Chapter 5

Discussion
Ecosystems naturally consist of 3-5 trophic levels with chlorophyll bearing green plants at base constituting 1st trophic level. The herbivores consume plant parts and rebuild animal proteins form 2nd trophic level. The carnivores constitute 3rd to 5th trophic levels. In all ecosystems, the partitioning of energy from 1st to highest trophic level is naturally balanced. The highly evolved process of checks and balances in the grazing food-chain maintains a very delicate balance between the organisms at each trophic level and thus make the entire ecosystem self sufficient. An understanding of the key processes of checks and balances in energy (food) transfer may be of immense importance and use in agriculture and several other human welfares including ecology and environment. The aim of the present study was to understand various defense traits evolved and inherited by the plants and extent of counter offensive mechanisms in herbivores and their carnivores. In the present study, the defensive traits evolved and inherited by plant, their impacts on herbivory and predation; effects of herbivory on plant growth and population growth of herbivore and its predators have been studied. For this purpose, linear food-chain model of mustard-aphid-beetle (producer-herbivore-predator) was studied through five experiments.

The first experiment was conducted to study the relative sensitivity of five cultivars of mustard to herbivory by 40 aphids. The selected cultivars were grown in pots and at 45 days after sowing (DAS), each replicate plant was exposed to 40 aphids \( (Lipaphis erisimi) \). The growth responses of all the five cultivars were studied and one least and one most sensitive cultivars among the five were screened. The plants are directly exposed to environmental stresses including biotic stress caused by herbivores. The host plants have evolved several defense strategies including production and release of certain volatile chemicals with deterring or phytotoxic effects against aphids and to inviting signal the predatory beetles. All these traits are not equally evolved in plants. The types and levels of constitutive and induced defenses differ species to species and cultivar to cultivar and accordingly plants differ in growth responses to herbivory as well. Before proceeding for detailed studies on defense signaling and responses to herbivory, it was deemed fit to determine the least susceptible and most susceptible cultivar of \( Brassica juncea \) (referred as mustard) to
aphids (*Lipaphis erysimi*). It emerged from this experiment, that cultivar Alankar was relatively least susceptible to aphid herbivory and feasibly had better inherited traits of defenses than most sensitive cultivar Rohini.

Aphids are considered successful herbivore with soft bodies, membranous wings and a diet comprised of phloem sap (Dixon, 1998). In the present study, growth of plant (plant length, leaf area, fresh plant mass, dry plant mass, chlorophyll content and protein content) decreased in proportion to the increase in aphid population in all the five selected cultivars (Fig.2-12, and 14). Defense proteins increase resistance in plants (Elzinga and Jander, 2013). The protein contents were higher in cv. Alankar than in cv. Rohini. The growth attributes had a high degree of correlation with the aphid population (Fig. LR I and II). Aphids are phloem suckers and their water as well as nutrient requirements are fulfilled through the consumed phloem sap. The direct intake of phloem sap increase osmotic potential in aphids’ gut and to compensate it, aphid sucks water from the xylem tissues (Spiller et al., 1990; Walling, 2008). The cultivars with high population densities of aphids partitioned proportionately high quantities of phloem sap (water and photosynthates) to herbivorous aphids as is evident from correlation studies (Fig. LR I and II). On aphid attack, the selected cultivars suffered from abundant availability of photosynthates for cell division and adequate water for the turgor pressure required during cell expansion. The correlation coefficients and linear regression between per cent reduction in fresh plant mass (Fig. LR I and II) and per cent increase in aphid population (Fig. 1) explicitly explains the loss of water from plant tissues was due to consumption by aphids and thereby caused stress in plant tissues (Khattab, 2007; Sadek et al., 2013). It is also evident from the excess accumulation of proline that plant has suffered from water stress. Therefore, proline besides playing important role in plant defenses, also managed the osmotic imbalance in mustard (Oncel et al., 1996). The correlation coefficients between plant dry mass (Fig. LR I and II) and increase in aphid population established that growing aphid population consumed excessive quantity of photosynthates and this loss of carbon may have suppressed cell division in root and shoot. Walling (2000) reported that phloem sap herbivory adversely affected the plant productivity (Bak et al., 2013; Sadek et al., 2013).

