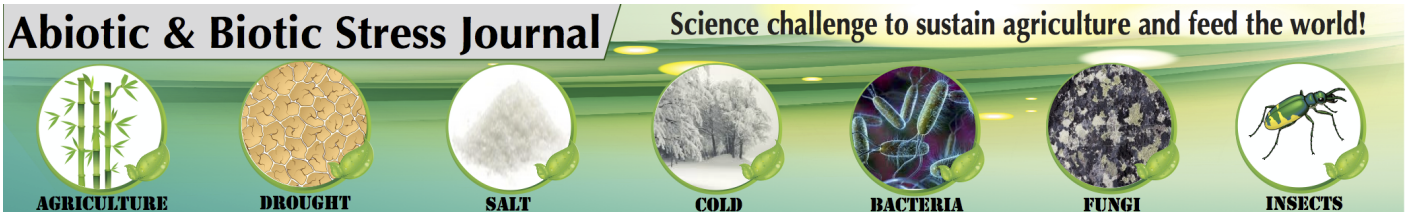

Responses of antioxidative defense system and photosynthetic pigment composition of Brassica juncea L. to imidacloprid stress

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Responses of antioxidative defense system and photosynthetic pigment composition of *Brassica juncea* L. to imidacloprid stress.

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Abstract:

The effect of insecticide imidacloprid on photosynthetic pigments and antioxidative enzymes of *Brassica juncea* L. cv. alankar was investigated. Forty-day-old pot plants were exposed to different concentrations of insecticide imidacloprid, ranging from 0 to 40 gram active ingredient hectare^{-1} (g.a.i ha^{-1}) through foliar spray. Analyses were done at Day 3, Day 07, and Day 15 after the treatment. Lipid peroxidation rate, proline content, ascorbate and glutathione content and antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were all assayed. Imidacloprid enhanced malondialdehyde (MDA) and proline content over their respective controls at all doses at all the observed growth stages, attaining maximum with 40 gram active ingredient hectare^{-1} at Day 15 after treatment. The antioxidative enzyme activity (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 7 days 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 dose dependence decline at higher concentations of 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 Asc, dehydroascorbate (DHA) and reduced glutathione (GSG) levels. The photosynthetic pigments showed slight increase over their respective controls at lower concentrations of 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 concentration of imidacloprid applied on the mustard

might have no severe effect on the mustard plant because of the regulation of anti- oxidative enzyme system and MDA, but provoked the state of oxidative stress when applied at higher dosages. Therefore pesticide application at higher concentrations can be considered as a kind of stress factor similar to other stresses such as salt, drought and low or high temperature.

Key words: insecticide; mustard; malondialdehyde; proline; stress.

1. 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, nonenzymatic (e.g., glutathione, tocopherols, ascorbate, proline and carotenoids) defense systems 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 entire Indian sub-continent, but infestation of this crop with insect pests is the 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, 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 of plants. Keeping in view the importance of oil seed crops, the present investigation was carried out to find out 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 to find out the optimum level of insecticide imidacloprid for efficient growth and photosynthetic pigments of *B. juncea*.

2. Results

2.1. Visual symptoms: Plants were keenly watched after the foliar spray of insecticide imidacloprid and at 50 days of growth stage (10 DAT), marginal necrosis was observed to occur in plants sprayed with 40 g.a.i/ha.

2.2. Lipid peroxidation rate.

The 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 (fig 1). 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. 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 at day 7 after treatment of imidacloprid respectively ($P < 0.05$). 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}.

2.3. 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 levels 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 (fig 2). The maximum enhancement was found with 40 g.a.i ha^{-1} imidacloprid at 15 DAT.

2.4. 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 (fig 3). The gain was maximum at 40 g.a.i ha^{-1} imidacloprid at 7 DAT followed by 3 DAT and 15 DAT.

The CAT activity also showed a similar variation trend (fig 4), as did SOD with respect to age of the plant, thus having its maxima 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 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. 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 (fig 5).

The activity of APX was also raised with increasing concentration of imidacloprid, gaining the most with highest dose of chemical (40 g.a.i. ha^{-1}) at 7 DAT, followed by 3 DAT and 15 DAT respectively (fig 6). The APX activity at 3 DAT 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 than in the control, respectively. No significant difference was observed between the control and 10 and 20 g.a.i ha^{-1} imidacloprid treatments at 7 DAT and between the control and 10 g.a.i ha^{-1} imidacloprid treatments at 15 DAT.

