIS Spectrometer).

Catalase activity

CAT activity was measured following Aebi (1984). 0.1 ml of enzyme extract was added to 2.8 ml 50 mM phosphate buffer (pH 7.0) and absorbance was taken at 240 nm. To this, 0.1 ml of H_2O_2 was added and again absorbance was taken at the same wavelength. The extinction coefficient value of 0.039 mM^{-1} cm^{-1} was used for calculations.

Ascorbate peroxidase activity

For the estimation of APX activity, 0.1 ml of enzyme extract was added to 2.8 ml reaction mixture composed of 0.5mM ascorbic acid in 50 mM phosphate buffer (pH 7.0). Changes in absorbance at 290 nm were recorded after addition of 0.1 ml of H_2O_2 (Asada, 1984).

Glutathione reductase activity

The leaf samples for estimation of GR were extracted in 100mM potassium phosphate buffer (pH 7.0) containing 1 mM Na_2-EDTA and 4% polyclar AT (w/v). Activity of GR was determined following the method of Sgherri et al. (1994) by measuring decrease in absorbance at 340 nm and using extinction coefficient of 6.2 mM^{-1} cm^{-1}.

Ascorbate content

Fresh leaves (0.5 g) was ground in 2 ml of 0.1 M Na-phosphate buffer (pH 7) and 1 mM EDTA and centrifuged at 10,000 rpm for 15 minutes. Ascorbate (ASC) and dehydroascorbate (DHA) were estimated by a method of Law et al. (1983).

Glutathione content

Fresh leaf tissue (0.5 g) was homogenized in 2 ml of 5% sulphosalicylic acid at 4 °C. The homogenate was then centrifuged at 10,000 rpm for 15 minutes. Reduced (GSH) and oxidized (GSSG) glutathione were determined by the method of Anderson (1985).

Estimation of Chlorophyll and Carotenoids

Chlorophyll and carotenoid contents were estimated by the method of Arnon (1949).

Statistical analysis

The data were analyzed by employing analysis of one-way variance (ANOVA) followed by a Duncan's multiple range test (DMRT) to determine whether the values were significantly different from the control. Analysis of variance (ANOVA) for all the measured variables was performed by SPSS Ver. 10, Inc., Chicago, USA. The data are expressed as mean ± standard error (SE) (n = 4).

Results

Visible symptoms

Plants were keenly observed after the foliar spray of insecticide Imidacloprid and at 50 days of growth stage (10 DAT), marginal necrosis started occurring

in plants sprayed with 40 gram active ingredients per hectare (g.a.i ha^{-1}) Imidacloprid.

Lipid peroxidation rates

The Melondialdehyde (MDA) content was increased with increase in the Imidacloprid concentration as well as with plant age as observed at 3, 7 and 15 DAT. The maximum accumulation of MDA took place at 40 g.a.i ha^{-1} Imidacloprid, compared to the respective control (i.e., the plants sprayed with deionized water and 0.5% tween 20) (Figure 1). The MDA content in plants at day 3 after treatment increased significantly ($p < 0.05$) and was 16.41, 18.75, 22.35, 31.18 and 46.13 nmol/g at 0, 10, 20, 30 and 40 g.a.i ha^{-1} Imidacloprid respectively. The MDA content at day 3 after treatment was 14.25%, 36.2%, 90% and 181.1% higher in the plants exposed to 10, 20, 30 and 40 g.a.i ha^{-1} Imidacloprid respectively than in the control plants. The MDA content in control plants at 7 DAT was more than the MDA content observed in control plants at day 3 after treatment. At 7 DAT, the MDA content also showed increased trend with increase in Imidacloprid treatments and was 22.77, 24.58, 34.22, 45.30 and 55.36 nmol/g at 0, 10, 20, 30 and 40 g.a.i ha^{-1} respectively. Interestingly, except for the 10 g.a.i ha^{-1} of Imidacloprid, the MDA contents at 20, 30 and 40 g.a.i ha^{-1} were 50%, 99% and 143.1% higher ($p < 0.05$) respectively than the respective control at day 7 after treatment of Imidacloprid. No significant difference was observed between the control and 10 g.a.i ha^{-1} 7 DAT with the MDA content being the highest at 40 g.a.i ha^{-1}. At 15 days after Imidacloprid treatment, the MDA content in plants was significantly increased ($p < 0.05$) at all the doses than the respective control. The MDA content in plants at 15 days after Imidacloprid treatment was 28.44, 35.35, 45.80, 59.88 and 72.82 nmol/g at 0, 10, 20, 30 and 40 g.a.i ha^{-1} Imidacloprid. Moreover, the control plants at 15 days after treatment (DAT) showed highest MDA content (28.44 nmol/g) and were 73% and 30% higher than the control plants at day 3 and day 7 after Imidacloprid treatment respectively.

Proline content

Proline content did not show any significant alteration in the leaves of the plants treated with 10 g.a.i ha^{-1} Imidacloprid as compared to control at all the growth stages. However, the presence of Imidacloprid at higher concentrations (30 and 40 g.a.i.ha^{-1}) enhanced proline level 63.82%, 107.82% 3 DAT, 60.2% and 102.52% at 7 DAT, and 32.4% and 57.88% 15 DAT respectively in comparison with that of control plants (Figure 2). The maximum enhancement was found with 40 g.a.i ha^{-1} Imidacloprid at 15 DAT.

