शीर्षक: ब्रासिका की जंगली प्रजातियों में अंतर एफिड्स संक्रमण के आणविक और जैव रासायनिक आधार

Molecular and biochemical basis of differential aphid infestation in wild species of *Brassica*

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Molecular and biochemical basis of differential aphid infestation in wild species of *Brassica*

By

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CERTIFICATE

This is to certify that the thesis entitled **"Molecular and Biochemical basis of differential aphid infestation in wild species of *Brassica*"** submitted to the Faculty of the Post-Graduate School, **Indian Agricultural Research Institute, New Delhi**, in partial fulfillment of the requirements for the degree of **Master of Science** in **Molecular Biology and Biotechnology**, embodies the results of bonafide research work carried out by **Mr. Naresh Kumar Samal, Roll No. 20997** under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help availed during the course of investigation as well as source of information have been duly acknowledged.

Place: New Delhi
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(Dr. Ramcharan Bhattacharya)
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DEDICATED

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1. INTRODUCTION

The genus *Brassica* belongs to the family Brassicaceae which includes 51 genera. *Brassica* species constitute a group of oilseed and vegetable crops that are cultivated worldwide. Rapeseed-mustard, the group of oilseed crops in *Brassica* represents the second most important oilseed crop in the world next to only soybean. Annual worldwide production of *Brassica* oilseeds exceeds 75 million tons (2016-17, FOP, 04-11, USDA). In the year 2016-17, rapeseed-mustard contributed 71.28 million metric tons amounting 12.31% of the total oilseed production of 575.29 million metric tons (source: Foreign Agricultural Service, Circular Series FOP 04–11, USDA). India is one of the leading producers of *Brassica* oilseeds along with Canada, USA, EU, Australia and China. In India the acreage under rapeseed-mustard is 6.412 mha with a total production of 6.33 mt (source: Solvent Extraction Association of India https://www.business-standard.com) which amounts to the average productivity of 6979 kg/ha. Among the several species of *Brassica* in rape-mustard group of crops, Indian mustard (*Brassica juncea*) fits well in cropping system of rain fed areas and accounts for >75% of the total area under rapeseed-mustard cultivation in India.

The productivity of mustard is crippled with several biotic and abiotic stresses. Among the biotic stresses, damage caused by mustard aphids (*Lipaphis erysimi*) is the most economically significant. Aphids belong to family *Aphididae* and order Hemiptera. These are small soft bodied insect-pests, with colour ranging from light yellow to dark grey. Aphids are found in colonies and occasionally possess wings. Aphid feed on the plants by extracting the sugary liquid straight from the phloem through their hardened stylet. During feeding aphids also transmits many plant viruses, and thereby acts as a potential virus transmitting agent. Prolific parthenogenetic reproduction and short generation time allow aphids to cover the whole plant in small time causing significant phloem sap diversion. The various taxonomic groups of aphids have been found capable of causing damage to crop yield ranging from 9- 91 per cent (Singh and Sachan 1994).

There are several control measures available to prevent crop damage due to aphid infestation. These control measures can be categorised as agronomical, physical, biological and chemical methods. Physical methods of aphid control involve use of sticky traps and other methods to restrict the spread of aphids to floral parts. Biological methods involve introduction of natural parasites or competitors of aphids which reduce the aphid population by feeding on aphids. However, these methods are insignificant. The most prevalent way of aphid control is through application of systemic insecticides.

The chemical control method has various shortcomings as the insecticides are toxic and environmentally hazardous. These chemicals lead to potential risk to cattle and poultry industry also as the oil cakes are used to feed the cattle and poultry birds (Singh 2001, Singh and Sharma 2002). Therefore, it becomes imperative to develop pest management tactics that will provide effective and economical control of the pest without any adverse effect on the environment. In this context, developing genetic resistance in the host plant is important. However, development of an insect resistant cultivar is a long process. The first requirement in the development of insect resistant cultivar is precise knowledge of possible sources of resistance (Stoner and Shelton 1988). The plant resistance to insects may be caused by antixenosis, antibiosis or tolerance or a combination of these (Painter 1951, Kogan and Ortman 1978). The combination of these mechanisms can increase the effective life of an insect resistant cultivar. At the same time combination decreases the likelihood of a pest to overcome the resistance as long as alternative sources of preferred hosts are available (Gould 1984). Breeding for genetic resistance against aphids has not been possible owing to the non-availability of resistance source(s) within the crossable germplasms and lack of knowledge of the genetics of the trait (Bhatia et al. 2011).

Wild relatives of cultivated species have been attractive sources of resistance genes in many instances. The wild relatives of *Brassica* can serve as potential source of resistance which can be subsequently brought in to the cultivated species through breeding methods. The family Brassicaceae has a total of 349 genera, and the genus *Brassica* has about 37 species. These large numbers of genus and species under the Brassicaceae family forms a huge pool of wild relatives for the cultivated species *B. juncea*. This large reservoir of wild relatives has never been systematically explored for possible source of aphid resistance. If a potential source of resistance is identified even within a wild relative which is not traditionally crossable with *B. juncea*, there could be ways and means for introgressing those useful genes into the primary germplasms of *B. juncea*.

Therefore, the following objectives were undertaken in the present investigation with an aim to identify wild relatives of *B. juncea* with genetic resistance to mustard aphids.

Objectives:

I. Screening for aphid resistance in wild germplasms of *Brassica*

II. Biochemical and gene-expression analysis of defense related genes and enzymes in selected wild germplasms showing resistance to aphid infestation

2. REVIEW OF LITERATURE

2.1 *Brassica* crops

Brassica crops are angiosperm plants and are placed under Brassicaceae family. *Brassica* crops are grown worldwide as a source of edible oil or vegetables. *Brassica* crops are also being used as green manure crops, biofuel crops and bio fumigant crops. The domestication of *Brassica* crops can be traced back to 6th century BCE to 4th A.D in various Greek and Latin literatures. (Maggioni et al. 2010). In Indian scenario, *Brassica* crops have a long tradition and believed to be 1st cultivated by Aryans in 1800 BCE. In 2017-18, the worldwide production of rapeseed-mustard was 74.67 million metric tons contributing 12.9% of total oilseed production which is 578.41million metric tons. In India annual production of rapeseed-mustard contribute 6.33 mt out of total 35.41mt oilseed (Foreign Agricultural service, circular series 2019).

2.2 Biotic stresses on *Brassica* crops

Despite being the 3rd largest producer (11.3%) of oil seed crops next to Canada and China, India has become 7th largest importer of edible oil. Substantial crop loss due to various biotic and abiotic stresses has restricted the productivity of rapeseed-mustard. The present productivity can meet only 57% of domestic edible oil requirement and rest of the demand is complemented by import from neighbouring countries (Singh et al. 2019).

Among the various factors inflicting crop losses, crop loss due to aphid infestation is a major concern and it alone account for 9-96% in a year. It is imperative to minimise the yield loss in order to enhance the productivity and self-sufficiency in edible oils. In this regard for controlling aphid infestation there has been less progress either in creation of resistance varieties or use of eco-friendly pesticides having less detrimental effect towards environment. Also, use of natural predators for aphid control has seldom showed promising results.

2.3 Aphids

Aphids are soft bodied slender organisms often classified in *Aphididae* family and order Hemiptera. Aphids feed on the host plants by inserting their hardened stylet into the phloem column and suck the phloem sap from it. A short lifespan and high fecundity rate enable them to multiply quickly on plants. A small handful initial population can rapidly colonise the whole plant especially if the environments are congenial. Aphids can cause a damage ranging from 9-91% (Singh and Sachan 1994). The range of damage caused by aphids is influenced by growth of crop during infestation, duration and intensity of infestation but the greatest damages are caused if the plants are infested during flowering and pod filling stage.

2.4 Agriculturally important genera of aphids

Aphids has emerged as serious insect-pests of several agriculturally important crops throughout the world (Bhatia et al. 2011). The 1st emergence of aphids on earth is speculated to be in early Permian era some 200 million years ago. There are about 4000 species of aphids reported out of which 250 species constitute damage to agricultural crops. Most of the damage causing aphids are classified to 13 genera. Oil seed *Brassica* crops are infested by green peach aphid (*Myzus persicae S*), cabbage aphid (*Brevicoryne brassicae* L) and turnip aphids (*Lipaphis erisimi Kalt*). Indian mustard (*B. juncea*) is predominantly affected by *L.erysimi*. The winter conditions, October to March are congenial for *L. erysimi* under Indian condition.

2.5 Life cycle and reproduction of aphids

Two distinct life cycles are exhibited by major taxas of aphid, holocyclic and anholocyclic life cycle (Agarwala 2007). Both life cycles are exhibited at the same time at different geographical locations by aphids showing cosmopolitan distribution. *Myzus persicae* overwinters as eggs in primary hosts during holocyclic cycle during winter time. During spring or summer, the winged stem mother return to secondary host and multiplies into apterous vivipary and alate vivipary (Moran 1992). The apterous vivipary female are termed as “the multiplier” produces numerous offspring’s by parthenogenesis in a very short span of time. During the winter time some apterous vivipary turns into apterous ovipary and alate vivipary into alate male. Following sexual reproduction, the mated females return to secondary host to lay fertilised eggs (Stern 1995). Following spring, the eggs are hatched to produce stem mother and begin parthenogenesis again. In areas having mild winter there is no overwintering and parthenogenesis is seen throughout the year. Reproductive habit of aphids exhibits an alternation of generation from a bisexual generation to parthenogenesis associated viviparity leading to telescoping one generation into another. It essentially reduces the generation time of aphid. Parthenogenesis combined with telescoping of generation provide aphid an exceedingly rapid turnover of generation which enable them to multiply very quickly (Bhatia et al. 2011).

2.6 Feeding mechanism of aphids and evasion of host defense

Aphids and some other homopteran insect inject their stylet into phloem sieve element and feed on it delicately without killing the cell. Plants secrete “P” proteins and callose as a wound response in the phloem. P proteins and callose forms coagulation in the sieve element and prevent phloem sap loss. In order to suck sap from phloem, breaking of wound response is necessary. Aphids are able to break the wound response by injecting watery saliva before they actually start sucking phloem sap. The exact mechanism of interaction is not known. However, certain biochemical interactions happen between the aphid ingested saliva and plant secreted P proteins as well as callose that prevent their coagulation, thereby keeping the perforation open in phloem for uptake for hours (Tjallingii et al. 2005). In comparison to chewing insects, the sap sucking insects inflict less mechanical damage as they insert their stylet through epidermal and parenchymal cells to reach the sieve element as a result the plant often fails to perceive the damage. Additionally, the aphid saliva contains reducing compounds which interact with the phytochemicals secreted as wound response by plant and prevent their action by inactivating them (Miles et al. 1999). In another mechanism, the calcium containing proteins in aphid saliva interact with the calcium of plant tissues and prevent it triggering calcium mediated defence mechanism (Will et al, 2007). Feeding habits of aphids are very precise and selective. They selectively avoid plants containing allelochemicals and indigestible compounds (Shoonhoven et al. 2007).

2.7 Endosymbiont in aphids

Aphids feed on the phloem sap that they divert from the host plant. Although phloem saps are very nutrient rich food for animals but their ingestion as food poses two major nutritional barriers. Nitrogen barrier and sugar barriers (Douglas et al. 1999). The nitrogen barrier to phloem sap utilization is due to low availability of most of the essential amino acids, which are crucial for protein synthesis. Sugar barrier to phloem sap utilization is caused by the high concentration of phloem sap due to presence of sugar alcohols such as mannitol, sorbitol and raffinose series such as stachyose and raffinoses (Ziegler et al. 1975). The high concentration of phloem sap often exerts a high osmotic pressure 2-5 time greater than the insect body. Ingestion of such a high osmotic pressure sap can cause transfer of body fluids and osmotic collapse of insect.

To overcome the nitrogen barrier the aphids, harbour a group of symbiotic bacteria called *Buchnera sp*. These obligate endosymbiont bacteria are present inside the cytoplasm of a specialised cell called bacteriocytes in the aphid hemocoel. The *Buchnera sp*. has a small genome of 64MB and it contains most of its genes committed to synthesis of essential amino acids (Shigenobu et al. 2000). These bacteria are maternally inherited to the next generation and play a key role in the genetic differentiation, evolution of host-plant specialization and further speciation (Leonardo et al. 2006). To overcome the sugar barrier, aphids exhibit higher sucrase activity in their guts. (Ashford et al. 2000). It is proposed that the breakdown of sucrose into oligosaccharides reduces the osmotic pressure of ingested foods, in addition the trans-glucosidation activity of sucrase enzyme which produces oligosaccharides from other sugars and further decreases the osmotic pressure in phloem sap (Walter et al. 1988).

2.8 Chemical control measures against aphid infestation

For most of the crops the host-pathogen resistance against aphids are historically unavailable. Therefore, integrated pest management mostly relies on agrochemical-based control and biological control or a combination of both.

Agrochemical based approach is the most widely used aphid control system adopted by crop growers across the world. Both contact as well as systematic insecticides are used for aphid control. However, aphid control using contact insecticides is less effective as aphids feed directly from phloem sap rather from abaxial leaf surfaces. Systemic insecticides are absorbed by plant and integrated into phloem sap and kill aphids. Although use of systemic insecticides is quite efficient in controlling aphid infestation but they do suffer from many draw backs. One of the major drawbacks of using systemic insecticides is their persistence in plant parts and bio-magnification which could ultimately enter into human food chain. Beside residual effects, indiscriminate use of insecticides leads to development of resistance in strains of aphids against organo phosphates (Sudderuddin 1973). Major groups of chemicals used as systemic insecticides include carbamates, organo-phosphates, pyrethroids, and cyclodienes (Bahlai et al. 2010, Cameron and Fletcher 2005). Residual effects of insecticides and their detrimental effect on beneficial organisms such as pollinators, nutrient cyclers and natural pest controlling agents can be minimised to certain extent by avoiding their heavy dose usage and by limiting their use to need based application at appropriate growth stages. Overall the detrimental effect of systemic insecticides on beneficial organisms, environmental hazard, associated heavy cost of purchasing insecticides impelling the researchers to breed genetic resistance into the crop cultivars against aphids.