In the present study, as compared to control a dose dependent reduction in Asc and DHA by 56.65 and 31.19 %, respectively, at 15DAT was observed under the highest concentration of (40 g a.i ha^{-1}) imidacloprid used (fig. 7A and 7b). A dose dependent decrease was also observed in GSH content by 61.39 % under the highest (40 g a.i ha^{-1}) imidacloprid concentration as compared to its control at 15 DAT (fig 8A). But on the other hand as

compared to its respective control, an increase in GSSG by 93.2 % at 3 DAT under 40 g a.i ha^{-1} insecticidal treatment was observed (fig 8B).

2.5. Photosynthetic pigments

The photosynthetic pigment chlorophyll 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 treatment, in a dose dependent manner over their respective controls at all the growth stages (fig 9A).

Like chlorophyll content, the carotenoid content also increased at lower concentrations of the insecticide treatment over their respective controls at all the observed growth stages, showing maximum enhancement 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 (fig 9B).

3. 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 application, 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. Fig 1 showed age and dose dependent increase in lipid peroxidation rate in imidacloprid-treated plants than that of the untreated plants. Compared to control, the average values 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 amino acid- 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 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 present study, suggests little or even no oxidative injury occurring in plants (fig 2). It might be due to the presence of chloropyridene side chain in imidacloprid that structurally resembles 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 highest dose of imidacloprid (Zhang et al., 2011). Similar results in proline content was also reported by other workers under insecticide stress (Parween et al., 2011).

Plants have evolved 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 defence against toxic reactive oxygen species (ROS) and constitute the first line of defence with in 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 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 leaf 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 defence 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).

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 defence 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 (fig 4). As a consequence, severe oxidative damage occurred as is evident from the marginal leaf necrosis at higher concentrations 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 cereale. 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 be also 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 (fig 5). 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). 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 oxidative 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 (Wingate et al., 1988) or GSSG (Winglse and Karpinski, 1996) or a change between GSH and GSSG (Foyer et al., 1997) may function as signal for activating stress-responsive gene expression under stress situations.

In this 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 increment in chlorophyll content after foiar 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 caused due to 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 undress

insecticide dimethoate stress (Mishra et al. 2008). Carotenoid is an important component of defence mechanism of plants and protect 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 carotenoid at lower doses of imidacloprid (10 and 20 g.a.i ha^{-1}) (fig 9B) occurred to protect the plant against the photodynamic damage. Similar to this, increase in the level of carotenoid at low dose 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 Chl 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 higher 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 us to develop tactics for reducing the risk caused due to insecticide contamination in the crop production.

4. 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 ^{o}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^{TM} 200 SL), ranging from 0 to 40 g a.i ha^{-1} with 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 leaf. 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 (approx.) in one sprinkle and each plant was sprinkled thrice. Accordingly, each plant received 3 ml of DDW or insecticide solution. All the plants were healthy and disease free at the time of spraying. The maximum and minimum temperatures during the mustard crop season were 27°C and 13°C, respectively

with relative humidity of 30%. Average sunshine recorded was 8.50 hours. There was no rainfall during the period of this study.

Each experiment was repeated three times with four replicates in completely randomised 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 analyse the effect of different concentrations of insecticide on photosynthetic pigments, proline content, and 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).

4.1. 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 sample.

Lipid peroxidation was estimated 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.

4.2. 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 (1mM EDTA, 0.05% Triton X- 100, 2% PVP, 1 mM ascorbate in 50mM phosphate buffer of pH 7). The homogenate was centrifuged at 15000 rpm for 20 min at 4^{o}C. Supernatants obtained were used for enzyme determinations.

4.3. Superoxide dismutase activity

SOD activity was assayed according to the method of Beauchamp and Fridovich, (1971) in terms of ability to inhibit the photochemical induction of nitroblue tetrazolium (NBT) to formazon 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).

4.4. 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.

4.5. 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).

4.6. Glutathione reductase activity

The leaf samples for estimation of GR were extracted in 100mM potassium phosphate buffer (pH 7.0) containing 1mM 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 340nm and using extinction coefficient of 6.2mM^{-1} cm^{-1}.

4.7. Estimation of Chlorophyll and Carotenoids

Chlorophyll and carotenoids were estimated by the method of Arnon, (1949).