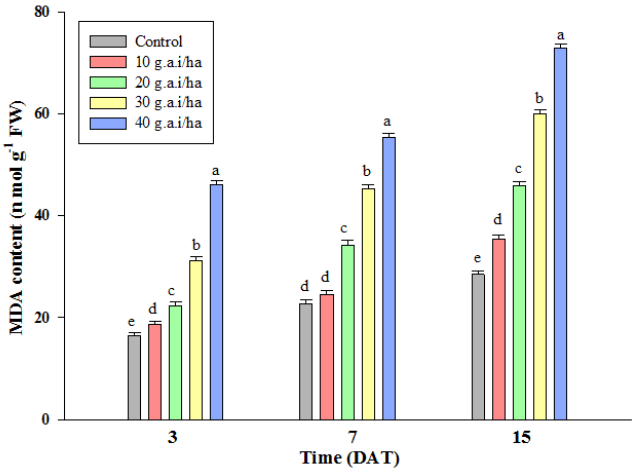

Figure 1: Changes in MDA content (nmol g^{-1} FW) at various growth stages in *Brassica juncea* L. cv. alankar exposed to different concentrations of imidacloprid

Values with different small letters at each time point are significantly ($p < 0.05$) different from each other (Duncan's multiple-range test). The values represent Mean ± SE (n =4).

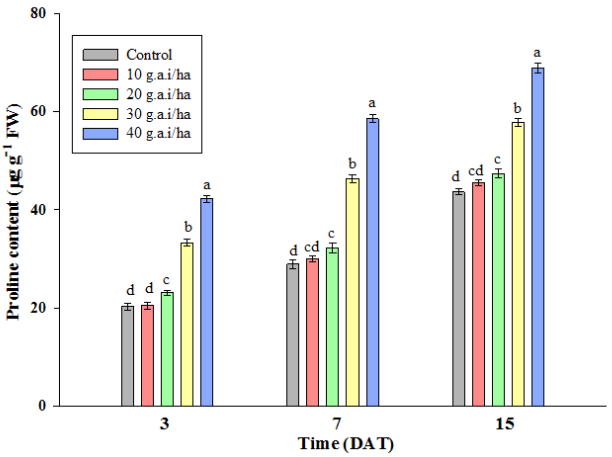

Figure 2: Changes in proline content (µg g^{-1} FW) at various growth stages in *Brassica juncea* L. cv. alankar exposed to different concentrations of imidacloprid

Values with different small letters at each time point are significantly ($p < 0.05$) different from each other (Duncan's multiple-range test). The values represent Mean ± SE (n =4).

Antioxidant enzyme activity

No significant difference in the SOD activity was observed among the control, 10 and 20 g.a.i ha^{-1} Imidacloprid treatments at 3 DAT. However, it increased significantly with all the insecticide treatments as observed at 7 and 15 DAT, compared with the control (Figure 3). The gain was maximum reaching 37.33 units g^{-1} fresh weight at 40 g.a.i ha^{-1} Imidacloprid treatment 7 DAT followed by 3 DAT and 15 DAT.

The CAT activity also showed a similar variation

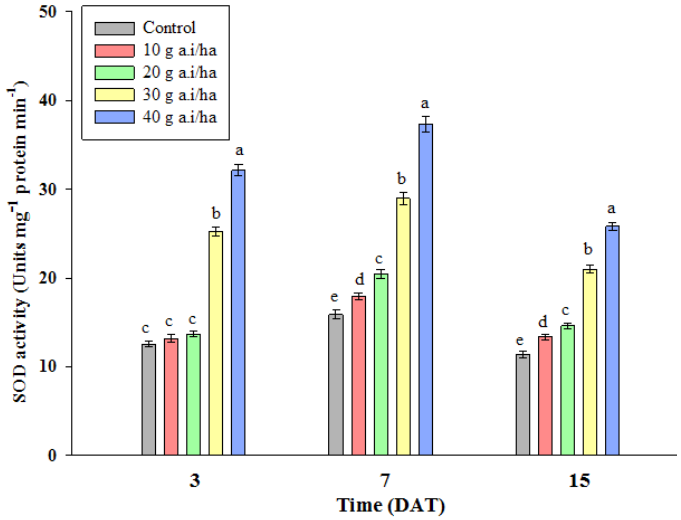

Figure 3: Changes in SOD activity (Units mg^{-1} protein min^{-1}) at various growth stages in *Brassica juncea* L. cv. alankar exposed to different concentrations of imidacloprid
Values with different small letters at each time point are significantly ($p < 0.05$) different from each other (Duncan's multiple-range test). The values represent Mean ± SE (n =4).

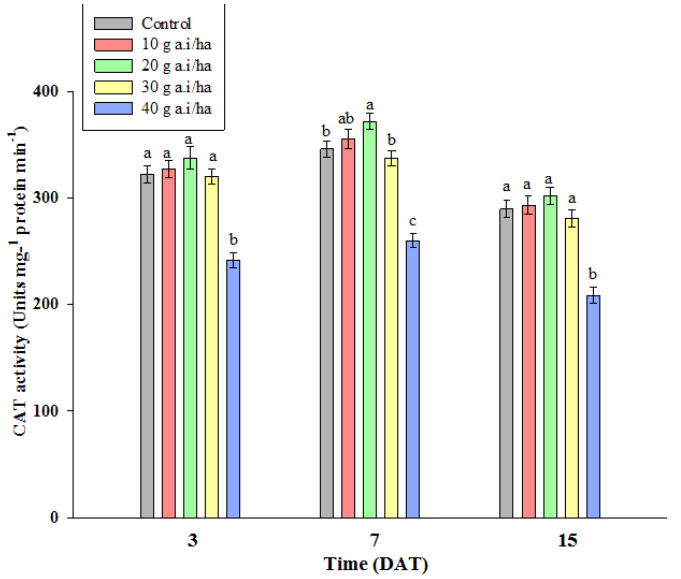

Figure 4: Changes in CAT activity (Units mg^{-1} protein min^{-1}) at various growth stages in *Brassica juncea* L. cv. alankar exposed to different concentrations of imidacloprid
Values with different small letters at each time point are significantly ($p < 0.05$) different from each other (Duncan's multiple-range test). The values represent Mean ± SE (n =4).