The presumption that phloem sap directly consumed by aphids caused water stress in the plant tissues is further supported from high degrees of correlation
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Proline content and aphid population in varying cultivars (LR I and II). The proline in water stressed conditions acts as water stress adjuster in plants (Khattab, 2007). The photosynthetic pigments, leaves and plant mass in both selected cultivars were more severely affected than roots. It may be due to severe cell damage caused by aphid in above ground leaves, stem and inflorescence. The minute loss to roots may have been due altered carbon partitioning and loss of carbon in above ground plant parts. Aphid infestation reduced photosynthetic pigments level (Fig. 9-12), proteins (Fig. 14) and other growth attributes (Fig. 2-8) in all the cultivars of *Brassica juncea* (El-Khawas and El-Khawas, 2008). This finding is in agreement with the earlier report that insect infestations in wheat crop inhibited chlorophyll biosynthesis (Heng-Moss et al., 2003).

Usually, dose-response variables are in common practice of correlation studies. The responses of one species to varying doses of aphids establish relationship at individual species level only. The cultivars of any single species differ in the inherited traits for resistance, defensive strategies, growth and yield potentialities. The treatment of five cultivars with 40 aphids per plant and correlation of cultivar-response variables establish a community level response.

In this experiment, growth responses of the two selected mustard cultivars (Alankar and Rohini) to varying quantities of aphids (50, 100, and 150 aphid per plant) were studied. The reduction in shoot and root length, leaf number and area of both the cultivars increased with the number of aphids (Fig. 15, 16, 17, 18). The reduction in growth parameters was higher in cv. Rohini than in cv. Alankar. It is reported that, aphid devitalized the crop by sucking the cell sap (Bakhetia 1991, Atri et al. 2012). In the present study, the fresh mass reduced at every aphid infestation level. The reduction in fresh and dry mass was higher on infestation with 150 aphids per plant (Fig. 19, 20). It is evident from the Fig. 43 that the population of aphid multiplied more quickly on Rohini than in Alankar. The aphid count corresponded with the initial number of aphids inoculated. The aphids are phloem sucker and directly consumed photosynthates. The concentration of carbohydrates in the phloem sap create an osmotic imbalance in the aphid gut (Walling, 2008). To avoid dehydration, aphids maintain water balance by occasional feeding through the xylem (Spiller et al., 1990). It is evident from the finding on fresh mass and dry mass of both the cultivars at selected aphid infestation levels. The fresh mass in the plant is due to
the water content and dry mass is the water organic matter. The reduction in both fresh and dry mass indicates the loss of carbohydrate and water from the tissues in proportion to aphid numbers. The higher degree of weight loss in Rohini than in Alankar appears to be due to the difference in inherited defense traits (Fig. 19, 20). The proline level increased in both the cultivars in proportion to the number of aphid inoculated (Fig. 27).

The proline is a universal stress marker in plants (Oncel, 1996) and hence increased with the infestation level. Similar increase in free proline has been reported by (Khattab, 2005, 2007). In cabbage and eucalyptus leaves infested with aphid and therefore the proline accumulation was considered as biotic stress marker. The proline accumulation is speculated to play a defensive role (Kuznetsov and Shevyakova, 1997). The greater amount of proline in cv. Alankar than in cv. Rohini indicates that the former cultivars had some inheritance resistant traits than the later cultivars.

The nitrogen (N) and phosphorus (P) content also decreased in proportion to the number of aphid inoculated (Fig. 28, 29). The insect feeding alters translocation pattern and growth of host plant (Miles, 1999) and reduced the nutrients uptake form the root (Wu et al., 2004). In the present study the N content decreased significantly with increase infestation level in both the cultivars (Fig. 28). It is reported that N enhance the reproductive capacity and resistance against insect damage (Forno and Semple, 1987). From the linear regression analysis it is evident that N content had strong negative relationship with the aphid infestation level in both cultivars (Fig. LR III and IV).

The reduction in nutrient contents due to aphid infestation many have directly affected the chlorophyll a, b, total chlorophyll and carotenoid content (Fig. 21-24). The loss of chlorophyll may have been caused by consumption of nutrients by the aphid, as pigment biosynthesis is dependent upon water and minerals. The aphid infestation reduced stomatal number in both the cultivars (Fig. 31, 32). The relative stomata closure index (RSCI) increased with the aphid infestation level but more prominently in cultivar Rohini (Fig. 33, 34).