4.8. Ascorbate content

Fresh leaf (0.05 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).

4.9. Glutathione content

Fresh leaf tissue (0.05 g) was homogenized in 2 ml of 5 % sulphosalicylic acid at 4 ^oC. The homogenate was then centrifuged at 10,000 rpm for 15 minutes. Reduced (GSH) and oxidised (GSSG) glutathione were determined by the method of Anderson, (1985).

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 ± SE (n = 4).

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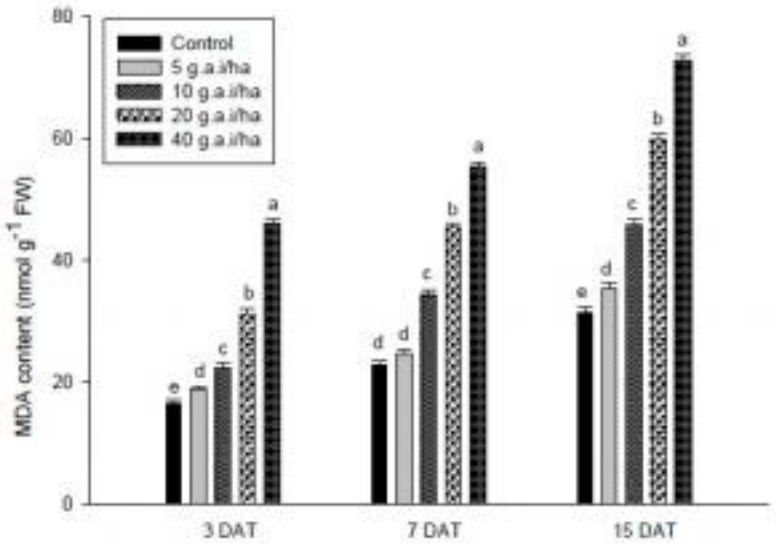

Fig 1

Fig 1 Change in MDA content (nmol g^{-1} FW) at various growth stages of *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)

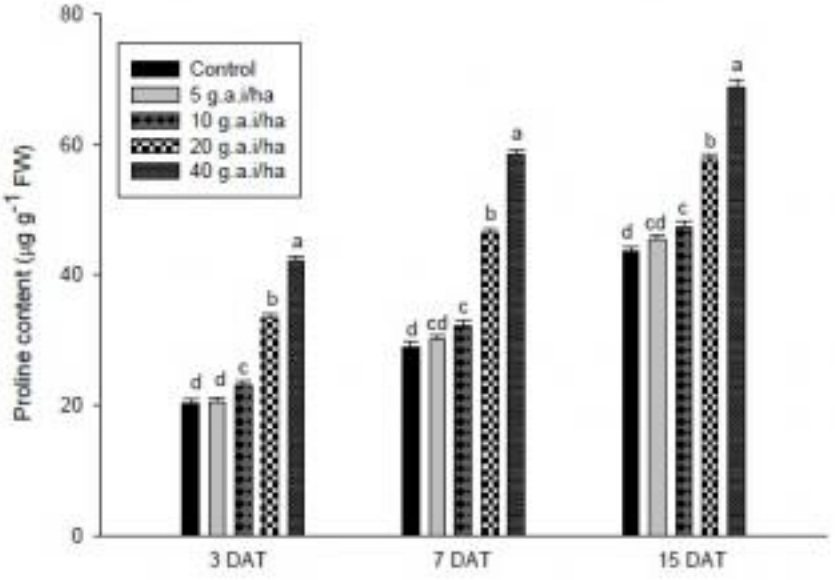

Fig 2

Fig 2 Change in proline content (µg g^{-1} FW) at various growth stages of *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)