trend (Figure 4), as did SOD with respect to age of the plant, thus having its maximum at 7 DAT. It increased marginally at the lower concentrations of Imidacloprid (10 and 20 g.a.i ha^{-1}) with respect to control at all the observed growth stages, but started declining sharply at higher doses of the chemical 30 and 40 g.a.i ha^{-1}. The CAT activity was the highest at 20 g.a.i ha^{-1} during the whole experimental duration touching 371.75 units g^{-1} protein min^{-1}.

The activity of APX was also raised with increasing concentration of Imidacloprid, gaining the most with the highest dose of the chemical (40 g.a.i ha^{-1}) at 7 DAT, followed by 3 DAT and 15 DAT respectively (Figure 5). The APX activity at 3 DAT was 1.06, 1.39, 1.60, 2.38 and 3.14 units g^{-1} fresh weight at 0, 10, 20, 30 and 40 g.a.i ha^{-1} Imidacloprid. The magnitude of increase in APX activity was 32.58%, 69.66%, 128.08% and 178.6% higher in the 10, 20, 30 and 40 g.a.i ha^{-1} Imidacloprid treatments respectively than in the control. No significant difference ($p > 0.05$) was observed between the control, 10 and 20 g.a.i ha^{-1} Imidacloprid treatments at 7 DAT and between the control and 10

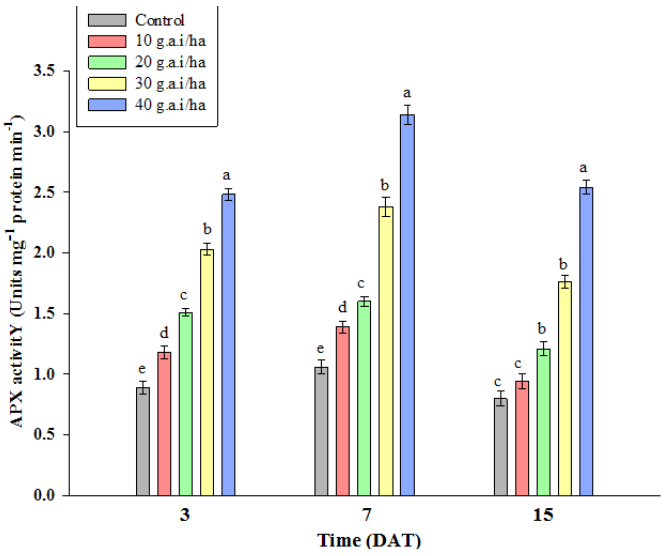

Figure 5: Changes in Apx activity (Units mg^{-1} protein min^{-1}) at various growth stages in *Brassica juncea* L. cv. alankar exposed to different concentrations of imidacloprid
Values with different small letters at each time point are significantly ($p < 0.05$) different from each other (Duncan's multiple-range test). The values represent Mean ± SE (n =4).

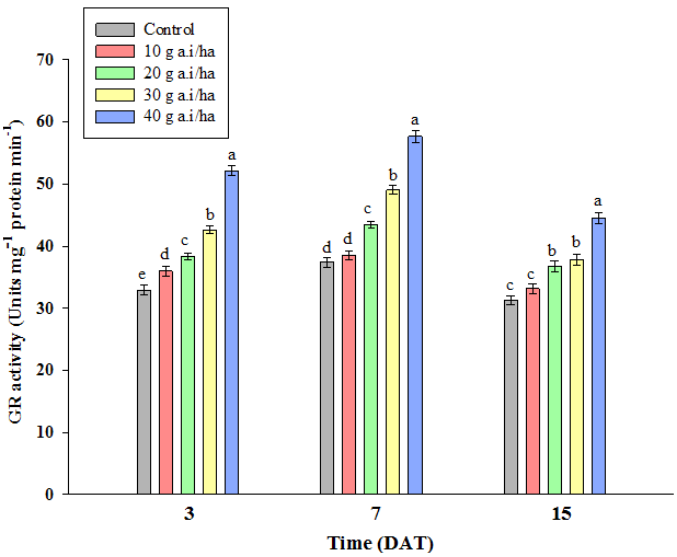

Figure 6: Changes in GR activity (Units mg^{-1} protein min^{-1}) at various growth stages in *Brassica juncea* L. cv. alankar exposed to different concentrations of imidacloprid
Values with different small letters at each time point are significantly ($p < 0.05$) different from each other (Duncan's multiple-range test). The values represent Mean ± SE (n =4).

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g.a.i ha^{-1} Imidacloprid treatments at 15 DAT. Like the SOD activity, GR activity increased considerably under the lower doses of Imidacloprid treatments, and attains the maximum with (40 g.a.i ha^{-1}) 7 DAT, followed by 3 DAT and 15 DAT respectively (Figure 6).