2.9 Biological control of aphid population using parasitoids and biopesticides

The method of reducing the population density of an organism by allowing growth of another antagonistic organism is termed as biological control (DeBach 1964). The antagonistic organism can be a natural predator, a parasite or a pathogen. For aphids there are various natural predators available in environment which can be used to control aphid population on plants. Most of these predators fall into the category of parasitic wasps, predatory beetles, predatory mites, lacewings, midge, predatory bugs and parasites. Sometimes aphids show secondary resistance to use of parasitoids through the presence of endosymbiotic bacteria inside them (Gwynn et al. 2005). Thus, dissociating the symbiosis from aphids can enhance the biological control (Bhatia et al. 2011). Certain viruses e.g. RhPV infestation to aphids is seen to reduce the aphid populations. Recombinant baculoviruses expressing small RNA from RhPV viruses has been constructed and found to effectively control aphid population (Pal et al, 2007). In the biopesticide approach the use of insecticidal toxin from *Dickeya dadantii*, a phytopathogenic bacteria producing cyst family toxins causes' septic injury or oral infection on aphid instars (Grenier et al. 2006). Additional biopesticide approaches include use of *Photorhabdus luminescens* toxins, a symbiotic enterobacteria found in the entomophagous nematodes of *Heterorhabdis sp*. Through nematode invasion the bacteria reach into the guts of insects and releases three classes of toxins into insect gut. The three classes of toxins differ in their activity and can collectively bring about the death of the insects.

2.10 Insecticidal proteins against aphids

Proteinase inhibitors (PIs) are long known to be involved in plant defence mechanism. New proteinase inhibitors are still being discovered that target several new gene families and today there are about 13 different proteinase inhibitors known (Rawlings et al. 2006). Plants exhibit different PIs having variable specificities against digestive proteinases of insects as defensive response to the invading insects. PIs are found to be accumulated as wound response of the plants and also when exogenous JAs are applied on plants (Farmer and Ryan 1992). PIs can be induced and their accumulation by inducible promoters has been explored as a potential avenue to generate plant defence response against attacking pests and has been explored against cereal aphids e.g. *Diuraphis noxia*, *Schizaphis graminum* and *Rhopalosiphum padi* by transgenic expression of potato proteinase inhibitors I and II (Tran et al. 1997). Similarly, by expressing cysteine-proteinase inhibitors *M. persicae* and *A. pisum* performance has been deterred (Carrilo et al. 2011). Plant also accumulates amylase inhibitors as a defence response. Although there are several examples of pest control by expressing amylase inhibitors but so far there are no major success achieved against sap sucking insects. Ribosome inactivating proteins has been used (type 2 RIP) to control two aphid species e.g. *A. pisum* and *M. nicotianae* by feeding artificial diets supplemented with RIPs (Shahidi-Noghabi et al. 2008). Lectins form another class of proteins which can be used to control aphids through genetic engineering. Lectins bind reversibly and non-enzymatically to specific carbohydrates in insect gut and act as feeding deterrent against insects. Several lectins such as pea lectins garlic leaf and bulb lectins has been successfully found to control aphids (Melander et al. 2003, Hossain et al. 2006).

2.11 Secondary metabolites of plants

Plant secondary metabolites mediate insect-host interaction in two ways, chemical derived substances and volatile substances. Terpenoids, phenolics, glucosinolates, quinine’s, alkaloids and flavonoids etc. forms the chemical derived substances (Mello and Silva-Filho 2002). Volatile substances involved in plant defence response against aphids are mainly constituted by *cis*-jasmones. Volatile compounds act as insect repellents or as an olfactory signal (Baldwin 2010) or play an indirect role by attracting insect predators and parasitoids, thus helping in plant defence. Volatile compounds used by insects for communication are transgenically expressed and shown to act as an insect repellent (Bhatia et al. 2011). The core secondary metabolites which are having antibiosis effect on aphid and their biosynthesis differing only in few branch points among plants can be utilized to identify new genes for aphid resistance.

2.12 Resistant crop cultivars

There has not been much progress in identifying aphid resistant crops although resistant sources have been greatly explored throughout the world. The detrimental effects of wide use of insecticides has urged researchers to find alternative sources of resistance against aphid infestation. In this regard, the resistance present in intrinsically resistant plants can be considered as the most eco-friendly strategy for pest management. However, non-availability of sources of resistance has made development of resistant cultivars nearly impossible through conventional breeding techniques. In the absence of genetic resistance among cultivated species, there is a shift of focus towards the natural source of resistance in wild relatives of *Brassica*. Resistance to mustard aphid *L. erysimi* has been reported in a wild crucifer *B. fruticulosa* (Kumar et al. 2011). *B. fruticulosa* showed strong antibiosis response against mustard aphids. In addition, variable resistance against mustard aphids were also observed in various accessions of *B. montana* (Kumar et al. 2011). The resistance genes identified in wild Brassica crops are potential source and can be introgressed to cultivated species through con\ventional or nonconventional breeding procedures.

2.13 Plant resistant genes against aphids

Non-availability of resistance sources for aphid resistance in cultivated crops has resulted in identification of only few resistance genes and their homologues. Such examples include *Mi-1.2* gene from *Lycopersicon peruvianum* confers resistance against potato aphid (Rossi et al. 1998) and the *vat* gene from *Cucumis melo* against cotton aphid. *Mi-1.2* and *vat* genes are both single dominant genes but differ in their mechanism of action for resistance. *Mi-1.2* causes aphid starvation while *vat* gene retards the aphid growth. Recent study has identified *pti1 like kinase* gene in wheat conferred resistance against Russian wheat aphid (Boyko et al. 2006).

2.14 Biochemical factors of insect resistance.

Apart from various morphological characteristics such as plant height, trichrome, pubescence hair, stem hardiness, leaf texture, glossiness (Durbey and sarup 1982, Kumar1997, Rao and Panwar 2000, 2001), biochemical characteristics *viz.*, tannin, phenol, flavonoids, chlorophyll, carotenoids, protein, sugar, starch have also been reported to be involved in imparting resistance to various insect pests (Kumar and Saxena 1985, Bhanot et al. 2004, Yele 2014, Dhillon and Chaudhary 2015). These biomolecules when expressed either constitutively or in inducible pattern govern insect-plant interaction, which ultimately results into plant defence against insects (Sharma 2009). The quality of defence can be determined by specific allelochemicals, nutrients and anatomical factors present in the host plant (Baldwin et al. 2001). The sum of all the morphological, biochemical and anatomical plant features confer plants a durable resistance against insect pests (Dhillon et al. 2005; Huang et al. 2013). Anti-nutritional factors like lignin and phenolic compounds also play an important role in plant defence against herbivores (Dhillon et al. 2015, Rasool et al. 2017). Plant chemicals influence the resistance/susceptibility to insect pests in various ways: (i) determining the orientation, feeding and oviposition behaviour of the insects; and (ii) determining the metabolism of insects, which could be either to enhance the normal metabolic processes confirming insect's normal survival, development and egg production or can lead to production of plant toxins interfering with survival, development and egg production.

In plant defence response against herbivores, the production of "Reactive oxygen species (ROS)" also plays a very important role. The ROS produced in plants acts as a secondary messenger and activates various defence related pathways. The production of ROS in plants is highly co-ordinated as when produced in sufficient amount they promote beneficial oxidation, generate energy and kill invaders but when produced in excess ROS causes pigment co-oxidation, lipid peroxidation, membrane destruction, protein denaturation and DNA mutation. (Mittler 2002). In order to protect from the detrimental effect of ROS action the plants also produce many ROS scavenging enzymes so as to prevent oxidation (Howe and Schilmiller 2002). Antioxidant enzymes form an important component in the scavenging system of ROS. The induced resistance in host plants is regulated by oxidation, lipid peroxidation, membrane destruction, protein denaturation, and DNA mutation. Induced resistance in host plants is regulated by various antioxidative defence enzymes such as peroxidases (PODs), polyphenol oxidases (PPO), phenylalanine ammonia lyase (PAL), superoxide dismutase (SOD), and catalase (Gulsen et al. 2010, Usha Rani and Jyothsna 2010; War et al. 2011, 2012).

3. METERIALS AND METHODS

The present research work has been carried out at teaching discipline of Molecular Biology and Biotechnology (MBB), National Institute for Plant Biotechnology, IARI Campus, New Delhi.

Plant material: Wild relatives of Brassica sp. were grown in the net house facility of National Institute for Plant Biotechnology, New Delhi. The list of the species, included in present research has been enclosed as appendix. The plants were grown in the small plots (size 1×1 m^2) under the natural conditions of mustard season during October to March for consecutive two years, 2017-18 and 2018-19.

3.1 Screening for aphid resistance

3.1.1 Aphid collection

For aphid inoculum aphids were collected from *Brassica* field maintained at Division of Entomology. Heavily aphid infested twigs were cut from the plants and left on the butter paper sheet for few hours. The aphids descending from the twigs naturally were used for fresh infestation on the experimental plants. Natural migration ensured no damage to stylet of the aphids used for inoculation.

3.1.2 Aphid inoculation on experimental plants

The plants used for the experiments were surrounded by nets. The pore size of net was sufficient enough to allow penetration of light and wind without inhibiting growth of the plants. Three plants of 45 days old from each species/accession were chosen for the aphid infestation experiments and covered with cotton bags. Funnel shaped structure was prepared using blotting sheets and placed just above the last node and leaving 6-8 inches from apical tip on the selected branches. Aphids were released on this funnel which migrated and colonized the branch above the funnel. For confining wingless aphids, the node of the infested branches was wrapped with double adhesive tapes. For aphid inoculation, cotton bag cover was lifted and roughly around 100 aphids were released inside each blotting sheet funnel. All the inoculations were performed in the early hours of the days and date of infestation was noted. Aphid count was recorded in a time course manner (7th, 10th, 14th, 21st, 24th and 30th day) on each of the plants. The plant which did not show infestation was reinoculated three times at 3 days interval.

3.2 Preparation of enzyme extracts for biochemical enzyme assays

Crude extracts for enzyme assays of catalase, phenylalanine lyase, peroxidase, and superoxide dismutase and polyphenol oxidase were prepared from leave samples as per following steps:

1) Leaf tissue was collected from the plant and immediately frozen in liquid nitrogen in order to prevent any proteolytic cleavage.
2) The leave samples were grinded and suspended in 5ml of extraction buffer. The composition of extraction buffer was 0.2M phosphate buffer pH 7.8, 0.1Mm EDTA and 5% PVP.
3) After suspending the grinded powder in extraction buffer for few minutes the mixture was centrifuged at 15000g for 20min.
4) Centrifugation separated the mixture into two layers. The supernatant was taken in 2 ml eppendorf tubes and used for protein estimation as well as spectrophotometric analysis in enzymatic assays using Evolution300 UV–Vis Spectrophotometer (Thermo Scientific).

The protein concentration was estimated by Bradford (1976) method using bovine serum albumin (BSA) as a standard.

3.2.1 Protein estimation

The Bradford reagent consists of Coomassie Brilliant blue G250 in phosphoric acid and methanol. In this method the protein concentration is measured on the basis of complex formation between protein and coomassie blue under acidic conditions that results in a colour change from brown to blue and a shift in absorption maximum to 595nm.The absorbance is proportional to proteins present in solution.

Materials required:

- Bradford reagent
- Glass cuvettes
- Standard protein solution of known concentration
- Unknown sample whose protein concentration is to be measured.

Preparation of Bradford reagent

1. 50mg of coomasie blue G250 was dissolved in 50ml of methanol.
2. 100 ml of 85% (w/v) Phosphoric acid (H_3PO_4) was added to solution from step 1.
3. The resulting solution was added again with 500ml of water.
4. The solution was passed through whatman#1 filter to remove precipitates.
5. Additional 150ml water was added to make the final volume to 1liter and stored in brown bottle in 4°C.

Preparation of standards and samples:

A series of different dilutions of BSA was prepared from a stock of BSA (1mg/ml).0.1ml protein of known concentration was taken and added with 3ml of Bradford reagent and incubated for 5min. Absorbance was taken at 595nm and plotted against a graph to generate BSA standard curve. The Standard curve was prepared to calculate protein content in the samples.

3.2.2 Superoxide dismutase

Principle of the assay: SOD catalyses the dismutation of superoxide radical ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2).

$$O_2^- + 2H^+ \xrightarrow{\textbf{SOD}} H_2O_2 + O_2$$

The principle of SOD activity assay is based on the inhibition of nitro blue tetrazolium (NBT) reduction. Riboflavin in the presence of O_2 and methionine (electron donor) generates superoxide anions and these superoxide anions reduce the NBT to blue coloured formazan, which absorbs at 560nm. The enzyme (SOD) decreases this absorbance due to reduction in the formation of O_2^- radical. The blank, without enzyme, consequently gives highest absorbance, which decreases with increase in enzyme activity (Dhindsa et al.1981).

Reagents:

1) Methionine (200 mM)
2) Nitro blue tetrazolium chloride (NBT) (2.25 mM)
3) EDTA (3.0 mM)
4) Riboflavin (60 µM)
5) Sodium carbonate (1.5 M)
6) Phosphate buffer (100 mM, pH 7.8)

Preparation of 100 mM phosphate buffer:

<u>Solution A:</u> Potassium dihydrogen phosphate 6.80 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

<u>Solution B:</u> Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Mix 8.5 ml of solutionA and 91.5 ml of solution B and final pH was adjusted to 7.8.

Enzyme assay:

The decrease in optical density of formazan made by superoxide radical and nitro-blue tetrazolium dye by the enzyme forms the basis of SOD enzyme assay. Three ml of the reaction mixture contained.