In the present study, aphids caused severe damages of wax layer deposited on the outer surface of leaf epidermal cuticle (Plate 7). The cell membrane around the area of injury is also affected adversely. The loss of membrane permeability results
Plate 7. Features of leaf surface with stomata under 10×10x light microscope (A1-G). Details of leaf surface scanning electron microscopic (SEM) image (A3, B3, C3, D3). (A1-A3) open stomata in non-infested leaves of Alankar (control), (B1-B3) partially closed stomata without much deformation of wax layer on herbivory with 150 aphids per plant, (C1-C3) the damaged stomata wax layer in cv. Rohini infected with 150 aphids, (D1-D3) highly damaged wax layer and almost deformed and dissolved stomata in cv. Rohini infected with 150 aphids, (E1) Relatively intact sieve tube cells in the leaf veins of cv. Alankar treated with 150 aphid, (F) damaged sieve tube elements in the leaf vein of cv. Rohini treated with 150 aphids, (G) highly damaged guard cells and wax layer in cv. Rohini infected with 150 aphids.
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into the spill of cell water and vital contents (Walling, 2000; Louis and Shah, 2013). The hypertrophy noted in the mustard pods (Plate 3, 7) may have been caused severe cell damages in developing pods. Despite partial or complete closure of stomata, the fresh mass of plant also decreased on various level of aphid infestation (Fig. 19). This indicate that the reduction in fresh mass of plant was due to the consumption of water from the xylem in addition to photosynthates as evident from loss of plant dry mass as well (Fig. 20). The partially closed stomata as noted in aphid infested mustard plants (Plate 5) may have not checked transpiration completely, instead reduced the stomata conductance (Fig. 36) and hence affected the photosynthetic rate (Fig. 35). The reduction in pod length, oil content as well as pod per plant and seed weight in proportion to the aphid infestation level appears to be the outcome of reduction in photosynthetic rate and consumption of phloem sap by the aphid in proportion to their population (Fig. 37-42).

From the experiment, it is inferred that both the selected cultivars had difference in their inherited defensive traits. The increase in the degree of loss in various growth parameters increased with the infestation level and growth stage in both the cultivars and had a direct relationship with the aphid numbers. The relationship between the loss of selected growth parameters and aphid number is evident from linear regression analysis (Fig. LR III and IV). There was a strong correlation between the losses of net photosynthesis with respect to aphid infestation level. The strong and positive correlation coefficients were recorded between proline level and aphid infestation level in both the cultivars (Fig. LR III and IV).

In the third experiment, the selected cultivars (Alankar and Rohini) of Brassica juncea were exposed to varying number of aphid with equal degree of predation (2 beetles per plant). Almost all the selected parameters showed reductions in proportion to number of aphids inoculated. The cultivar Rohini remained more susceptible than cultivar Alankar despite the predation of aphids by beetles. The decrease in fresh and dry mass caused by aphid infestation adversely affected the plant development in terms of shoot and root length, leaf number and area. (Fig. 45-48). The loss in dry mass corresponding to number of aphid feeding on the plant indicated that larger number of aphid neutralized the defensive responses of host plants and limited population of beetles may have not been able to predate excessively on multiplying aphids. The aphids, besides gelling saliva also secrete watery saliva
Discussion: After piercing stylet into the plant tissues (Bak et al., 2013). The watery saliva plays important role in testing the suitability of the cell sap of the host plant (Bak et al., 2013). Recent analysis of saliva protein reflected that some of the proteins known as elicitors trigger the plant defense responses while some other counteract the plant defense as affector molecules. Some of these molecules might be species specific and some other have broader specificity (Bos et al., 2010; Tian et al., 2012; Pitino and Hogenhout, 2013; Bak et al., 2013). It is likely that cultivar level variations in such inherited defensive traits may have caused varying levels of damages in the two selected cultivars. In the present study the degree of losses in the various growth and biochemical parameters were lesser on infestation with 50 aphids (Fig. 49-66). This indicate that the selected cultivars of mustard had limited constitutive and induced defensive mechanisms against aphids in the presence of only two predatory beetles (Coccinella septempunctata). The higher number of aphids could damage the plant to a greater extent. It is also reported that the plant degradation products are recognized by plant receptors during aphid herbivory; however, certain effectors from aphid saliva neutralize plant defense responses to re-established plant susceptibility (Liu et al., 2009; Pitino and Hogenhout, 2013; Bak et al., 2013). This immunity to plant defense caused by aphid saliva proteins acting as effectors in the larger number of attacking aphids enabling them to successfully consume greater amounts of carbon to substantially reduce plant growth at higher level of aphid infestation (Jones and Dangl, 2006; Liu et al., 2009; Bak et al., 2013). Despite equal level of predation the cultivar Rohini remained more susceptible to aphid infestation than Alankar. It is reported that Orthopod feeding change plant metabolism and gene expression associated with plant defense responses and aphid resistance gene (Moran and Thompson, 2001). It is evident from the data on aphid count on both the cultivars that population of aphid consistently increased with age of the plant (Fig. 73). The large aphid population on cultivar Rohini than cultivar Alankar may have been due to gene level variations between them.