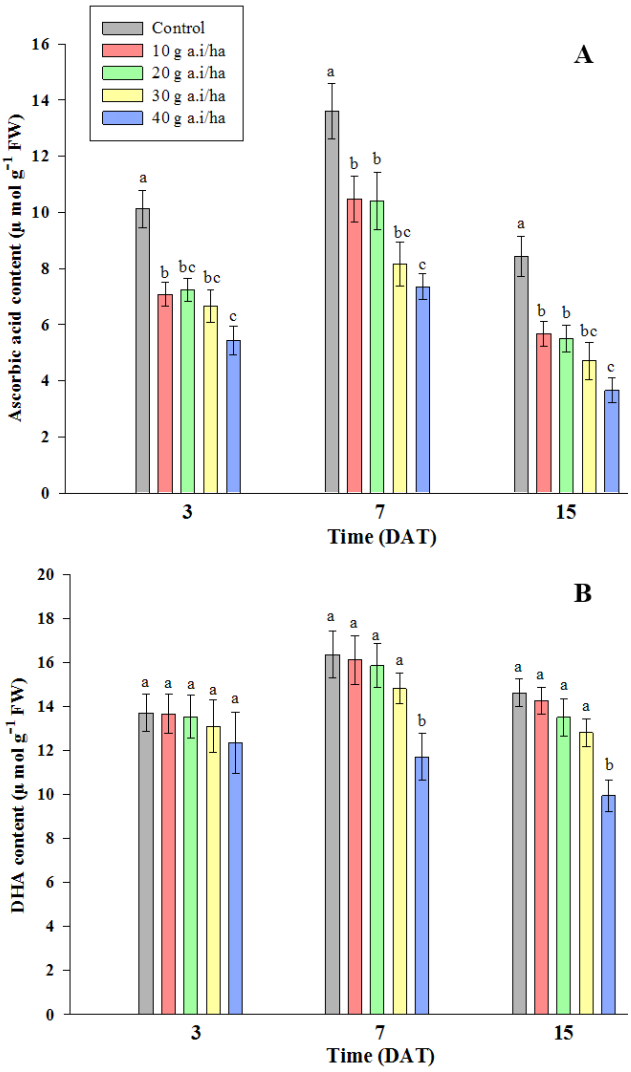

Figure 7: Variations in ascorbate content at various growth stages in *Brassica juncea* L.cv. alankar treated with different concentrations of imidacloprid. **(A)** Ascorbic acid (ASC) content (µmol g^{-1} FW) and **(B)** dehydroascorbate (DHA) content (µmol g^{-1} FW) Values with different small letters at each time point are significantly ($p < 0.05$) different from each other (Duncan's multiple-range test). The values represent Mean ± SE (n =4).

In the present study, as compared to control, a dose dependent reduction in Ascorbate (ASC) and Dehydroascorbate (DHA) by 56.65 and 31.19%, respectively, at 15 DAT was observed under the highest concentration of (40 g.a.i ha^{-1}) Imidacloprid used (Figure. 7A and 7B). A dose dependent decrease was also observed in reduced glutathione (GSH) content by 61.39% under the highest (40 g.a.i ha^{-1}) Imidacloprid concentration as compared to its control at 15 DAT (Figure 8A). But, on the other hand, as compared to its respective control, an increase in oxidized glutathione (GSSG) level by 93.2% at 3 DAT under 40 g.a.i ha^{-1} insecticidal treatment was observed (Figure 8B).

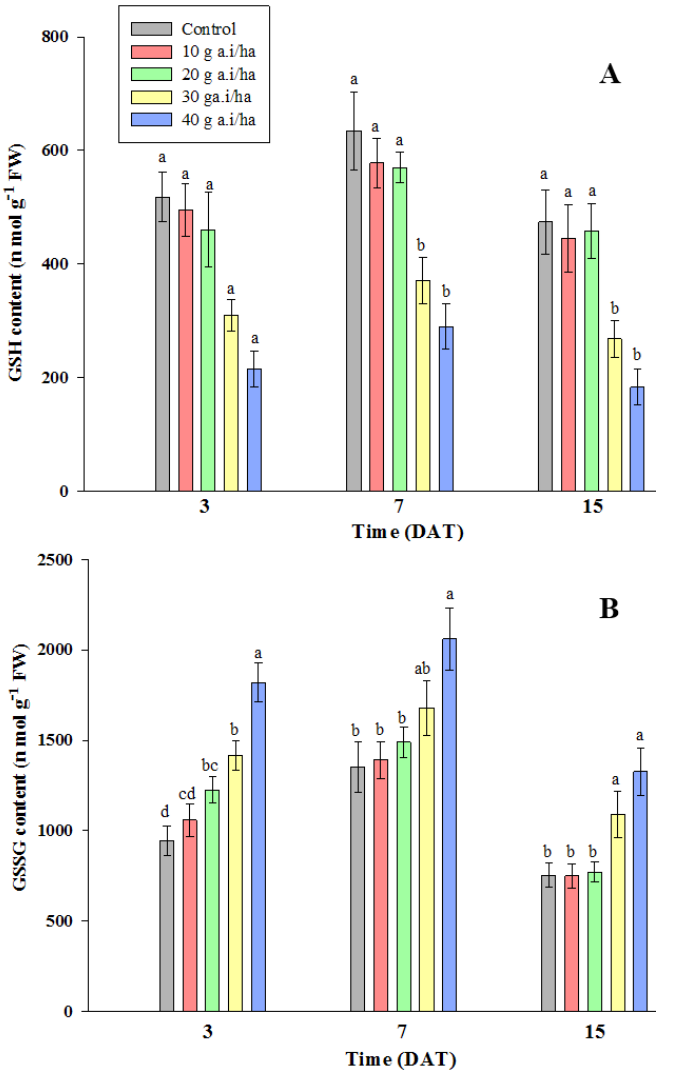

Figure 8: Variations in glutathione content at various growth stages in *Brassica juncea* L. cv. alankar treated with different concentrations of imidacloprid. **(A)** Reduced GSH content (nmol g^{-1} FW) and **(B)** oxidized GSSG content (n mol g^{-1} FW)
Values with different small letters at each time point are significantly ($p < 0.05$) different from each other (Duncan's multiple-range test). The values represent Mean ± SE (n =4).