13.33 mM methionine (0.2 ml of 200mM)
75 µM nitro blue tetrazolium chloride (NBT) (0.1 ml of 2.25 mM)
0.1 mM EDTA (0.1 ml of 3mM)
50 mM phosphate buffer (pH 7.8) (1.5 ml of 100 mM)
50 mM sodium carbonate (0.1 ml of 1.5M)
0.050 ml enzyme
0.950 ml of water (to make a final volume of 3.0 ml)

The reaction was initiated by addition of 2µM riboflavin (0.1ml) and placed under light for 40min. A complete reaction mixture without the enzyme is also kept as a control which supposed to give maximum colouration. The reaction was stopped after 40min by transferring the tubes to dark. Blank is constituted by placing a complete reaction mixture kept under dark. One unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of NBT by 50%.

Unit of enzyme = [Control – (Sample-Blank)]/ (control/2)

3.2.3 Catalase

Principle of the assay: Catalase catalyses the reduction of hydrogen peroxide to water and molecular oxygen. H_2O_2 in the process of degradation shows a continual decreasing trend in absorption in the UV range. The decomposition of H_2O_2 can be measured directly by the decrease in absorbance at 240 nm. The difference in absorbance (A_{240}) per unit time is a measure of the catalase activity(Aebi, 1984).Catalase shows high Km value as compared to other H_2O_2 scavenging enzymes such as ascorbate peroxidase apex (APX).The enzyme is localized in mitochondria and peroxisomes, but absent in chloroplast, one of the important sites of H_2O_2 generation.

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2$$

Reagents:

1) Hydrogen peroxide (30mM): Dissolve 349µl of 30 % H_2O_2 in double distilled water and make up the volume to 100 ml.
2) Phosphate buffer (100 mM, pH 7.0):

Preparation of Phosphate buffer:

Solution A: Potassium dihydrogen phosphate 6.80 g was dissolved in water and the volume was made up to500 ml with double distilled water.

Solution B: Dipotassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Mix 39 ml of solution A and 61 ml of Solution B. Final pH was adjusted with NaOH or HCl by pH meter. The leaf extract was diluted in 50 mM potassium phosphate buffer for 200 times.

The enzymatic reaction was set up as follows:

Test Reaction	For blank
Enzyme Extract 2ml	Enzyme extract 2ml
30mM Hydrogen peroxide 0.6ml	Potassium Phosphate buffer 0.6ml
Water 0.4ml	Water 0.4ml

The decrease in absorbance at 240 nm was recorded for 3 min, starting at 0min, 1min, and 2min. Enzyme activity was calculated by measuring the amount of H_2O_2 decomposed.

Enzyme activity is calculated as:-

Initial reading – Final reading = Quantity of hydrogen peroxide reduced per min per mg protein.

3.2.4 Peroxidase

Peroxidase (POD), also known as non-specific peroxidase or guaiacol-peroxidase. Peroxidase catalyses the reduction of hydrogen peroxide with simultaneous oxidation of a substrate. In plant cells it is located inside the cell wall and is involved in the oxidation of phenolic compounds towards the synthesis of lignins.

$$RH_2 + H_2O_2 \xrightarrow{\text{Peroxidase}} 2\,H_2O + R$$

The oxidation of guaiacol to tetra-guaiacol forms the basis of POD assay (Castillo et al. 1984). The reaction mixture of 3 ml contained 96 mM guaiacol, 50mM H_2O_2, 100 mM phosphate buffer (pH 6.1) and 0.1 ml enzyme extract diluted 10 times.

Reagents:

1) Phosphate buffer (100 mM, pH 6.1)

Solution A: Potassium dihydrogen phosphate 6.80 g dissolved in water and the volume was made up to 500 ml with double distilled water.

Solution B: Dipotassium hydrogen phosphate 8.71 g dissolved in water and the volume was made up to 500 ml with double distilled water.

Mix 15 ml of solution A and 85 ml of solution B. Final pH was adjusted using NaOH or HCL.

2) Hydrogen peroxide (50 mM): Dissolved 340 µl of 30 % H_2O_2 in double distilled water and volume was made up to 100 ml.

3) Guaiacol (96 mM): Dissolved 1075 µl of analytical grade guaiacol in distilled water and volume was made upto100 ml.

Enzyme assay: The 3 ml reaction mixture contained:

Test reaction	Blank
Phosphate buffer (50 mM, pH 6.1): 1.0 ml	Phosphate buffer (50 mM, pH 6.1):1.0 ml
Guaiacol (96 mM): 333.3µl	Guaiacol (96 mM): 333.3µl
H_2O_2 (50 mM): 80µl	
Enzyme extract: 0.1 ml	Enzyme extract: 0.1 ml
Water: 487µl	Water: 567µl

The tetra-guaiacol formed as a result of oxidation of guaiacol at 470 nm and enzyme activity of POD was calculated as per extinction coefficient of tetra-guaiacol $\in$ = 26.6 mM^{-1} cm^{-1}. Enzyme activity is expressed as µmol tetra-guaiacol formed per min per gram.

3.2.5 Polyphenol oxidase

Principle: The enzyme polyphenol causes oxidation of tyrosine into dihydroxy-phenylalanine which is further oxidized to o-Quinone. The o-Quinone formed shows an increase in absorbance at 470 nm. The rate of increase is proportional to enzyme concentration and remains linear during a period of 5-10 minutes after an initial lag. One unit of polyphenol oxidase is defined as the amount of enzyme required to cause a change in absorbance of 0.001at 470 nm per minute at 25°C, pH 6.5 under the specified conditions.

Enzyme assay: PPO (EC 1.10.3.2, EC 1.10.3.1, and EC 1.14.18.1) or polyphenol oxidase activity was measured according to the modified method of (Hori et al. 1997). The rate of increase in absorbance was measured at 470 nm for 1 min and the activity was expressed as $\Delta A470\ min^{-1}\ mg^{-1}$ protein.

Test reaction	Blank
Enzyme extract 200 µl	Enzyme extract 200 µl
Catechol (1.6%)	
(200 mM) HEPES Buffer pH 6.0 200µl	(200 mM)HEPES Buffer pH 6.0 200µl
Water 1.6 ml	Water 2.6 ml

3.2.6 Phenylalanine ammonia-lyase

Principle: Phenyl alanine ammonium lyase was first described by Koukol and Conn (1961). It is involved in the non-oxidative deamination of phenylalanine which breaks down into trans-cinnamic acid and ammonia. It is the initial step in the biosynthesis of phenolic compounds. PAL activity was analysed as the rate of conversion of L-phenylalanine into trans-cinnamic acid at 290nm in UV-VIS spectrophotometer. The reaction mixture contains 50µl of enzyme extract, 1ml of 0.01M sodium borate buffer (pH 8.5) and 200µl of L-phenylalanine (100 mM) and the blank was constituted by replacing L-phenylalanine with water. One unit of PAL is defined as the amount of enzyme required to deaminate 1µmole of L-phenylalanine into trans-cinnamic acid and ammonia per minute. The enzyme activity was measured by calculating the change in absorption per minute per mg of protein at 290 nm.

3.3 Total RNA isolation

Total RNA was isolated as per following method.

1. 100 mg of leaf tissue was ground into fine powder using liquid nitrogen and transferred immediately into 2ml micro centrifuge tube.
2. 1 ml of RNAiso plus (TAKARA, Bio Inc. Japan) reagent was added to each tube and mixed by vigorous vortexing for 1-2min. The RNA samples were stored in -20 ^{0}C and RNA isolation was done as and when required.
3. For RNA isolation the samples stored with RNAiso plus were thawed at room temperature for 5 minutes
4. 0.2 ml of chloroform was added per 1 ml of RNAiso plus reagent and shaken vigorously for about 15 s. The tubes were incubated at room temperature for 2-3

minutes, followed by centrifugation at 12000 g for 15 min at 4 ^{0}C. The cocktail formed three phases, lower red phase containing DNA and protein, a whitish inter phase and an upper aqueous phase containing total RNA.

5. The upper phase was transferred to a new 1.5 ml eppendorf tube and RNA was precipitated by adding 0.5 ml of isopropanol. After adding isopropanol, the tubes were incubated for 10 min at 4 ^{0}C. Tubes were centrifuged for 10 min at 10,000 rpm. The RNA formed transparent pellet at the bottom of the tubes.

7. The supernatant was discarded and the RNA pellet was washed with 1ml75%ethanol prepared in DPEC- treated water and centrifuged at 7500 g for 5 min at 4 ^{0}C. The supernatant was removed and the pellet was air dried until the ethanol evaporated completely.

8. The dried pellet was resuspended in 20 µl of DEPC water and was heated at 55 ^{0}C until the pellet dissolved. Total RNA was treated with DNase I (Invitrogen, USA) to remove residual genomic DNA contamination.

3.4 DNase I Treatment of RNA

Total RNA was treated with DNase I (Invitrogen, USA) to remove genomic DNA contamination.

1. 0.1 volume 10x TURBO DNase buffer and 1µl TURBO DNase was added to RNA and mixed.

2. After mixing with DNase buffer and DNase, the mixture was incubated at 37ºC for 20-30 min.

3. After completion of incubation 0.1 volume resuspended DNase inactivation reagent was added and mixed well and kept for incubation for 5min.

4. After incubation, the mixture was centrifuged for 1.5min and RNA was transferred to a fresh tube.

3.5 Quantification of Total RNA

The concentration and quality of total RNA was determined by using Nano Drop 1000 (Thermo Scientific, USA). The quality was ensured by measuring the ratio of absorbance (OD) at 260 and280-nm. If the ratio is between 1.8 and 2.0, the RNA quality is acceptable.

- RNA concentration was calculated by using the formula:

  RNA concentration in µg/ml =40×OD260×dilution factor.
- The isolated RNA sample was stored in -20ºC till further use.

3.6 Gel electrophoresis of RNA samples for checking DNA contamination

A. Preparation of formaldehyde gel

1. 1.8 gram of agarose was transferred in a 200ml conical flask containing 100ml of 1x MOPS (3-morpholinipropane-1sulphonic acid) buffer.
2. The solution was then boiled to dissolve the agarose completely, after dissolving the agarose the solution was then allowed to cool to ~65ºC.
3. After cooling of the solution 1µl of ethidium bromide (4µl/ml) and 18ml of formaldehyde was added.
4. The gel was then poured into a casting tray and combs are placed for formation of wells. After solidification of gel the gel was transferred to gel electrophoresis tank.

B. Preparation of RNA samples for loading on formaldehyde gel

The following components were added:

Components	Volume
RNA sample	1µg
Formaldehyde loading dye	3µl
RNase free water	Vol. made up to 10 µl
Total	10µl

After mixing all the components, the RNA samples were denatured at 65ºC for 15-20min and immediately the tubes were transferred to ice and kept for 5-7min. Then the RNA samples were loaded on the agarose gel.

3.7 Preparation of cDNA

After RNA quantification and quality check cDNA was prepared from each RNA samples. 2.5 µg of DNase treated RNA was used for cDNA synthesis using PrimeScript™ first strand cDNA synthesis kit (TAKARA, Bio Inc. Japan). Reaction mixture was prepared by adding following components in a sterile PCR tube.

Component		Amounts
I.	Oligo dT primer (50 µM)	1 µl
II.	dNTP mixture (10mM)	1 µl
III.	Template RNA (2 µg)	8 µl
Total volume		10 µl

After adding the oligo dT primer and dNTP to the RNA, the mixture was incubated at 65^{0}C for 5 min and then immediately cooled on ice for 2 min. Now, the other components viz.5XPrimeScript buffer, RNase inhibitor and PrimeScript RTase were added to the reaction mixture and the tubes were briefly centrifuged to bring the content at the bottom of the tube. The tubes were incubated at 42 ^{0}C for 1 h. At the end of the reaction the tubes were kept at 85 ^{0}C for 5 min for inactivating the enzyme and transferred on ice.

The reaction for second strand cDNA synthesis was prepared as follow:

Component		Amounts
I.	Template RNA mixture (from above step)	10 µl
II.	5X PrimeScript buffer	4 µl
III.	RNase Inhibitor (40 U/ µl)	0.5 µl
IV.	PrimeScript RTase (200 U/ µl)	1 µl
V.	RNase free water	4.5 µl
Total volume		**20 µl**

3.8 Determination of quality of cDNA

Integrity and uniformity of cDNA in each sample was confirmed by amplification of a housekeeping gene encoding GAPDH (Glyceraldehyde 3-phosphate dehydrogenase).

The PCR reaction mixture for amplification of GAPDH gene in the cDNA samples was prepared as follows:

Components	Volume
Taq buffer	2µl
Taq polymerase	.2µl
dNTP(10Mm)	1µl
Forward primer	1µl
Reverse primer	1µl
Template cDNA	1µl
RNase free water	13.8µl
Total	20µl

The reaction cocktail was mixed thoroughly and placed on the thermal cycler for PCR

Thermal cycling steps of cDNA-PCR

Cycle steps	Temp.	Time	Cycle(s)
Initial denaturation	94ºC	1min	1
Denaturation	94ºC	15 s	40
Annealing	60ºC	30s	
Extension	72ºC	30s	
Final extension	72ºC	5min	1
Hold	4º	∞	1

Agarose gel electrophoresis of PCR products

After PCR amplification the PCR product was mixed with 5µl of 6X loading dye and loaded on 2% Agarose gel for electrophoresis using TBE buffer. After checking the quality of cDNA, the cDNA samples were diluted 5times and stored at -20ºC for further use.

3.9 Expression analysis of defence related genes

A. Designing of primers for qRT-PCR analysis

As there is no sequence related information available for most of the wild relatives included in this study, we used the sequence information of cultivated *Brassic*a sp. For designing of primers for amplification of defence related genes. For primer designing FASTA sequences were first obtained from www.ncbi.nlm.nih.gov and used for primer designing using PRIMER 3 online software. PRIMER 3 generated several possible primers taking into consideration of different properties like primer length of 20-24bp, annealing temperature, GC content etc. The primer sets so obtained are again checked by PRIMER BLAST software in www.ncbi.nlm.nih.gov and primer sets generating a optimum product length of 150-200bp were finally selected.