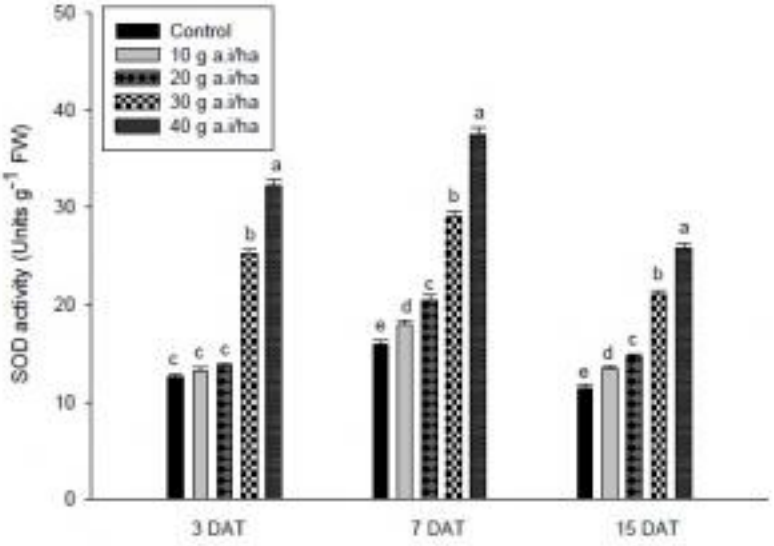

Fig 3

Fig 3 Change in SOD activity (Units mg^{-1} protein min^{-1}) at various growth stages of *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)

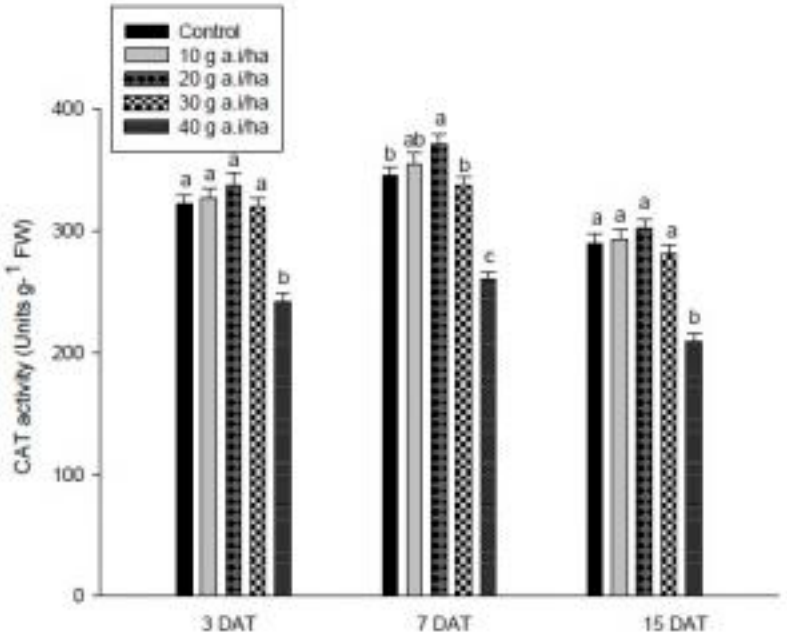

Fig 4

Fig 4 Change in CAT activity (Units mg^{-1} protein min^{-1}) at various growth stages of *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)

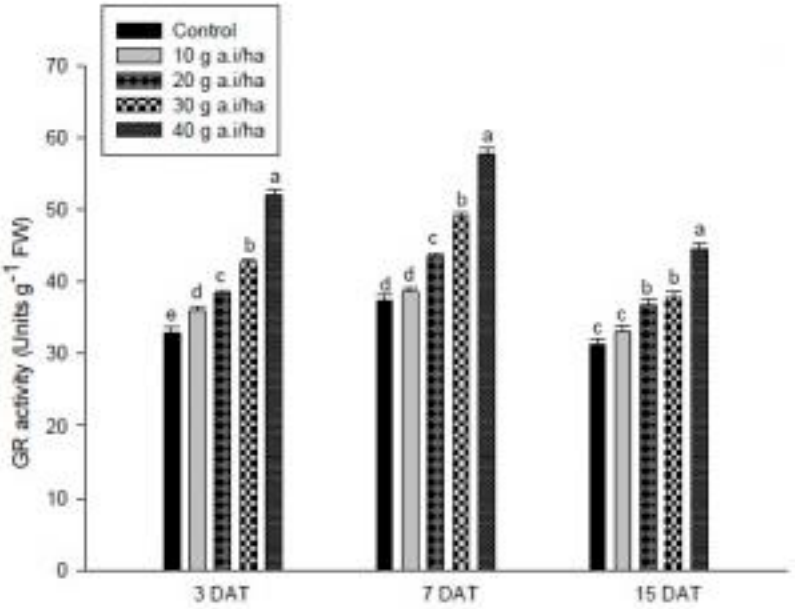

Fig 5

Fig 5 Change in GR activity (Units mg^{-1} protein min^{-1}) at various growth stages of *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)