Photosynthetic pigments

The photosynthetic chlorophyll (Chl) pigment showed marginal increase 1.6% and 4.8%; 5.4% and 10.4%; and 4.2% and 13.2% over their respective controls at lower concentration of Imidacloprid treatment at 3 DAT, 7 DAT and 15 DAT respectively, touching maximum with 20 g.a.i ha^{-1} at 7 DAT followed by 3 and 15 DAT respectively. However, it showed a consistent decreasing trend at higher concentrations (30 and 40 g.a.i ha^{-1}) of Imidacloprid, in a dose dependent manner over their respective controls at all the three growth stages (Figure 9A).

Like chlorophyll content, the carotenoid (Car) con-

tent also increased at lower concentrations of the insecticide treatment over their respective controls at all the observed growth stages, showing maximum enhancements at 20 g.a.i ha^{-1} at 7 DAT followed by 3 and 15 days respectively, and then decreased considerably at higher concentrations at all the observed growth stages with maximum decrease at 40 g.a.i ha^{-1} at 15 DAT followed by 3 and 7 DAT respectively (Figure 9B).

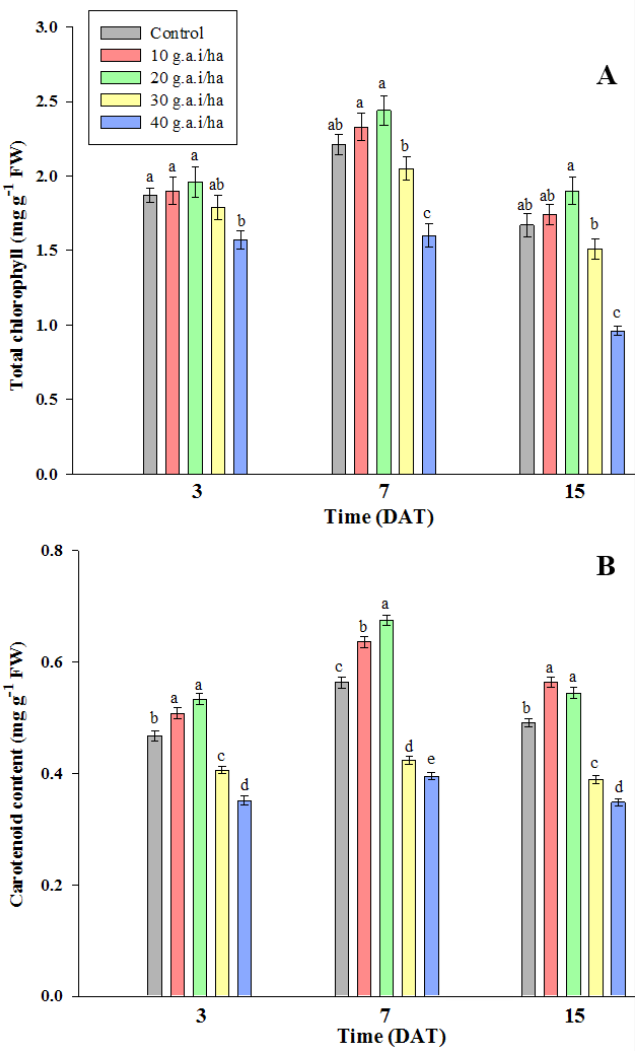

Figure 9: Effects of different concentrations of imidacloprid on photosynthetic pigments in *Brassica juncea* L. cv. alankar at different growth stages. **(A)** Total chlorophyll content (mg g^{-1} FW). **(B)** Carotenoid content (mg g^{-1} FW)
Values with different small letters at each time point are significantly ($p < 0.05$) different from each other (Duncan's multiple-range test). The values represent Mean ± SE (n =4).

Discussion

Imidacloprid is a systemic insecticide that moves rapidly through plant tissues after the foliar application or with soil (Fossen 2006). Kumar and Dikshit (2001) observed that Imidacloprid was not detected in *Brassica* plants after 7 and 15 days of foliar treatments from lower and higher rates of applications respectively. Therefore its effects on plants could be reflected at least for this time duration.

In our study, the occurrence of marginal necrosis at higher concentrations of Imidacloprid was similar to the previous studies (Wallace 2000; Bucholz and Nauen 2002). These symptoms may be due to the translaminar and acropetal (movement towards leaf margins) movement of topically applied Imidacloprid (Bucholz and Nauen 2002).

Lipid peroxidation may be the first step of cellular membrane damage caused due to insecticide stress, and has been extensively used as a marker of oxidative stress (Hazarika et al., 2003). Increase in lipid peroxidation rate is regarded as a general response to many stresses like heavy metals (Vanaja et al., 2000), and salt stress (Hernandez et al., 2000). The increased concentration of MDA in *Brassica juncea* L. plants particularly at higher concentrations of Imidacloprid (30 and 40 g.a.i ha^{-1}) suggests that these are highly susceptible to insecticide stress. Table 1 depicts that MDA content in plant increased with the increase in Imidacloprid dose at all the studied growth stages (3, 7 and 15 DAT), thus *B. juncea* plants in present study showed age and dose-dependent increases in lipid peroxidation rate compared to control, the average value of MDA content increased by 57.7% at day 15 after Imidacloprid treatment under 40 g.a.i ha^{-1}.

Exposure of *Glycine max*. L. to insecticide deltamethrin or other pesticides led to increase in lipid peroxidation in leaves and roots (Bashir et al., 2007b; Song et al., 2007; Parween et al., 2012). The increased lipid peroxidation in the present study suggested that ROS-induced damage may be one of the main toxic effects of Imidacloprid.