Table M1. Primers used for studying the defence related gene expression in RT-PCR:

S.L No	Path way involved	Gene or Acc. no**.**	Forward primer (5’>3’)	Reverse primer(5’>3’)	Tm(ºC)	Product in bp.
1.	SA signalling	PR1	CCAGGCTAAGTTTTCCCCGT	AGCGATGTTTACGAACCCCA	60	>200
2.	Defence	PR2	GACCGACTAAGAGGCGACTG	TTTGCCACATCCAATTCTCA	60	>200
3.	ROS signalling	Bra038281 (mapk3 gene)	TCTTCTCCTGAACGCCAACT	CGCATCTGATGGACATGGTC	60	>200
4.	Transcription factor	Bra022786 (WRKY25 gene)	CCAAGTTCAAGACGGCTCAG	CTGCAAACTCACGAGGAACC	60	>200
5.	Ethylene signalling	ERF1 (Bra02374)	GAGCAGTCCACACAGCAAAA	CGAGCCTCTCATGGAAAAAG	60	>200
6.	Defence	ERF1 (Bra02374)	AAACGTTCCGGTCAAAACAG	ACGAGCCTCTCATGGAAAAA	60	>200
7.	JA signalling	VSP2 (Bra02047)	TACCTGCGCCTAAGCTCATT	AGCTTCTTTGCACACCGTTT	60	>200
8.	JA signalling	VSP1	TACCTGCGCCTAAGCTCATT	GGAGTCAGCACGGTACTGGT	60	>200
9.	Glucosinolate pathway	(Bra01593)	AGCTCGTCGATTACCAGGAA	GAGCTGATGATGGCCCTAAG	60	>200
10.	Glucosinolate pathway	(Bra013009)	TGCAGGTCGGAGAAAGAGTT	ACATATACCGGCGATTGCAT	60	>200
11.	Lectin gene	(Loc106401296)	GTTTGATGGGTTCGCAAAGT	ACCAACCTCATTAGGCATCG	60	>200
12.	Myrosinase gene	(LOC106382674)	ACCCTCTTTCACTGGGACCT	AGGAGAACATCGACCTGGTG	60	>200
13.	Aphid resistant gene	(X64653.1)	GATCAATGCATTCCCAAGTG	GATGGTGCCATCTCCATCTT	60	>200

B. Optimization of primers

As all the primers are designed using genomic sequence information of cultivated species, so a final primer optimization of primer annealing was carried out using the genomic DNA isolated from wild Brassica plants**.**

3.10 Genomic DNA isolation

Genomic DNA was isolated following CTAB method as mentioned below.

1. 200mg of leaf tissue were weighted and homogenised in prechilled mortar and pestle using liquid nitrogen. For each 200mg of leaf tissue 1ml of CTAB buffer was used.
2. The homogenates were then centrifuged at 10,000g for 10min.
3. After centrifugation the supernatants were transferred to a new tube & RNase solution was added and incubated for 30min.
4. Equal volume of chloroform/isoamyl-alcohol (24:1) was added and vortexed well and then centrifuged at 10,000g for 20min.
5. The aqueous phase was then transferred to a fresh tube and 0.6 volume of chilled isopropanol was added and kept for incubation for 30min at -20^{o}C for 15min.
6. After incubation is over the samples were centrifuged at 10,000g for 10min.The supernatants were decanted and the pellets collected were washed with ice cold 70% ethanol.
7. After washing the pellets were dissolved in nuclease free water.

3.11 PCR Amplification of Genomic DNA using designed primers.

The preparation of PCR reaction mixture, thermal cycling and agarose gel electrophoresis were carried out as described earlier in section 4.11.

3.12 Quantitative real time – PCR

cDNA prepared from 2500.ng of RNA in 20 µl volume was diluted in 1:5 times for use in qPCR. GAPDH was taken as normalizer or internal control and ROX was used as reference dye as per SYBR green chemistry. The qPCR reaction was carried out using SYBR premix Ex taq^{TM} (T1i RNase H plus).

Composition of 10.0µlPCR reaction is given below.

Components	**Volume**
SYBR	5.0µl
ROX	0.2µl
cDNA	1.0µl
Forward primer	0.4µl
Reverse primer	0.4µl
Nuclease free water	3.0µl
Total	10.0µl

The master mix was prepared using forward primer, reverse primer and nuclease free water according to the concentrations listed above. From this master mix 3.8µl was taken and pipetted in each selected well of 96 well PCR plate. After that the PCR plate was centrifuged at 1000 rpm for 3min.Another master mix was prepared using SYBR, ROX and cDNA and 6.2µl volume was pipetted in already specified wells so that each well contained 10µl of reaction volume. Third master mix for negative control was prepared using all the components except cDNA. In each negative control reaction nuclease free water was added instead of cDNA. 6.2µl of negative control sample was also pipetted in specified wells. After adding all three master mixes the PCR plate was sealed with the help of sealer and again centrifuged at 1000 rpm for 3min and kept on ice till transferred to thermal block.

The qPCR programme was carried out as follows:

PCR cycle	Conditions
Initial denaturation	95°C for 1 min
Denaturation Annealing Extension	95°C for 10 s 60°C for 30 s 72° C for 30 s
Data collection and continuous melt curve analysis	

The PCR cycling was carried out for 40 cycles and at the end relative amplification in each case was studied based on the software available in the machine.

3.13. Transcriptome analysis of *Eruca sativa* leaf samples

For transcriptome analysis *E. sativa* leaf samples were collected before aphid infestation and 30 days after infestation. Transcriptome Sequencing was carried out using Illumina Genome Analyzer (101x2) bp Chemistry through outsourcing to NxGenBio Life Sciences, India.

4. RESULTS

4.1 Objective 1: Screening for aphid resistance in wild germplasms of *Brassica*.

A total of 22 wild *Brassica* species were screened for variable level of aphid infestation in this study (Table 1; Appendix 1). For aphid inoculation, 3 branches from two plants of each species was selected. Approximately 100aphids were released on the funnel shaped structure fixed above the nodes of the branches (please see Material method for details) and allowed to walk through the desired branch under study (Fig. 4.1).

Table 1. List of *Brassica* wild species screened for aphid resistance

1	*Brassica chinensis*	12	*Erucastrum cardaminoides*
2	*Brassica fruticulosa*	13	*Erucastrum gallicum*
3	*Brassica fruticulosa* (Spain)	14	*Orychophragmus violaceous*
4	*Brassica spiniscens*	15	*Oxycampus sp.*
5	*Brassica tournefortii* (RBT 2003)	16	*WDKY* (wild collected by N.singh)
6	*Camelina sativa*	17	*Crambe abyssinica* (EC 145)
7	*Diplotaxis cretacia*	18	*Crambe abyssinica* (EC 159)
8	*Diplotaxis erucoides*	19	*Brassica cysimbrium*
9	*Diplotaxis gomezcampoi*	20	*Eruca sativa* (selection (80-3))
10	*Diplotaxis tenuisilique*	21	*Lepidium sativum*
11	*Erucastrum canariense*	22	*Crambe abyssinica* (EC071)

Downward movement of aphids were restricted using double adhesive tape thus infestation was confined only in the upper portion of the plant (Fig. 4.2). The rough surface of blotting sheet allowed the aphids to climb easily toward the upper leaves and flowers.

Fig 4.1 Blotting sheet funnels

Fig 4.2 Use of double adhesive tape

Fig 4.3 Experimental plants in the net house

The study of aphid bioassay on wild relatives of *Brassica* species showed that the growth and multiplication rate of aphids varies when reared on one species to another species. The aphid counts were recorded in a time course manner at 7th, 10th, 14th, 21st, 24th and 30th days after inoculation on each plant. According to the rate of multiplication of aphids the wild plants were categorised into 3 different groups viz Resistant, moderately resistant and susceptible to aphid infestation. A population demograph was constructed by plotting the number of aphids recorded (on X axis) at different time intervals (Y-axis) (Fig. 4.4). In a typical susceptible species, the number of aphids increased continuously from ~100 to 3000-4000 at first 30 days of infestation. In case of moderately resistant species increase in aphid population was moderate and varied between 500 -700 (Fig. 4.5). The demography of aphid multiplication on resistant plants showed severely restricted aphid multiplication within 7-10 of inoculation and a consequent decrease in number of aphids at the end of 30th day even compared to the number of aphids used for initial infestation (Fig. 4.6).

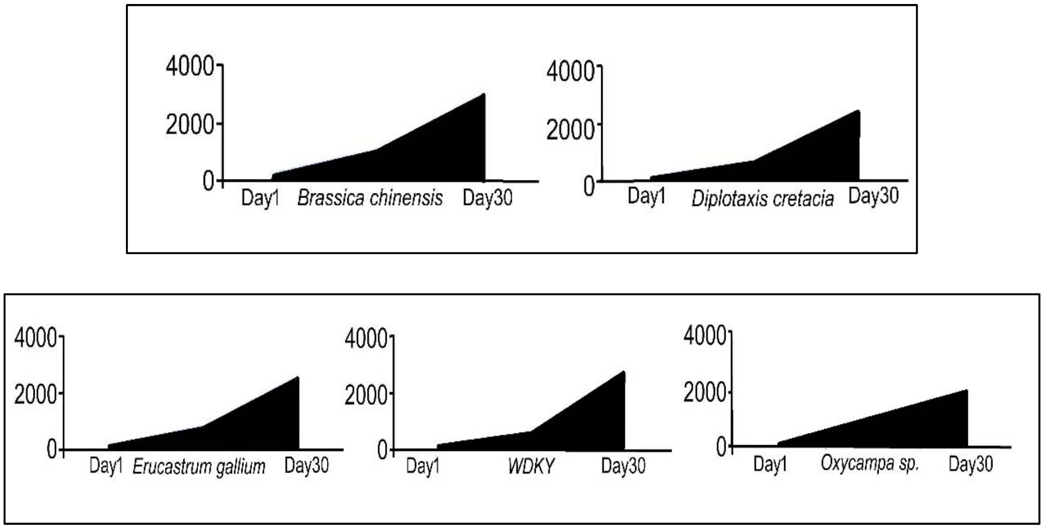


Fig 4.4 Demograph of aphid multiplication on five representative susceptible plants.

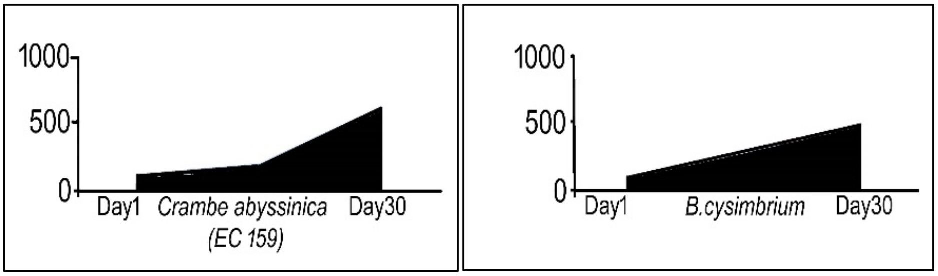


Fig 4.5 Demograph of aphid multiplication on moderately susceptible plants.

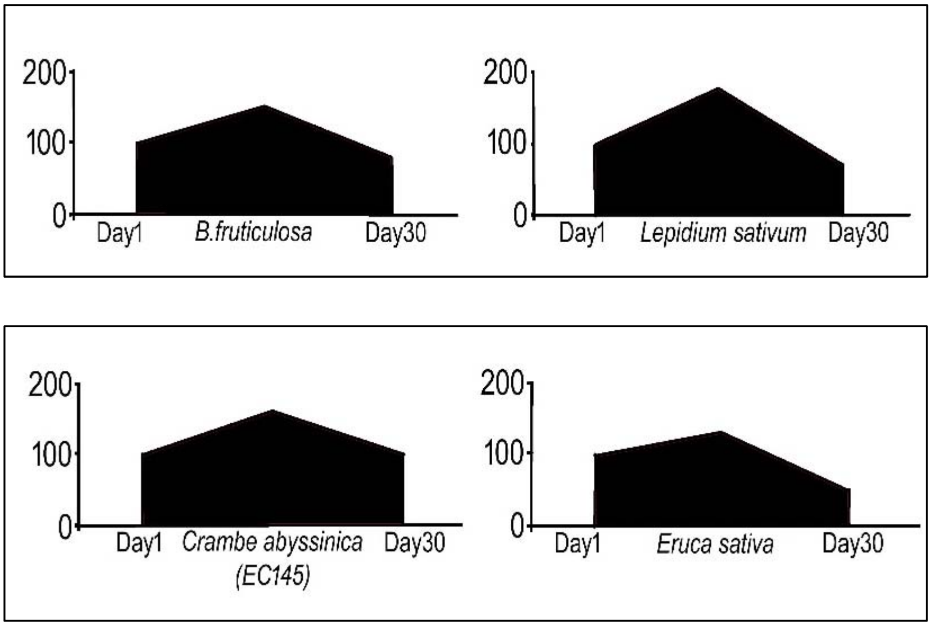


Fig 4.6 Demograph of aphid multiplication on resistant wild species of *Brassica*

Table 2. List of *Brassica* wild species showing resistance to aphid infestation

1	*Brassica fruticulosa*
2	*Lepidium sativum*
3	*Crambe abyssinica* (EC 145)
4	*Eruca sativa*
5	*Crambe abyssinica* (EC159)
6	*Brassica cysimbrium*

4.2 Objective 2: Biochemical analysis of wild species showing variable level of aphid infestation

4.2.1 Total soluble protein

The total soluble protein content before infestation and after infestation in samples of different wild *Brassica* species varied from 355.42µg/g to 593.2µg/g and 359.86µg/g to 671.42µg/g respectively (Table: 3).