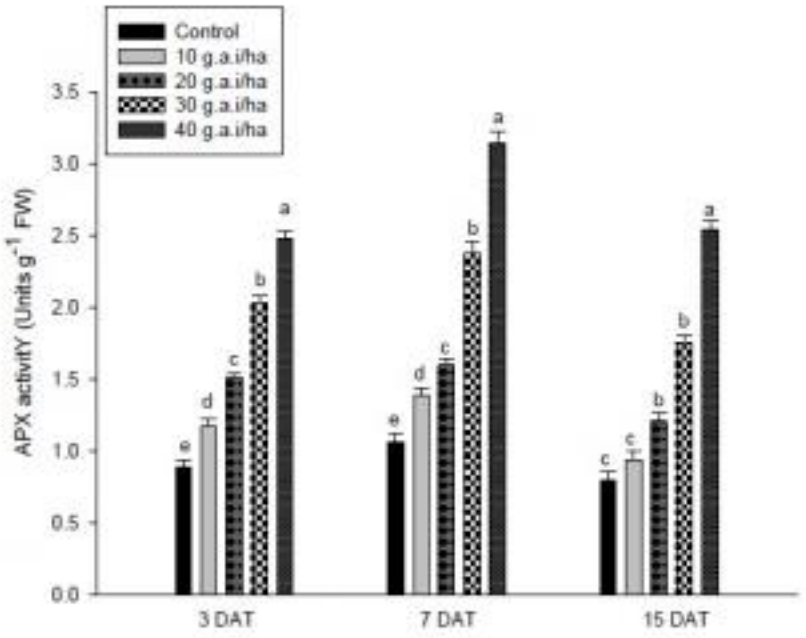

Fig 6

Fig 6 Change in Apx activity (Units mg^{-1} protein min^{-1}) at various growth stages of *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)

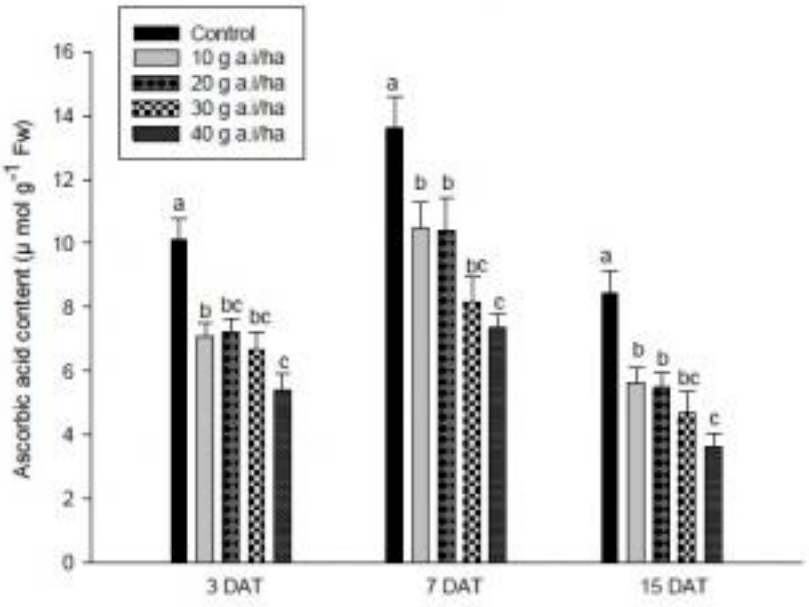
Control
10 g a.i/ha
20 g a.i/ha
30 g a.i/ha
40 g a.i/ha
Ascorbic acid content (µ mol g-1 Fw)
0
2
4
6
8
10
12
14
16
a
b
bc
bc
c
a
b
b
bc
c
a
b
b
bc
c
3 DAT
7 DAT
15 DAT

Fig 7A

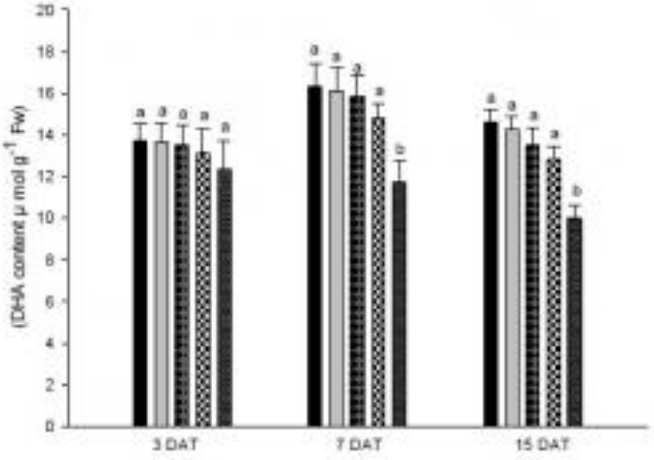

Fig 7B

Fig 7 Variation in ascorbate content at various growth stages of *Brassica juncea* L. treated with different concentration 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)