Plants respond to a variety of stresses by accumulating certain specific metabolites; the most conspicuous of them being the proline (Jaleel et al., 2007). Proline may affect the solubility of various proteins, thus protecting them against denaturation under stressful conditions and in plant cells it acts as an osmoregulator, a soluble nitrogen sink, signal of senescence and an indicator of plant resistance to stress (Bashir et al., 2007a). Compared to the control, no significant change in the proline content in plants treated with 10 and 20 g.a.i ha^{-1} Imidacloprid in the present study, suggests little or even no oxidative injury occurring in plants (Figure 2). It might be due to the presence of chloropyridene side chain in Imidacloprid that

Table 1: Concentrations and activity of components of the antioxidative defense system (MDA, Proline, SOD, CAT, APX, GR, ASC, DHA, GSH, GSSG) and photosynthetic pigments (chlorophyll and carotenoid) in *Brassica juncea* L. at 3, 7 and 15 days after treatments (DAT) with the insecticide Imidacloprid at different concentrations (0 - 40 g.a.i ha^{-1})

Imidacloprid Treatments (g.a.i ha^{-1})	3 DAT	7 DAT	15 DAT
MDA content (nmol g^{-1} FW)			
0	16.41 ± 0.70	22.77 ± 0.71	31.44 ± 0.76
10	18.75 ± 0.59	24.58 ± 0.77	35.35 ± 0.88
20	22.35 ± 0.78	34.22 ± 0.90	45.80 ± 0.85
30	31.18 ± 0.85	45.30 ± 0.78	59.88 ± 0.83
40	46.13 ± 0.66	55.36 ± 0.77	72.82 ± 0.86
Proline content (µg g^{-1} FW)			
0	20.32 ± 0.70	28.90 ± 0.85	43.62 ± 0.60
10	20.46 ± 0.68	29.96 ± 0.67	45.46 ± 0.60
20	23.10 ± 0.52	32.20 ± 0.95	47.35 ± 0.90
30	33.29 ± 0.65	46.30 ± 0.88	57.76 ± 0.75
40	42.20 ± 0.69	58.53 ± 0.78	68.87 ± 1.00
SOD activity (units mg^{-1} protein min^{-1})			
0	12.57 ± 0.32	15.88 ± 0.50	11.41 ± 0.35
10	13.20 ± 0.40	17.92 ± 0.41	13.37 ± 0.30
20	13.70 ± 0.32	20.46 ± 0.51	14.61 ± 0.32
30	25.24 ± 0.47	28.95 ± 0.66	20.98 ± 0.45
40	32.16 ± 0.65	37.33 ± 0.90	25.80 ± 0.42
CAT activity (units mg^{-1} protein min^{-1})			
0	321.75 ± 7.83	345.62 ± 7.37	289.3 ± 8.17
10	326.62 ± 7.99	355.25 ± 9.21	293.12 ± 8.3
20	337.25± 10.54	371.75 ± 7.30	301.7± 8.13
30	320.00± 7.35	337.12 ± 7.14	280.8 ± 7.80
40	241.50 ± 7.05	260.00 ± 6.17	208.6 ± 7.17
APX activity (units mg^{-1} protein min^{-1})			
0	0.89 ± 0.05	1.06 ± 0.06	0.80 ± 0.06
10	1.18 ± 0.05	1.39 ± 0.05	0.94 ± 0.07
20	1.51 ± 0.03	1.60 ± 0.04	1.21 ± 0.05
30	2.03 ± 0.06	2.38 ± 0.08	1.76 ± 0.05
40	2.48 ± 0.05	3.14 ± 0.08	2.54 ± 0.06
GR activity (units mg^{-1} protein min^{-1})			
0	32.93 ± 0.80	37.38 ± 0.80	31.29 ± 0.73
10	35.95 ± 0.75	38.56 ± 0.69	33.17 ± 0.76
20	38.33 ± 0.59	43.43 ± 0.59	36.75 ± 0.87
30	42.62 ± 0.58	49.04 ± 0.70	37.82 ± 0.84
40	52.15 ± 0.77	57.65 ± 0.93	44.54 ± 0.85
Ascorbic acid (ASC) content (µmol g^{-1} FW)			
0	10.12 ± 0.65	13.60 ± 0.99	8.42 ± 0.70
10	7.08 ± 0.41	10.47 ± 0.81	5.67 ± 0.43
20	7.23 ± 0.41	10.40 ± 1.00	5.50 ± 0.47
30	6.66 ± 0.59	8.15 ± 0.77	4.7 ± 0.65
40	5.42 ± 0.51	7.35 ± 0.45	3.65 ± 0.43
Dehydroascorbate (DHA) content (µmol g^{-1} FW)			
0	13.70 ± 0.85	16.34 ± 1.06	14.60 ± 0.62
10	13.65 ± 0.88	16.10 ± 1.10	14.25 ± 0.61
20	13.52 ± 0.97	15.85 ± 1.00	13.50 ± 0.85
30	13.10 ± 1.18	14.80 ± 0.69	12.80 ± 0.63
40	12.33 ± 1.39	11.70 ± 1.05	9.94 ± 0.71
reduced GSH content (nmol g^{-1} FW)			
0	518.04 ± 44.1	635.18 ± 68.7	474.08±56.7
10	494.9± 45.98	577.8 ± 43.45	443.56±59.2
20	460.2± 65.86	569.89±26.76	458.4±48.01
30	310.3± 27.65	370.4± 40.80	267.78±32.1
40	214.79 ± 31.9	289.98 ± 40.5	183.3± 31.8
oxidized GSSG content (nmol g^{-1} FW)			
0	942 ± 82.09	1350 ± 139.7	755 ± 65.51
10	1058 ± 90.01	1387 ± 104.3	749 ± 65.97
20	1225 ± 72.25	1490 ± 83.00	766 ± 55.73
30	1416 ± 79.03	1678 ± 151.3	1088± 129.4
40	1820 ± 108.54	2057 ± 173.3	1326± 128.7
Total chlorophyll content [mg g^{-1} FW]			
0	1.87 ±0.05	2.21 ± 0.07	1.67 ± 0.08
10	1.90 ±0.09	2.33 ± 0.09	1.74 ± 0.07
20	1.96 ±0.10	2.44 ± 0.10	1.90 ± 0.09
30	1.79 ±0.08	2.05 ± 0.08	1.51 ± 0.07
40	1.57 ±0.06	1.60 ± 0.08	0.96 ± 0.03
Carotenoid content [mg g^{-1} FW]			
0	0.46 ± 0.009	0.56 ± 0.01	0.49 ± 0.008
10	0.50 ± 0.01	0.63 ± 0.01	0.56 ± 0.009
20	0.53 ± 0.01	0.67 ± 0.009	0.54 ± 0.01
30	0.40 ± 0.006	0.42 ± 0.007	0.38 ± 0.008
40	0.35 ± 0.008	0.39 ± 0.007	0.34 ± 0.007