Table 3. Protein concentration (µg/g) in wild *Brassica* species before and after aphid infestation

Wild *Brassica* species	**µg/g protein before infestation**	**µg/g Protein after infestation**
Brassica chinensis	542.53	671.42
Brassica fruticulosa	588.75	630.53
Diplotaxis cretacia	496.75	490.08
Erucastrum gallium	480.75	462.97
Lepidium sativum	543.86	577.2
WDKY	396.75	371.42
Crambe abyssinica (EC 145)	560.75	610.53
Crambe abyssinica(EC 159)	528.75	583.42
Brassica cysimbriyum	429.2	454.97
Eruca sativa	593.2	683.86
Oxycampa sp.	355.42	359.86

4.2.2 Enzymatic analysis

4.2.2.1 Peroxidase

POD activity was found to be increased in four wild *Brassica* species viz *Brassica chinensis, Brassica cysimbrium, Eruca sativa* and *Oxycampa sp* after aphid infestation as compared to POD activity observed before infestation (Fig. 4.7). However, the fold change in enzyme activity was found to be different for each of the wild species. A decrease in POD enzyme activity was observed in six wild *Brassica* species viz *Brassica fruticulosa, Diplotaxis cretacia, Lepidium sativum, Erucastrum gallium,Crambe abyssinica* (EC159) and *Crambe abyssinica* (EC 145). No change in POD enzyme activity was observed in wild *Brassica* species WDKY between before and after infestation samples.

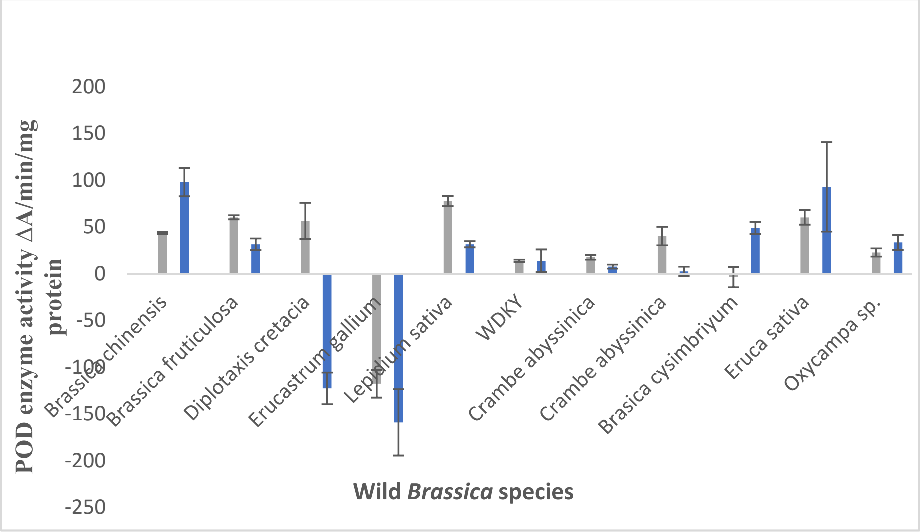


Fig 4.7 Relative change in POD enzyme activity before and after aphid infestation samples.

4.2.2.2 Catalase

The catalase enzyme activity was found to be increased in seven wild *Brassica* species after infestation as compared to before infestation (Fig. 4.8). These include two from susceptible viz *Brassica chinensis* and *WDKY*, two from moderately susceptible and three from resistant group viz *Brassica fruticulosa*, *Crambe abyssinica* (EC 145) and *Eruca sativa* resistant. However, a much higher fold change in catalase activity was observed in *Brassica chinensis*.

In four wild *Brassica* wild species a decrease in catalase enzyme activity was observed after infestation as compared to before infestation samples viz *Diplotaxiscretacia*,*Erucastrum gallium*, *Lepidium sativum* and *oxycampa sp.*

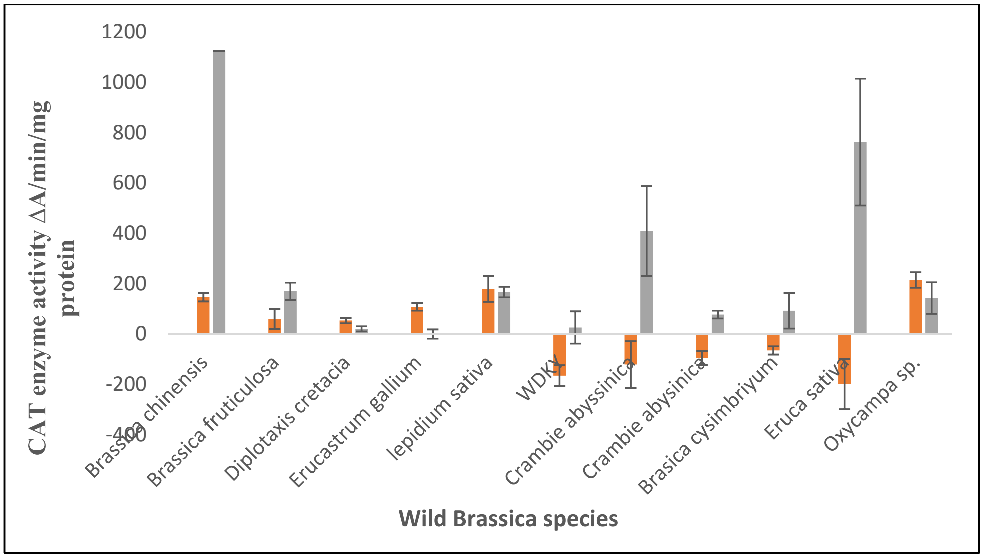


Fig 4.8 Relative change in CAT enzyme activity between before and after aphid infestation

4.2.2.3 Superoxide dismutase

The superoxide dismutase (SOD) activity was found to be increased in six wild *Brassica* species after infestation as compared to before infestation samples. Viz. *Brassica fruticulosa, Diplotaxis cretacia, Erucastrum gallium, Lepidium sativum,WDKY* and *Brassica cysimbrium* (Fig. 4.9). However, the fold change in increase activity was different for each wild species and was found to be highest for *Lepidium sativum.*

A decrease in SOD enzyme activity was observed in five wild Brassica species after infestation as compared to before infestation samples. viz; *Brassica chinensis, Crambe abyssinica* (EC 159), *Crambe abyssinica* (EC 145), *Eruca sativa* and *Oxycampa sp.*

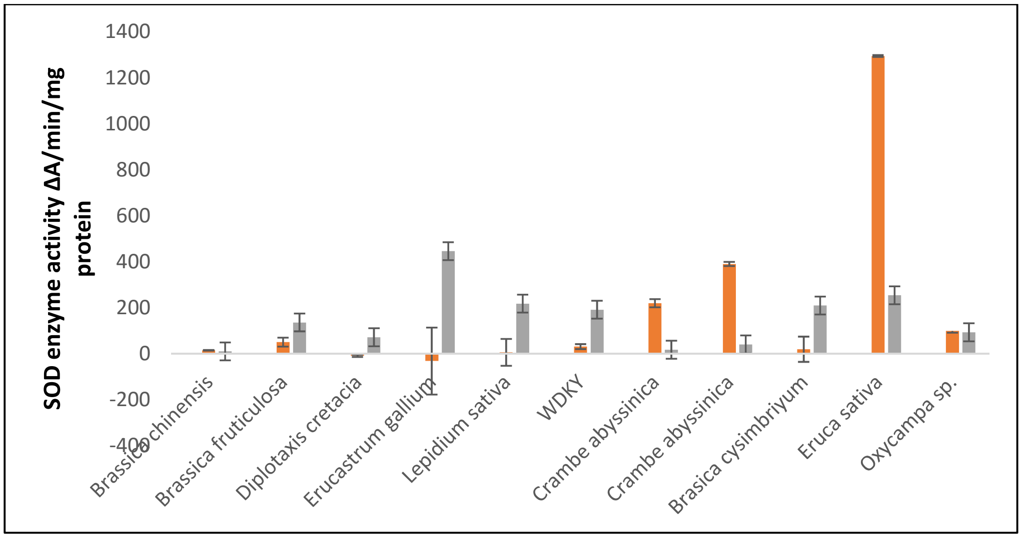


Fig 4.9 Relative change in SOD enzyme activity between before and after aphid infestation

4.2.2.4 Polyphenol oxidase

The polyphenol oxidase activity was found to be decreased in ten wild *Brassica* species after infestation as compared to before infestation samples viz *Brassica chinensis*, *Brassica fruticulosa*, *Diplotaxis cretacia*, *Erucastrum gallium*, *Lepidium sativum*, *Crambe abyssinica* (EC 159), *Crambe abyssinica* (EC 145), *Brassica cysimbrium*, *Eruca sativa* and *Oxycampa sp* (Fig. 4.10). A decrease in PPO enzyme activity was observed in one wild *Brassica* species *Oxycampa* sp.

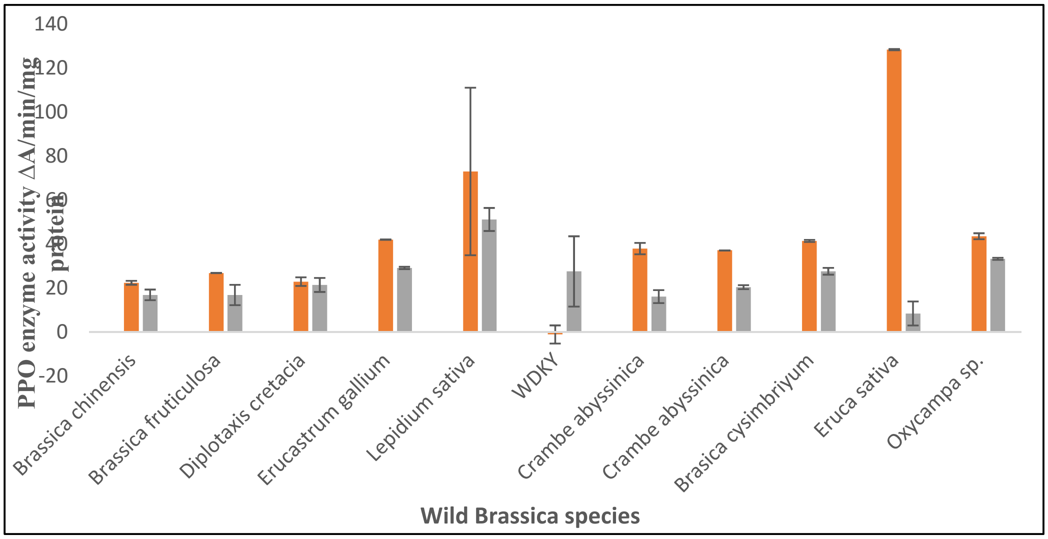


Fig 4.10 Relative change in PPO enzyme activity before and after aphid infestation

4.2.2.5 Phenylalanine lyase

The enzyme phenylalanine lyase activity was found to be increased in three wild Brassica species viz *Crambe abyssinica* (EC 159*)*, *Eruca Sativa* and *Oxycampa sp.*after infestation as compared to their before infestation samples (Fig. 4.11).

A decreasing PAL enzyme activity was observed in eight wild Brassica species after infestation as compared to before infestation samples. These include *Brassica chinensis, Brassica fruticulosa, Diplotaxis cretacia, Erucastrum gallium, Lepidium sativum, WDKY, Crambe abyssinica* (EC 145) and *Brassica cysimbrium*.

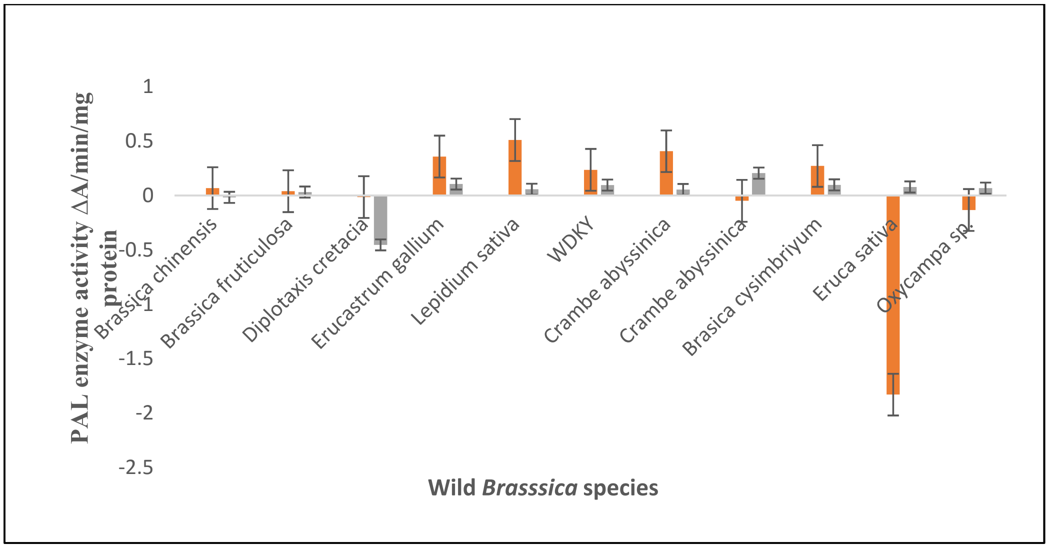


Fig 4.11 Relative change in PAL enzyme activity before and after aphid infestation

4.2.2.6.1 Defence related enzyme activity before and after aphid infestation in representative susceptible wild *Brassica* species.

Assay of defence related enzymatic activity insusceptible wild *Brassica* species, before and after aphid infestation, showed an increase in enzymatic activity for one or two enzymes (Fig. 4.12). For example, in *Oxycampa sp*. CAT activity was found to be increased after infestation. In *WDKY* and *Brassica chinensis* CAT and SOD activity was found to be increased after infestation. In *Erucastrum gallium* and *Diplotaxis cretacia* SOD activity was found to be increased after infestation. Interestingly, in most of the susceptible *Brassica* species CAT activity was found to increase after infestation. POD and SOD enzyme activity was found to be increased in two wild susceptible species, while PPO and PAL enzyme activity remained same before and after infestation in all the wild Brassica species.