It may be noted that the limited number of beetles (2 beetles per plant) could reduced the aphid population to a limited extent as compare to the infestation by aphid without beetles in Experiment 2 and eventually the growth and yield of selected cultivars (Fig 49-72). On comparison of results of the Experiment 2 and 3, the growth of both cultivars was relatively better under predation by beetles (Experiment 3) than
Discussion: without predation (Experiment 2). The beetles also reduced the aphid population in Experiment-3, but insufficient number of beetles and possible reversal of plant immune and defensive system by larger number of aphids reduced the growth of mustard in Experiment 3.

The selected cultivars (Alankar and Rohini were treated exogenously) with varying doses of jasmonic acid (JA) to study the effect on the growth, physiology and yield as well as extent of inviting signal to beetles. The JA application of 0.1 mM enhanced the growth and yield of both the cultivars (Fig. 74-101). Jasmonic acid is a key molecule of octadecanoid signaling pathway (Meyer et al., 1984, 2003, Markunas et al., 2011, Nabity et al., 2013) which strongly stimulates photosynthetic pigment synthesis (Poonam et al., 2013). It also protects the cell membrane in accordance with the dose of JA (Poonam et al., 2013). Jasmonate treatment reduces the proline level in the plants as compare to the aphid attacked ones (with or without predation). This indicates that JA itself reduced plant stress, thereby; proline did not accumulated in larger proportions in JA treated plants. Since proline is considered as a stress marker (Oneel, 1996), reduced proline accumulation indicated that JA treatment stimulated and protected the cellular stress and relatively healthy cells in selected cultivars defended the plants better. In earlier studies JA treated stressed and non stressed plant had mixed response on accumulation of proline (Poonam et al., 2013, Jamalomidii et al., 2013). The increased chlorophyll content in JA treated plants, increased plant growth specifically the fresh and dry mass (Fig. 80-95). The JA treated cultivars Alankar and Rohini attracted larger number of beetles in the present experiment at 60 DAS than at 75 DAS (Fig. 102). This indicates that 1.0 mM of JA signaled beetles very effectively only at 60 DAS and relatively to a lesser extent at 75 DAS as effect of this volatile chemical reduced with time. The JA is reported to play on important role in aphid resistance as well (Smith and Boyko, 2007, Morkunas et al., 2011). The JA treatment enhanced the plant’s immunity to aphid attack. It may be noted that JA application reduced the aphid reproduction in an earlier study (Zhu-Salzman et al., 2004).

In the fifth experiment, both the cultivars (Alakar and Rohini) pretreated with 0.1mM Jasmonic acid (JA) at 45 DAS were inoculated with 50, 100, and 150 aphid per plant at 50 DAS, and the growth performance was studied. On pre-treatment with JA, the plant growth enhanced far better than without JA treatment (Experiment 2).
The inoculations of aphids on JA pre-treated cultivars reduced shoot and root length to a lesser extent than in Experiment 2 (Fig. 103-104). The population of aphid on JA pre-treated cultivars was also lesser than without JA treated plant but exposed to beetles as recorded in Experiment-3. The population of aphid on pre-treated (JA 0.1 mM) plants was relatively lesser than in plants without JA. From this experiment (Fig. 129), it is evident that exogenous application of 1.0 mM of JA detracted the aphids due to adverse impact on their reproductive abilities (Zhu- Salzman et al., 2004) and substantial attraction of beetles had added advantages to safely predate on them before the aphids could introduce defensive chemicals in them. The JA also provided a cue to predators more effectively in presence of aphid.

The defensive volatiles synthesized and released by leaves of cultivar Alankar inoculated with 150 aphids per plant was studied in GC-MS analysis. The profile reflected that the content of total allyl-isothiocynate (AITC) was only 42.36% and 3-hexan-1-ol was 10.34%. When this cultivar was treated with 1.0 mM of JA prior to inoculation of 150 aphids, the contents of both these defensive and signaling volatiles increased. The AITC increased up to 46.59% and 3-hexan-1-ol up to 20.84% (Fig. 133). These findings firmly suggest that the exogenous application of JA on mustard plants improved plant’s ability to defend the aphid herbivory as AITC directly deter the aphids and JA spray worked as volatile signal to attract predatory beetles. The induced defenses in many plant species through volatile chemical arsenals have been reported earlier also (Lambrix et al., 2001; Kliebenstein et al., 2002; Pontoppidan et al., 2005; Kissen et al., 2009).