The values represent Mean ± SE (n =4).
Abbreviations: **MDA** = Melondialdehyde; **SOD** = Superoxide dismutase; **CAT**= Catalase; **APX** = Ascorbate peroxidase; **GR**= Glutothione reductase; **ASC**= Ascorbate; **DHA**= Dehydroascorbate; **GSH**= reduced glutathione; **GSSG**= oxidized glutathione

structurally resembles to nicotinamide and nicotinic acid (niacin), known as systemic plant resistance inducers and possess antioxidant activity (Ogata et al., 2002). By contrast, the proline content increased significantly at higher doses of Imidacloprid used, indicating that the oxidative damage caused by lipid peroxidation was never fully prevented. The other reason behind this could be the over production of ROS at high dose of Imidacloprid (Zhang et al., 2011). Similar results in proline content were also reported by other workers under insecticide stress (Parween et al. 2011). Moreover, the variation in MDA and proline content in control plants at different growth stages

in our study fully agrees with the results of Parween et al. (2012), who also reported the change in MDA and proline content in control plants at their different growth stages.

Plants have evolved with complex antioxidant system to mitigate and repair the damage caused by ROS. In the present study, Imidacloprid-treated seedlings showed a significant enhancement in superoxide dismutase (SOD) activity at higher doses, especially at 7 DAT. The ability of plants to mitigate oxidative stress partially relies on the induction of SOD activity and subsequently on the upregulation of other downstream antioxidant enzymes (Alscher et al., 2002). SOD is likely to play a central role in the defense against toxic reactive oxygen species (ROS) and constitute the first line of defense within a cell against ROS (Liang et al., 2003). SOD processing is known to be substrate inducible and an increase in the SOD activity may be attributed to the increased production of active oxygen species as substrate that lead to increased expression of genes encoding SOD (Abedi and Pakniyat, 2010). Reactive oxygen species of O^{2-} have been considered as the central components of signal transduction which triggers the defense genes responsible for oxidant enzymes, such as SOD (Arrora et al., 2002). Conversely, the increased enzyme activity contributes to the removal of Superoxide anion (O^{2-}) (Liu et al., 2011). The relatively low activity at 15 DAT could be because old leaves generally contain lower metabolic activities than younger leaves and this is why the old leaves are more prone to enhanced oxidative injury than the young leaves (Haddad et al., 2009). Our observations on SOD activity find support from several earlier findings concerning the effects of air pollutants (Hernandez et al., 2000), and salinity (Qureshi et al., 2007) on the antioxidant defense system in plants. It was also reported that SOD activity in rice and wheat exposed to 1,2,4-trichlorobenzene stress, wheat exposed to omethoate and in bitter gourd exposed to dimethoate were significantly increased compared with the control (Mishra et al., 2009; Zhang et al., 2011).

Hydrogen peroxide (H_2O_2) produced from the action of SOD diffuses rapidly across the membranes and is very toxic to the chloroplasts, nucleic acids, protein and lipids (Gille and Singler, 1995). Therefore, it is important that H_2O_2 be scavenged rapidly by the antioxidative defense system to water and oxygen which can be eliminated by CAT and a series of peroxidase (POD) (Guo et al., 2006). It seems to suggest that the cooperation between H_2O_2 scavenging enzymes and SOD plays a significant role in plant resistance to stressful environments.

Significant increase in the activities of CAT and APX in the present investigation suggests their role in constant detoxification of H_2O_2 in *B. juncea* L. seedlings under Imidacloprid toxicity. CAT activity was highly sensitive to low concentrations of Imidacloprid treatments, and was thus stimulated by Imidacloprid to scavenge H_2O_2 to alleviate lipid peroxidation. However, at higher levels of Imidacloprid, ROS load might have exceeded the antioxidant defense capacity in *B. juncea* plants as is evident by low activities of CAT (Figure 4). As a consequence, severe oxidative damage occurred as is evident from the marginal leaf necrosis at high concentrations (40 g.a.i ha^{-1}) in present study. Similar results were found by (Zhang et al., 2011; Parween et al., 2012) in wheat and *Vigna radiata* under omethoate and chloropyriphos stress respectively. Streb et al. (1993) have reported similar changes in CAT activity in the Paraquat–treated Secale cereal. Decline in CAT activity is regarded as a general response to many stresses and is supposedly due to inhibition of enzyme synthesis or change in assembly of the enzyme subunits (Somashekaraiah et al., 1992).