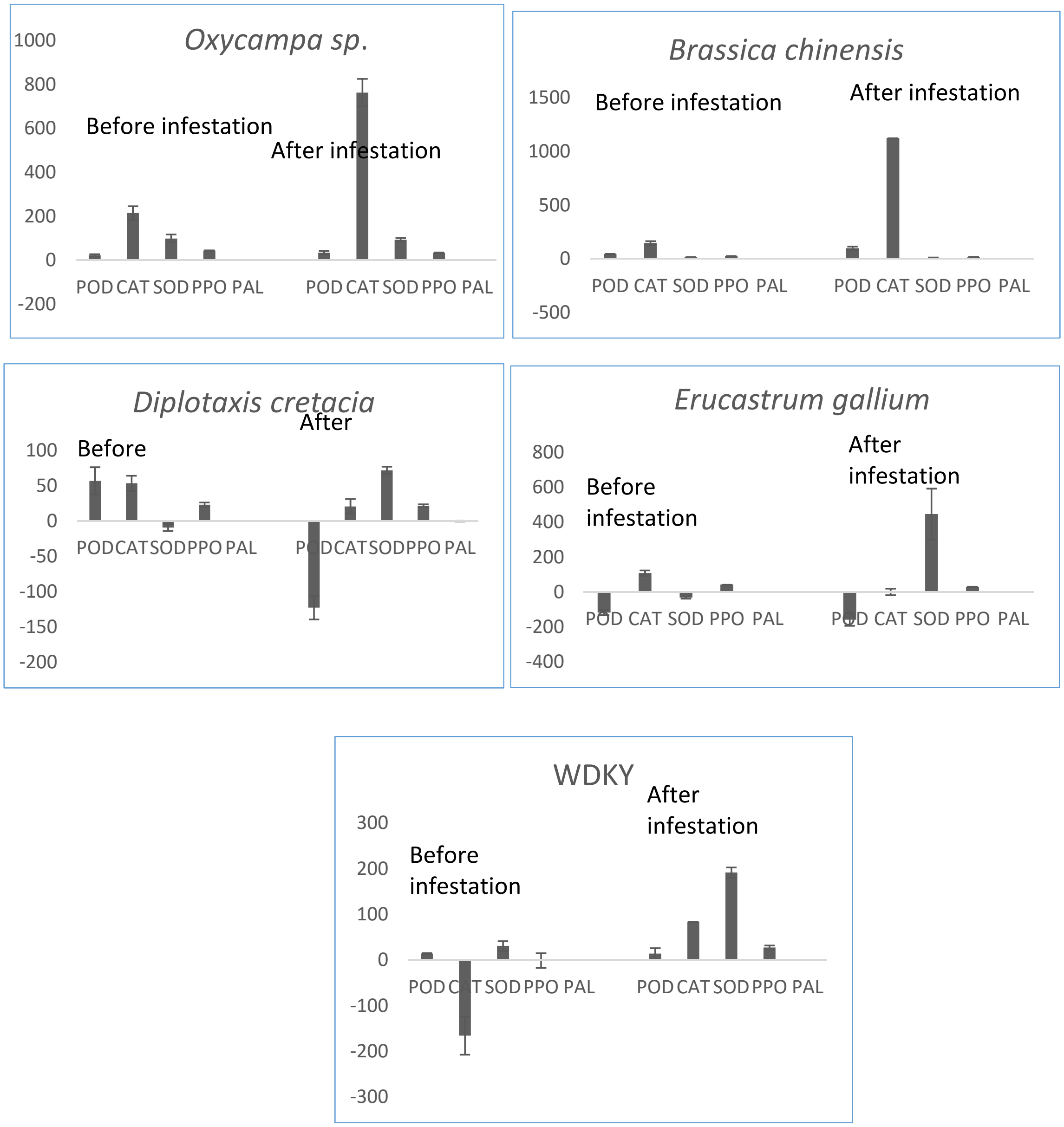
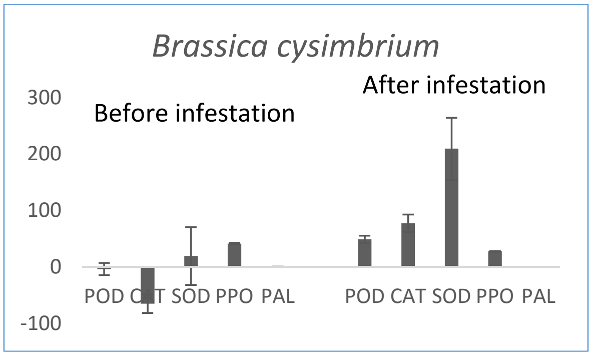


Fig 4.12 Defence enzyme activities in susceptible wild species of *Brassica*

4.2.2.6.2 Activities of defence related enzymes before and after aphid infestation in moderately resistant wild *Brassica* species

In moderately resistant wild *Brassica* species assay of enzymatic activities, before and after aphid infestation, showed that either CAT or SOD or both were increased in response to aphid infestation. For example, in *Brassica cysimbrium* CAT and SOD enzyme were found to be increased whereas in *Crambe abyssinica*only CAT activity increased after infestation (Fig. 4.13).



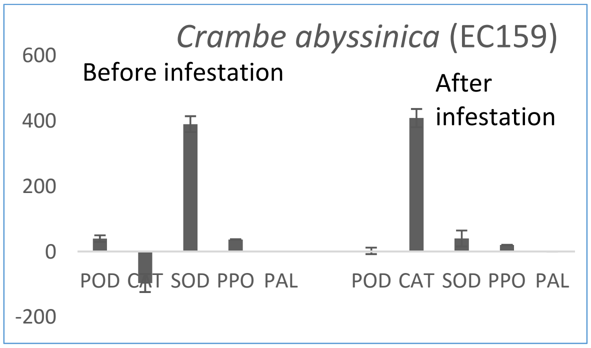


Fig 4.13 Defence related enzyme activity before and after aphid infestation in moderately resistant wild *Brassica* species

4.2.2.6.3 Activities of defence related enzymes before and after aphid infestation in resistant wild *Brassica* species

It is apparent from the status of enzymatic activities, before and after aphid infestation, in resistant wild *Brassica* species that the resistant species showed activation of multiple defence related enzymes in response to aphid infestation (Fig. 4.14). In *Eruca sativa* POD and CAT enzyme activity was found to be increased. In *Brassica fruticulosa* POD, CAT and SOD enzyme activity was found to be increased after infestation. *Lepidium sativum* (EC 145) showed a striking increase (40-fold increase) in SOD activity after infestation. In *Crambe abyssinica* catalase activity significantly increased in response to aphid infestation.

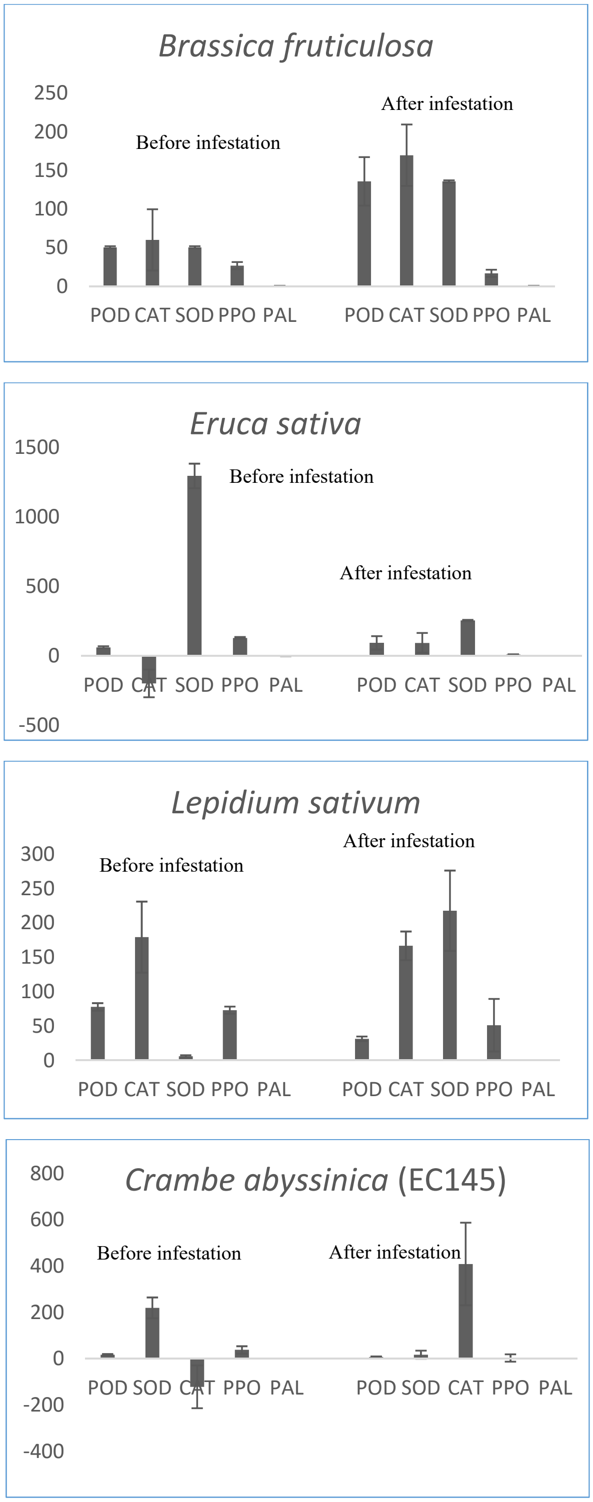


Fig 4.14 Defence related enzyme activity before and after aphid infestation in resistant wild *Brassica* species

4.2.3 Optimization of primer annealing temperature for PCR amplification of defence related genes

Genomic DNA was isolated from five representative wild Brassica species viz.*Brassica fruticulosa*, *Lepidium sativum*, *Crambe abyssinica* (EC145) and *Brassica chinensis***.** PCR amplification with the designed primers was performed separately using genomic DNA isolated from them. In primer optimization it was found that in *Brassica chinensis* which is a close relative of cultivated species *Brassica juncea* most of the designed primers showed single desired amplification, whereas successful amplification of all the primers in rest of the four wild Brassica species differed (Fig. 4.15 and Fig. 4.16).

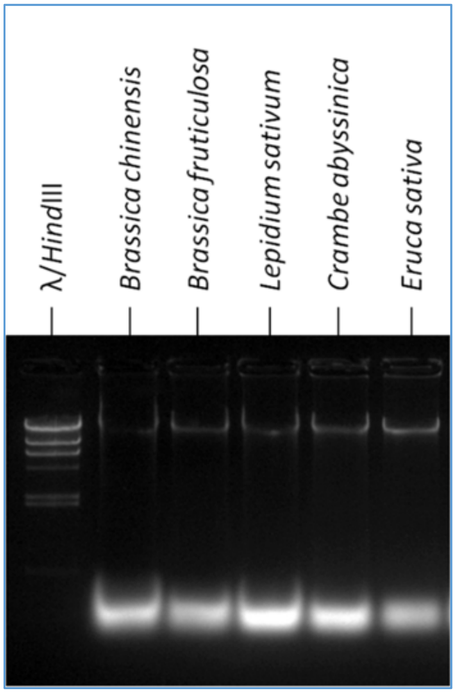
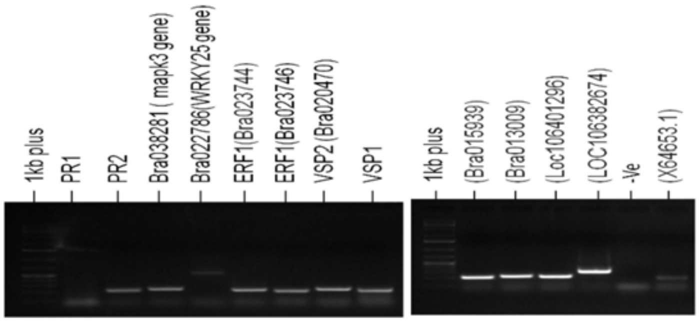


Fig 4.15 Genomic DNA isolated from five representative wild *Brassica* species viz; *Brassica fruticulosa*, *Lepidium sativum, Crambe abyssinica* (EC 145) and *Eruca sativa*.


1kb plus
PR1
PR2
Bra038281 (mapk3 gene)
Bra022786(WRKY25 gene)
ERF1(Bra023744)
ERF1(Bra023746)
VSP2 (Bra020470)
VSP1
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(Bra015939)
(Bra013009)
(Loc106401296)
(LOC106382674)
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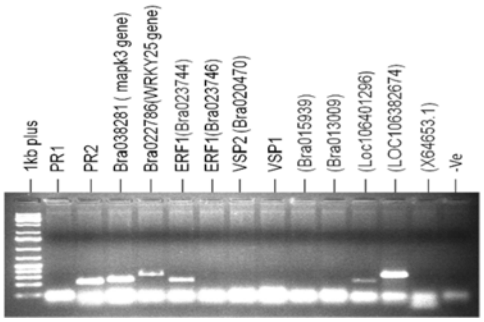
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Bra022786(WRKY25 gene)
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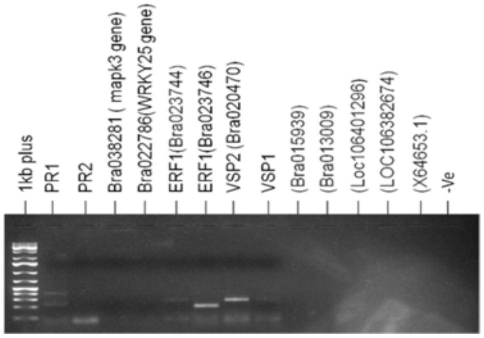
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Bra022786(WRKY25 gene)
ERF1(Bra023744)
ERF1(Bra023746)
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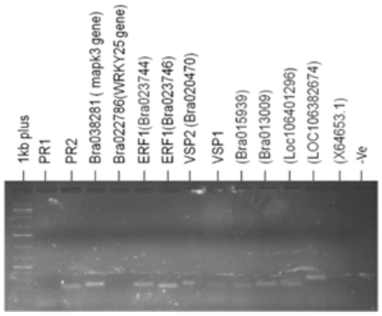
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Bra022786(WRKY25 gene)
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VSP2 (Bra020470)
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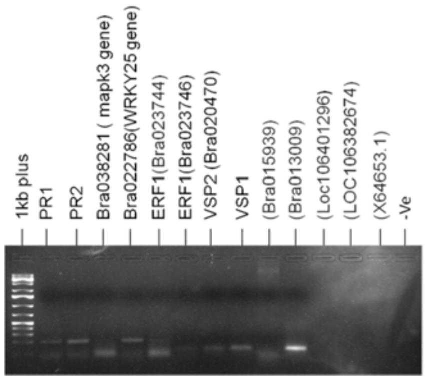


(5)

Fig 4.16 PCR amplification of defence genes using designed primers in genomic DNA isolated from (1) *Brassica chinensis,* (2) *Brassica fruticulosa*, (3) *Lepidium sativum*, (4) *Crambe abyssinica* (EC145) and (5) *Eruca sativa* respectively.