The overall outcome of the present studies (Experiment 1-5) has been explained through the Plate 8. The aphid attack is more common on mustard cultivars with least inherited defensive traits. The plants have evolved constitutive and induced defensive traits against herbivores. The herbivores in turn have co-evolved counter defenses against the defensive plant volatiles and thus developed the ability to alter certain blends of plant volatiles in their favor. Herbivores often alter the plant volatile signaling specialist predators or parasites of the attacking herbivore (Bak et al., 2013). In the present study it is evident that the selected aphid damaged the wax layer and feasibly secreted enzyme pectinase, dissolved the middle lamella between epidermal cells and subsequently the middle lamella of cells/tissues in leaf interior (mesophylls, cortex in stem, phloem cells, primary xylem cells in the vascular system of leaf and
Plate 8. Tri-trophic interaction of *Brassica juncea* – *Lipaphis erysimi* - *Coccinella septempunctata* (mustard plant – aphid – beetle). Attack of aphid on mustard plant results into loss of phloem and low of nutritional quality. However, this (A) led to release of volatile signals which attracts beetles (B1) which feeds on aphid to check their population (C1). Aphid feeding also induces octadecanoid pathway to release jasmonic acid (B2). Elevated level of jasmonic acid closes stomata (D2) to decrease photosynthesis (E2) further reduces nutritional quality (F2). Alternatively, pre-infestation external application of jasmonic acid (blue arrows) induces glucosinolates (B'2) which on infection quickly releases allyl isothiocyanates to deter aphids (C'2).
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The aphids thus easily enter their stylets through the macerated cells and immediately secrete the gelling saliva and fill in the injured tissues around stylet probing sites. The gelling saliva secures the stylets. The purple brown pigmenting substance filled in the injured portions of leaf and stem has been detected in the present study and shown in the plates (Plate 3). The probing aphid first tastes the contents of the cell sap by injecting watery saliva inside the cell and withdrawing the cell sap. On judging the suitability of cell sap, aphids enter the probing stylet into sieve tube and suck in the photosynthates synthesized in the leaves and unloaded in the phloem. The excessive sugar intake increases osmotic potential in the aphid guts. To nullify this effect, aphids enter their stylets into the xylem cell and withdraw water. The mustard cultivars with better defensive traits (a set of few genes) activate the sequestering several blends of volatiles. Some volatiles deterred the aphids and some other signalled the predatory beetles of third trophic levels for indirect control. The cycles of deterring and predator cueing volatiles are shown in the Plate 8. Certain blends of volatiles are known to signal the specialist predator about the suitability of egg laying sites. In the present study, the specialist beetle (Coccinella septempunctata) preferred to maintain its life cycle on cultivar Alankar pre-treated with 1.0 Mm of JA (Plate-6).

Conclusion

Present study draws following conclusions to answer the objectives framed (see Chapter 1) for the conducted experiments

1. The selected mustard cultivars (Alankar, Pusa Jai Kisan, Varuna, Sakha and Rohini) responded differently against selected aphid infestation (40 aphids per plant) at the two stages of growth. Alankar stood least sensitive against aphid herbivory amongst all the five mustard cultivars whereas the response of Rohini was most susceptible.

2. The number of aphids increased linearly once their selected numbers were inoculated on young leaves of plant, which caused increasing damage with the growth progression (60 to 75 DAS).

3. The herbivory damage increased from 60 to 75 DAS which was more pronounced in cv. Rohini as compared to cv. Alankar. Moreover, stress
progressively increased with increasing the level of aphid infestation (0-150 aphids).

4. Inoculation of predatory beetle controlled the aphid population which was observed as recovery of selected parameters as compared to aphid infestation alone. The damage declined with the age progression (60 to 75 DAS).

5. Amongst the three concentration of jasmonic acid (0.5, 1.0 or 1.5 mM) the response of 1.0 mM was most effective which furnished positive responses of selected parameters at the two stages of growth.

6. Pre-treatment (aphid pre-infestation spray) with jasmonic acid with JA solution effectively controlled the aphid infestation which resulted in better growth performance of cultivars as compared to aphid stressed plants, more at late stage of growth (75 DAS) than at early stage (60 DAS). Also the yield improved with the simulated jasmonic acid application.

7. Marked increase of proline in Alankar (least sensitive cv.) as compared to Rohini (most susceptible cv.) suggested its key role in protecting plants against herbivory induced damage.

8. Jasmonic acid (1.0 mM) application induced the proline level further confirms its protective role in herbivory induced plant growth regression.

9. Detection of higher level of allyl isothiocyanate (AITC) and 3-hexanal-1-ol in plants treated with JA pre-infestation to aphids as compared to aphid infestation alone confirms the applicability of JA against herbivores through the induction of direct and indirect defense.