GR is localized mainly in the chloroplast in which it represents about 80% of total GR activities in leaf tissues. It can also be found in cytosol, glyoxysomes, and peroxisomes (Jimenez et al., 1997). Like APX, GR is one of the major components in the ascorbate–glutathione cycle, by which the efficient recycling of glutathione is ensured by GR. Therefore, it plays an essential role in the protection of chloroplasts against the oxidative damage by maintaining a high reduced/oxidized glutathione (GSH/GSSG) ratio (Noctor and Foyer, 1998). The present study showed that the GR activity has increased with the increasing concentration of Imidacloprid (Figure 6). The reason behind this could be the high operating rate of ASC–GLU cycle to detoxify the ROS in these plants (Stolt et al., 2003). Another reason is maintenance of high levels of reduced glutathione pool to make high availability of tripeptides for the synthesis of phytochelatins (Stolt et al., 2003), the small peptides involved in the inactivation of pesticides by conjugate formation (Parween et al., 2012). It has been observed that plants under environmental stimuli tend to have high activities of GR (Batish et al., 2006). Hence, a critical role is played by GR in protecting plants against oxi-

dative stress.

The non-enzymatic cellular antioxidants, like ascorbate and glutathione, undergo changes during oxidative stress (Nakano and Asada, 1981). In the present study, a dose dependent reduction in ASC and DHA as compared to their respective controls was observed. Similar results were reported by (Qureshi et al., 2007) and (Parween et al., 2012) under heavy metal and insecticide stress respectively. Oxidative stress appears to cause decline in the ratio of ASC and dehydroascorbate (DHA) (Qureshi et al., 2007). Glutathione acts as an antioxidant and is believed to protect the cell against oxidative stress by maintaining cellular redox potentials. It may react directly with ROS, protect protein thiol groups and is believed to be involved in enzymatic detoxification of H_2O_2 (Kok De and Stulen, 1993). The enhanced levels of GSSG in *B. juncea* due to Imidacloprid toxicity suggest its involvement in the detoxification of reactive oxygen species and free radicals, directly (non-enzymatic) as well as through certain enzymes. It is believed that GSH (Wingale et al., 1988) or GSSG (Winglse and Karpinski, 1996) or a change between GSH and GSSG (Foyer et al., 1997) may function as a signal for activating stress-responsive gene expression under stress situations.

In the present study, the increase in chlorophyll content over their respective controls observed at lower concentrations of Imidacloprid at all the growth stages of plant is attributed to improved plant metabolism, but the evidence is lacking. The Imidacloprid molecule has a chloropyridine side chain which structurally resembles with nicotinamide and nicotinic acid (niacin). These molecules have been shown to possess antioxidant activity (Gonias et al., 2008). Our results fully agree with the findings of Baozhen et al. (2013), who also reported the increase in chlorophyll content after foliar application of Imidacloprid.

There was a significant reduction in total chlorophyll when concentration of Imidacloprid was increased from 30- 40 g.a.i ha^{-1}. Several studies reported that the decline in chlorophyll content might be due to a reduction in the synthesis of chlorophyll, possibly by increasing the enzyme activity of chlorophyllase (Raja et al., 2012) or breakdown of pigments or precursors under high Imidacloprid stress as suggested for cowpea seedlings under insecticide dimethoate stress (Mishra et al., 2008). Carotenoid is an important component of the defense mechanism of plants protecting chlorophyll and photosynthetic membrane from photooxidative damage (Mishra et al., 2008). Therefore, decline in carotenoid content could have serious consequence on chlorophyll, phycocyanin as well as thylakoid membrane (Prasad et al., 2005). The enhanced level of carotenoids at lower doses of Imidacloprid (10 and 20 g.a.i ha^{-1}) (Figure 9B) occurred to protect the plant against the photodynamic damage. Similar to this, increase in the level of carotenoids at low doses under dimethoate (Mishra et al., 2008), UV-B (Lingakumar and Kulandaivelu, 1993) and chloropyriphos (Parween et al., 2011) exposure were observed. High doses of Imidacloprid caused considerable reduction in carotenoid content and this condition results in serious consequence on chlorophyll and photosynthetic apparatus leading to reduced photosynthesis efficiency of the plant. Significant reduction in carotenoid content was reported under insecticide stress (Mishra et al., 2008). In our study, the decrease in the photosynthetic pigments at 15 DAT as compared to the 3 DAT and 7 DAT can be because of senescence of leaves (Sestak, 1981).

Conclusion

Mustard could tolerate Imidacloprid stress at lower concentrations as is evident from the enhancement of photosynthetic pigments at lower concentrations of insecticide. The possible mechanisms involved might be attributed to higher antioxidant enzyme activity and lower lipid peroxidation and membrane permeability. However, our results suggest that at high doses, a high lipid peroxidation rate indicates degradation of the cell-membrane system, which would disturb fundamental biochemical and physiological processes in plants and enhance the production of ROS. These biochemical results can be inferred as internal tolerance mechanisms and may help to develop tactics for reducing the risk of insecticide contamination in the crop production.

Acknowledgement

The authors are very thankful to University Grant Commission, New Delhi, for providing financial assistance during this research work.

Author's Contribution

Fareed Ahmad Khan designed the study and draft-

ed the manuscript. Mudasir Irfan Dar performed the experimental work and performed the statistical analysis. Farha Rehman participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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