4.2.4 RNA isolation

The total RNA was isolated as per the method described earlier in sec 4.9.After DNase I treatment the concentration and quality of total RNA was determined by using Nano Drop 1000, (Thermo Scientific, USA). The quality was ensured by measuring the ratio of absorbance (OD) at 260 and 280-nm. If the ratio is between 1.8 and 2.0, the RNA quality was considered good quality and accepted. (Table 4 and Fig. 4.17).

Table 4. Concentration of RNA samples isolated from *Brassica* wild species

Wild *Brassica* species	RNA Concentration in before infestation sample(ng/µl)	Absorbance ratio (260/280)	RNA Concentration in after infestation sample(ng/µl)	Absorbance ratio (260/280)
Brassica chinensis	1047.9	1.92	1651.8	1.97
Brassica fruticulosa	1981.1	1.87	907.2	1.85
Lepidium sativum	1277.9	1.92	1857	1.83
Crambe abyssinica(EC 145)	1040.5	1.85	1849.7	1.86
Eruca sativa	1123.4	1.96	1740.9	1.99

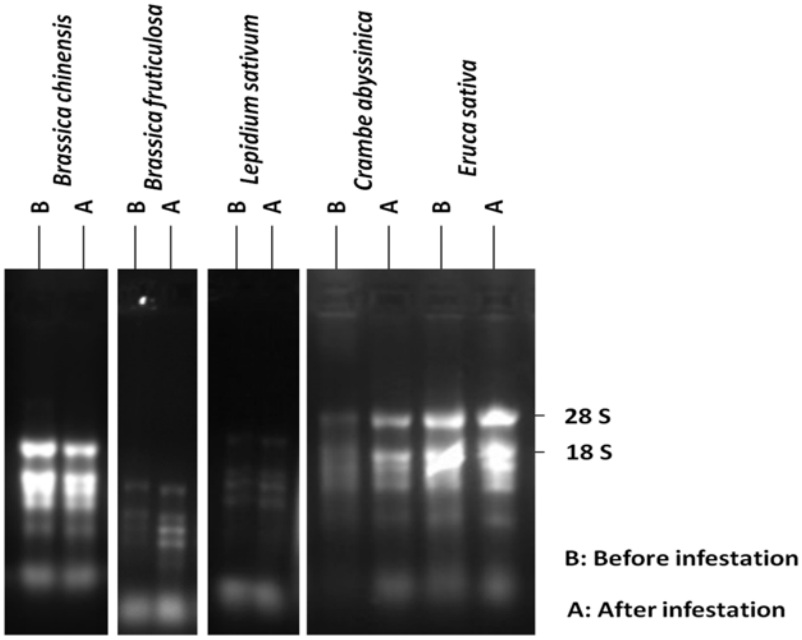


Fig 4.17 Total RNA isolated from five representative wild Brassica species before and after infestation viz;*Brassica chinensis*, *Brassica fruticulosa* , *Lepidium sativum, Crambe abyssinica (EC 145)* and *Eruca sativa* respectively.

4.2.5 cDNA preparation

After RNA quantification and quality check cDNA was prepared After RNA quantification and quality check cDNA was prepared from each RNA samples using 2.5 µg of DNase treated RNA synthesis using PrimeScript™ both for before and after infestation wild Brassica species (Fig.4.18) viz ; *Brassica chinensis*, *Brassicafruticulosa*, *Lepidium sativum*, *Crambe abyssinica* (EC 145)and *Eruca sativa* respectively. Further the quality of cDNA was checked by using housekeeping gene GAPDH.

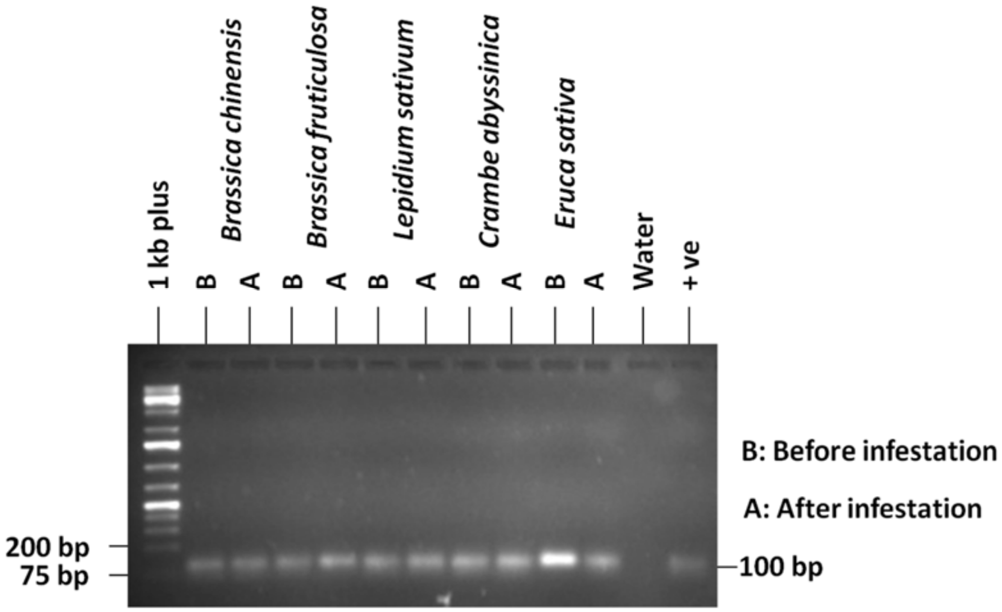
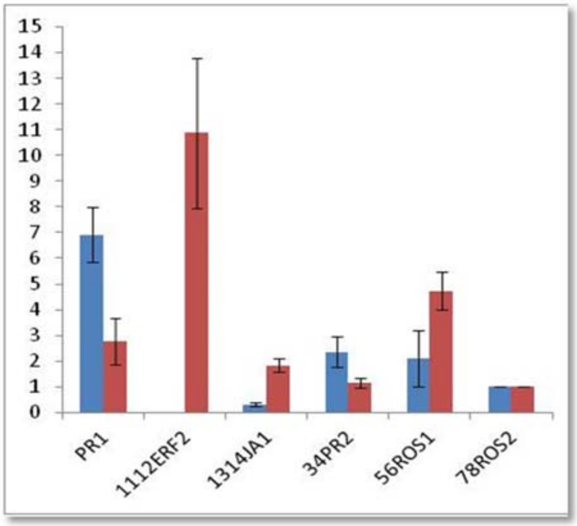


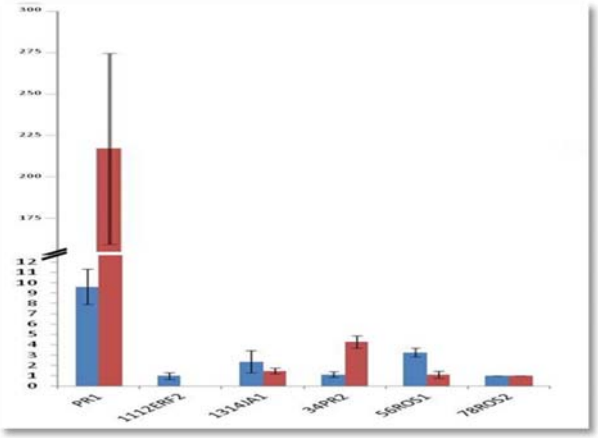
Fig 4.18 cDNA check using housekeeping gene GAPDH.

4.2.6 Real time qPCR based gene expression analysis

Quantitative real time PCR was carried out for expression analysis of defence related genes in five wild Brassica species (Fig. 4.19, Fig. 4.20). It was found four wild Brassica species showed upregulation for multiple defence related genes after infestation, while single gene upregulation was observed in *Eruca sativa*. In susceptible wild Brassica species *Brassica chinensis*two genes viz; PR1, PR2 genes involved in SA(salicylic acid) signalling pathways were up regulated after infestation. In *Brassica fruticulosa* three genes viz; ERF1, JA1 and map3 kinase genes involved in ethylene signalling, JA(Jasmonate) signalling and ROS were found to be up regulated after infestation. In *Lepidium sativum*four genes viz; PR1, PR2, JA1, map3 kinase genes involved in SA signalling, JA signalling and ROS pathway were found to be up regulated after infestation. In *Crambe abyssinica* (EC 145) two genes viz; PR2 and map3 kinase genes were found to be up regulated after infestation. In *Eruca sativa* a single gene PR2 involved in SA signalling was found to be up regulated after infestation.

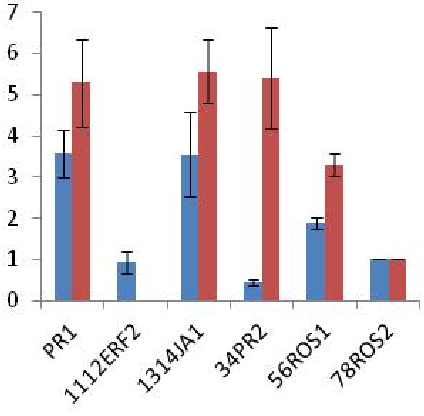


Brassica chinensis

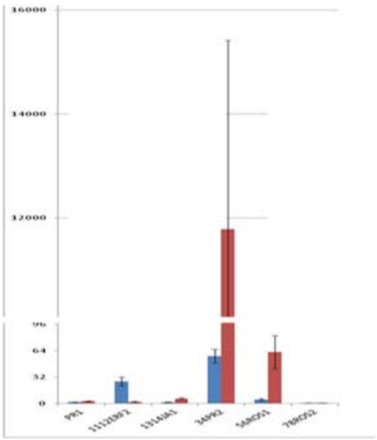


Brassica fruticulosa

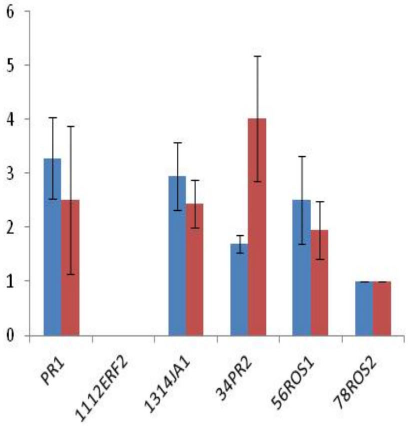
Fig 4.19 qPCR analysis of defence related genes in five wild Brassica species viz; *Brassica chinensis and Brassica fruticulosa* respectively



Lepidium sativum



***Crambe abyssinica* (EC 145)**



***Crambe abyssinica* (EC 145)**

Fig 4.20 qPCR analysis of defence related genes in wild Brassica species viz; *Lepidium sativum*, *Crambe abyssinica* (EC 145) and *Eruca sativa* respectively

4.3. Transcriptome analysis of *Eruca sativa* before and after aphid infestation

Transcript analyses of *Eruca sativa* leaf samples were carried out by using Illumina Genome Analyzer (101x2) bp Chemistry through outsourcing. Two samples constituted of the leaf sample before aphid infestation and the leaf sample after 30 days of aphid infestation. Total 10.92 GB raw data was generated (Fig. 4.21).

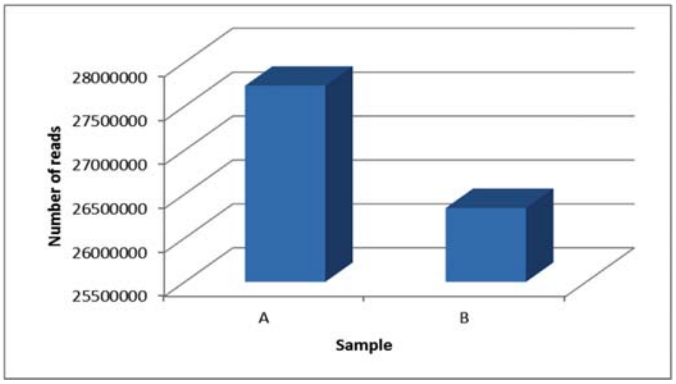


Fig. 4.21 Read length distribution

After removing the redundancy, a total of 29077 unigenes were used for differential expression analysis (Fig. 4.22). Out of which 4010 genes were having 2 fold change in transcript level when before and after infestation samples were compared. When 2 fold change and pvalue 0.05 both the filters were applied on all unigenes, 1307 genes qualified both filters. These 1307 unigenes were then used for further analysis. 852 genes were having expression in both samples, these genes were used for heatmap from 1307 significant unigenes (Fig. 4.23, Fig. 4.24)

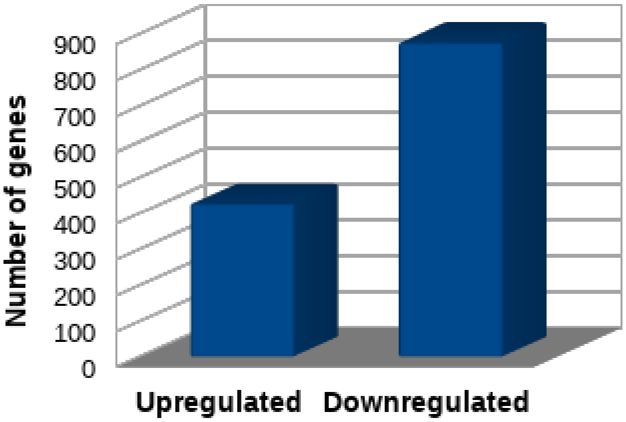


Fig. 4.22. The number of unigenes showing differential expression in *E. sativa* leaf sample in response to aphid infestation.