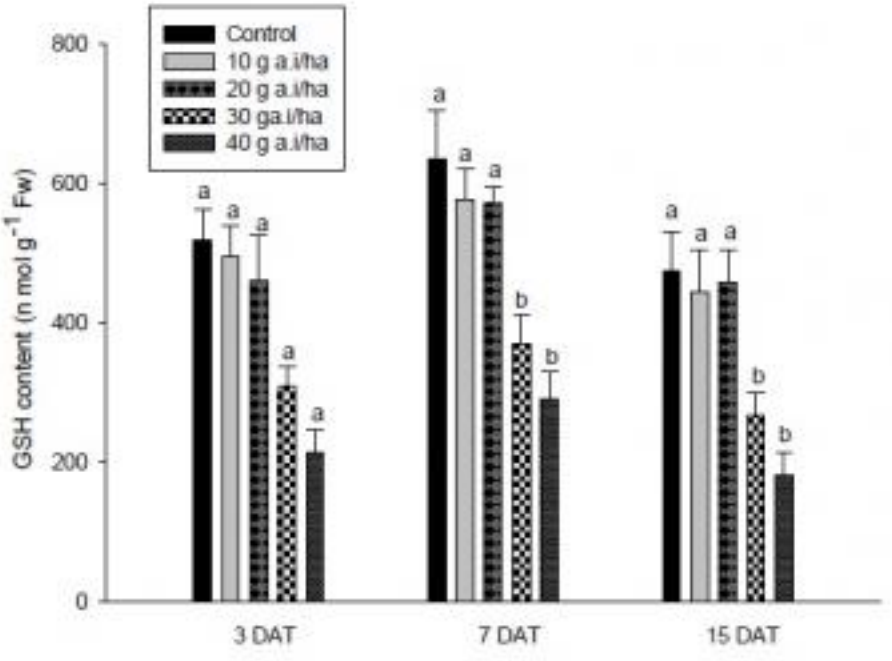
Control
10 g a.i/ha
20 g a.i/ha
30 ga.i/ha
40 g a.i/ha
GSH content (n mol g-1 Fw)
800
600
400
200
0
a
a
a
a
a
a
a
a
a
b
b
a
a
a
b
b
3 DAT
7 DAT
15 DAT

Fig 8A

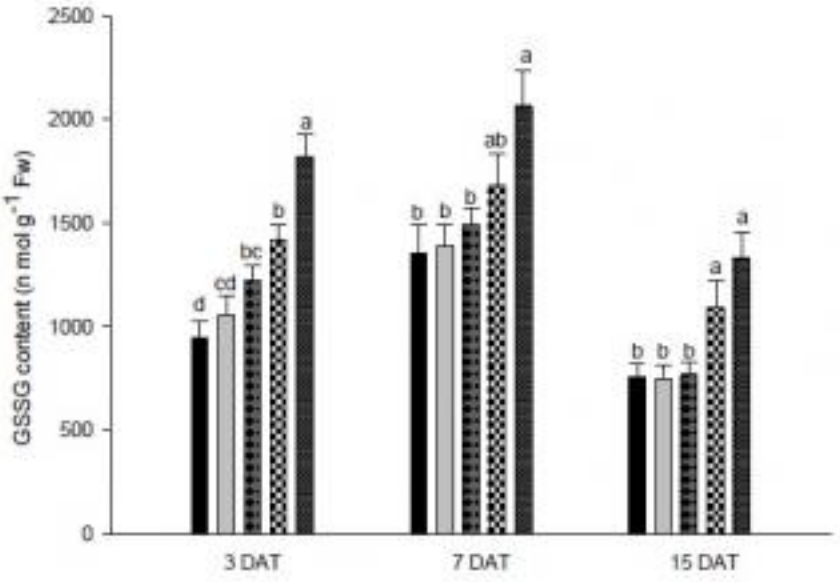

Fig 8B

Fig 8 Variation in glutathione content at various growth stages of *Brassica juncea* L. treated with different concentration of imidacloprid. **(A)** reduced GSH content (n mol g^{-1} FW) and **(B)** oxidised 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)

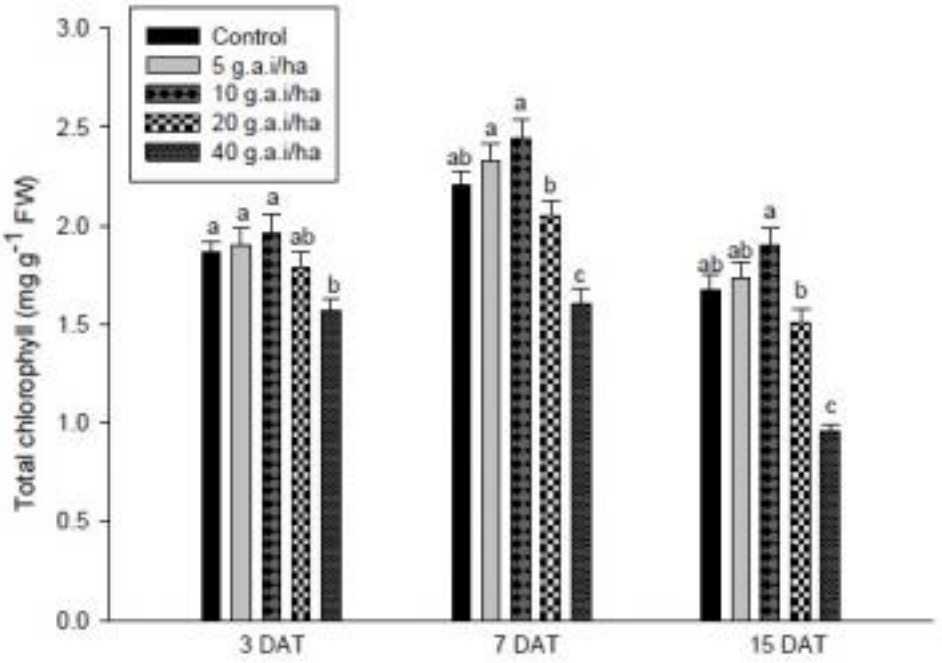
Control
5 g.a.i/ha
10 g.a.i/ha
20 g.a.i/ha
40 g.a.i/ha
Total chlorophyll (mg g-1 FW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
ab
b
ab
a
a
b
c
ab
ab
a
b
c
3 DAT
7 DAT
15 DAT

Fig 9A

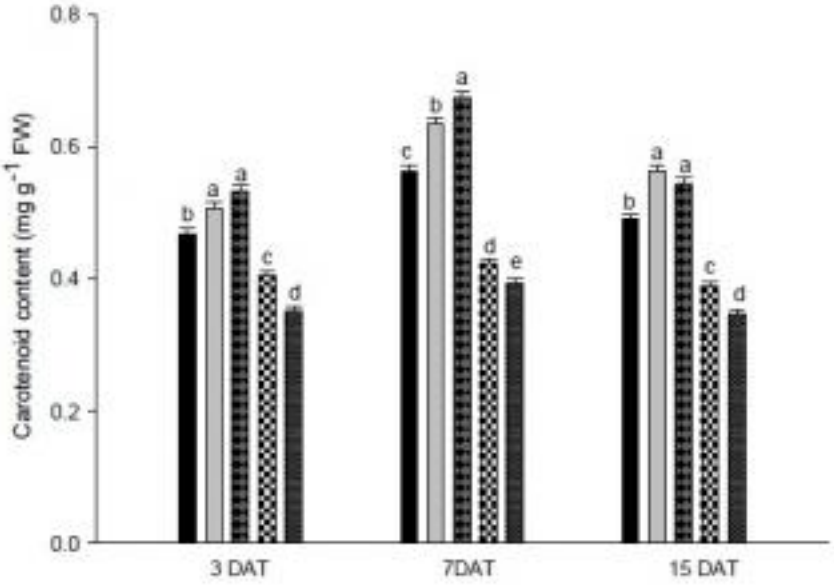

Fig-9B

Fig 9 Effect of different concentrations of imidacloprid on photosynthetic pigments of *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)