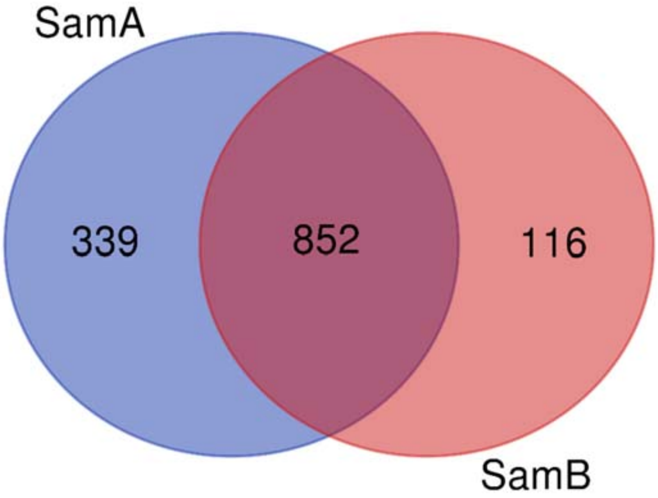


Fig. 4.23. Ven diagram showing commonly expressed genes in *E. sativa* leaf sample collected before and after aphid infestation.

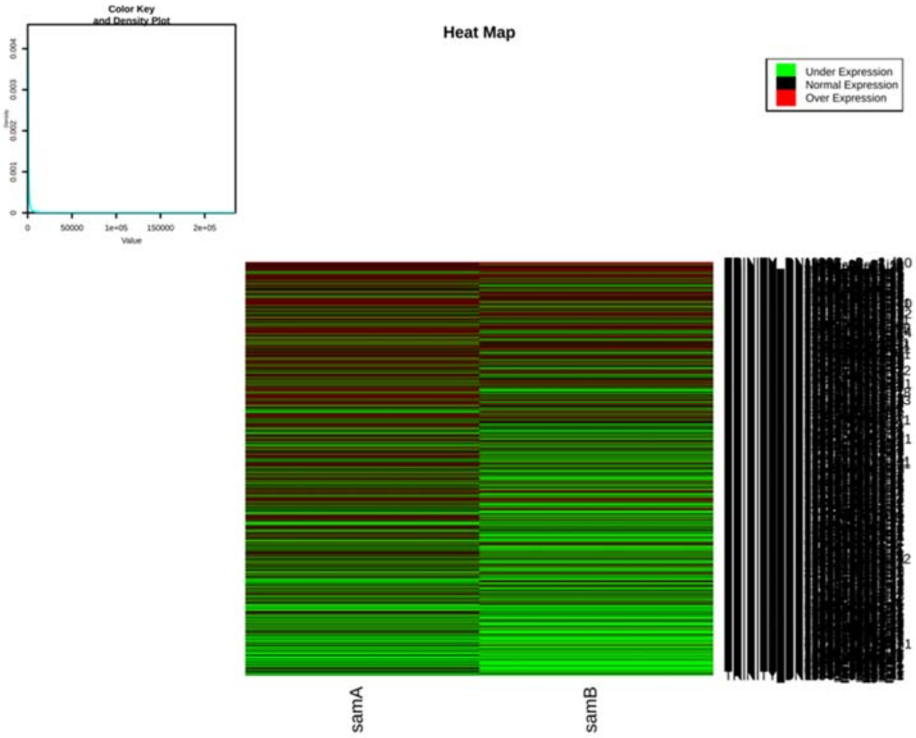


Fig. 4.24. Heat map showing differential expression of 1307 significant unigenes

However, due to paucity of time a detailed statistical analysis could not be done under the present study.

5. DISCUSSION

The genus *Brassica* constitutes an important group of vegetable and oilseed crops and possess great economic importance. The global production of rapeseed-mustard was 71.28 million metric tons, which constituted about 12.31% of the total oilseed production of 575.29 million metric tons in the year 2016-2017 (2016-17, FOP 04–11, USDA). In India, rapeseed-mustard contributes 28.6% of the total oilseed production and has the second highest production next to groundnut. The national production of rapeseed-mustard in 2018 was 6 million tons (SEA, India, 2018). The productivity of mustard is continuously decreasing over the years which has become a major impediment towards meeting the future projected demand of oilseed which is estimated to be 40 million tons by 2020. Because of rapid destruction of agricultural land in the name of development, horizontal increase in production by increasing acreage is not a viable option. Hence, quantum increase in productivity is to be achieved to meet the projected demand for oilseed. The present productivity of rapeseed-mustard in India is estimated to be 1183kg/ha (SOPA 2015-16), whereas average global productivity is 2144 kg/h (www.nmoop.gov.in, Status paper on rapeseed 2015-16). Thus it has become essential to fill up this gap in mustard productivity in India as well further enhance the productivity. In most parts of India, agriculture is resource poor and with limited availability of inputs major attention is given to rice and wheat. Therefore, we rely much on genetic potential of the crop varieties to perform in giving higher productivity in spite of suboptimal inputs. Indian mustard, *Brassica juncea* is the chief member of rapeseed-mustard group of crops. The productivity of mustard is affected due to stresses of biotic and abiotic nature. Among the biotic stresses damage due to aphid infestation is significant and wide spread (Raj et al. 2017). Aphids belong to the family aphididae. Aphids feed by diverting excessive phloem sap because of which the infested plants become devitalized and poor in yield. Aphids display a unique life cycle, which enables them to multiply rapidly and colonize the whole plant. Aphids poses a high potential for yield reduction of *Brassica* crops, which ranges from 9- 91% (Singh and Sachan 1994).

Among the available control measures for aphid control, the chemical control measures are widely practiced as the pesticide agrochemicals are easily available due to efficient marketing network of private companies. However, common farmers fail to realize hazardous implications of indiscriminate use of insecticides. Such implications include environmental pollution, residual effects of pesticides in plant body and eventual entry into the human food chain, secondary pest outbreak etc. in addition to involvement of high cost. Thus, it is responsibility of the scientific community to develop and provide safer alternative control measures to the farmers. Cultivation of aphid resistant mustard cultivars can potentially minimize use of chemical insecticides. However, developing aphid resistance in the cultivated varieties has been difficult as the source of resistance genes are not known or available to the breeders. The very little progress in these fields can be attributed to the non-availability of enough information about resistant sources and no systematic study regarding the biochemical basis of resistance. There have been limited efforts in developing aphid resistant transgenic plants by expressing lectin or protease inhibitor genes and also by utilizing gene silencing technology (Hilder et al. 1987, Hossain et al. 2006, Sadeghi et al. 2007, Saha et al. 2006) But the achieved resistance in all of these examples was not good enough to be applicable at the field level.

In absence of resistance gene(s) in the primary germplasms, wild relatives of the cultivated species may serve as potential source of useful genes in crop improvement (Ellis and Farrell 1995). There are many wild relatives of *Brassica* available in India. However, previously there has been no study on characterizing these wild relatives of Brassica in terms of aphid resistance. Hence, in the current study, a screening for evaluation of aphid resistance in a set of Brassica wild species was conducted. For the experimental purpose, total 22 species of wild relatives were grown in net house and screened for resistance to mustard aphid (*Lipaphis erysimi*). Screening was performed by artificial infestation of the plants in which an initial number of aphids (approximately 100) was released on each plant and success in infestation as well as population build up over a period of 30 days were monitored. According to the response of the wild species towards aphid infestation they were categorized into three groups viz. resistant, moderately resistant and susceptible to aphid infestation. All the species were scored in terms of their differential susceptibility/resistance to aphid infestation and entered into one of the three groups. In resistant species viz. *Eruca sativa*, *Brassica fruticulosa, Lepidium sativum, Crambe abyssinica* after inoculation either the infestation did not succeed or the multiplication was significantly restricted. Whereas in case of susceptible species viz. *B. chinensis*, *Diplotaxis cretacia* etc. following the inoculation the number of aphids rapidly increased to 30-40 folds due to parthenogenetic multiplication. The species which were comparatively resistant to mustard aphids are listed in Table 2. Further, the biochemical basis of differential aphid response was further studied in the three groups by enzymatic analysis of defence related enzymes such as POD, CAT, SOD, PPO and PAL and gene expression study of defence related genes based on RT- qPCR.

In response to insect and pathogen attack several reactive oxygen species (ROS) are generated in the host plant as a part of defence signalling process. Plants concomitantly activate antioxidant enzymes superoxide dismutase, catalase, peroxidase etc. which are primarily responsible for scavenging of ROS (reactive oxygen species) (Karpinski and Muhlenbock 2007). However, early activation of host-catalase may lead to retardation of H_2O_2 mediated signaling (Koramutla et al. 2014). The assay of defence related enzymes showed that most of the species which are susceptible to aphids showed increase in catalase activity after aphid infestation compared to species categorized as moderately resistant and resistant to aphids. Interestingly in resistant species viz. *Brassica fruticulosa, Lepidium sativum,* and *Crambe abyssinica* (EC 145) either the basal level of catalase was low or it decreased after aphid infestation. In endogenous defence response of plants against biotic stresses plant secondary metabolites play an important role. The levels of plant secondary metabolites are modulated by host defence enzymes peroxidase (POD), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) (Tian et al. 2014). The POD activity even without aphid infestation was higher in many of the resistant species viz. *Brassica fruticulosa, Lepidium sativum,* and *Crambe abyssinica* (EC 145) which also showed higher level of PPO activity either before or after aphid infestation. The PAL activity though widely varied across the wild species found to be low in most of the species irrespective of aphid resistance trait. Overall, wide variation in activity of different defence related enzymes and lack of clear trend in aphid induced activation across the resistant or susceptible species indicated involvement of more complex processes and mechanism of defence against aphids.

Since genomic sequence information was not available for the wild species, for gene expression study primers had to be designed based on heterologous sequence information. As a result, some of the primers failed to amplify the desired gene in many of the wild species. Therefore, an initial optimization of the PCR conditions particularly annealing temperature using the heterologous set of primers was necessary before RT-qPCR study. The gene expression analysis of selected defence related genes indicated their transcriptional activation in response to aphid infestation.

The wild species identified and categorized as resistant in this study can serve as source for aphid resistance genes. However, accessing those genes from any of these wild relatives of *Brassica* and introgression into the cultivated germplasms will require distant hybridization followed by several rounds of back crossing (Vasupalli et al. 2017). The breeding process for gene introgression from wild relatives will be tedious as well as time consuming. In this context, any genomic information on the set of genes involved in resistance in donor species will be helpful in developing molecular markers that will enhance the breeding efficiency and may reduce the no. of backcrosses required. Therefore, transcriptome data of *Eruca sativa*, one of the resistant type wild species has been generated under two conditions: before and after aphid infestation. However, due to paucity of time detailed statistical analysis of the transcriptome data could not be carried out. Nevertheless, the data of this research led to the foundation of gene introgression from wild relatives of *Brassica* for aphid resistance.

6. SUMMARY & CONCLUSIONS

The genus *Brassica* constitutes a very important group of vegetable and oilseed crops. In 2016-17, the global production of rapeseed-mustard was 71.28 million metric tons amounting about 12.31% of the total global oilseed production; whereas the national production was amounted to be 6.8 million tons in 2017-18. In India, *Brassica juncea* (Indian mustard) is the major oil producing crop among the *Brassica* oilseeds.

Among the many factors limiting the productivity of *B. juncea,* the biotic stress imposed by aphids are very noteworthy. Aphids, a member of the family aphididae, feed on the sugary sap derived straight from the phloem tissue through their hardened stylet. Aphids cause a significant damage to the crop yield at the national level. They can reduce the seed yield of *B. juncea* by 9-91%. The chemical methods are the most widely used control measures adopted by farmers as it is easily available to the farmers. The use of chemical control measures for aphid control has many adverse effects towards environment and animal health in addition to being costly. Also, there are chances of development of resistance in certain strains of aphid towards the agrochemicals frequently used. Unfortunately, there has been not much progress regarding development of aphid resistant cultivars of *B. juncea* through classical breeding procedures. For development of resistant cultivars, precise information about the sources of resistance are essential. Although, few resistant genes have been reported in other crops like *Mi-1.2* gene in tomato and *Pti like kinase* genes in wheat but resistant genes are yet to be discovered in *Brassica* crops. So, the non-availability of resistant gene within the crossable germplasms is the major bottleneck in developing aphid resistant cultivars in *B. juncea*. Under the present constraint, the wild relatives of *Brassica* can serve as potential source of resistance which can be subsequently brought in to the cultivated species through breeding methods. Large numbers of genus and species under the Brassicaceae family forms a huge pool of wild relatives. This large reservoir of wild relatives has never been systematically explored for possible source of aphid resistance. Therefore, the following objectives were undertaken in the present investigation with an aim to identify a wild relative of *B. juncea* with genetic resistance to mustard aphids.

1: Screening for aphid resistance in wild relative species of *Brassica.*

2*:* Biochemical and gene-expression analysis of defence related genes and enzymes in selected wild germplasms showing resistance to mustard aphids

Under the first objective screening for natural aphid resistance present in wild *Brassica* species was carried out. A total of 22 *Brassica* species (including accessions) were used in screening for aphid resistance. Depending on the outcome of the screening experiment these wild *Brassica* species were categorised into resistant, moderately resistant and susceptible groups. The demograph for aphid multiplication as generated for the 3 groups showed significantly different rates of aphid multiplication on the host. A rapid rate of multiplication and higher number of aphids were observed in susceptible wild *Brassica* species, whereas in resistant species the multiplication was either restricted or infestation failed. An intermediate response was observed in case of moderately resistant species. The classification generated in this study will be an important resource to the breeder to initiate breeding programme for introgression of resistant genes.

Under the second objective the biochemical analysis and gene expression studies were carried out for corroborating molecular basis of differential resistance shown by the wild species. In general, in biochemical assay as well as gene expression study the resistant species showed higher activity for a greater number of defense related enzymes compared to susceptible species.

The salient findings of the study are presented below:

- Out of 22 wild spices 4 found to be resistant and 2 are moderately resistant to aphid infestation.
- Biochemical analysis revealed more activation of redox signalling related genes and other defence genes in resistant species compared to their activation in susceptible species following aphid infestation.
- More number of enzymes shown higher activity in resistant species compared to moderate and susceptible types.
- Interestingly, catalase activity was found to be higher in susceptible plant types infested with aphids.

- The data of this research led to the foundation of gene introgression from wild relatives of *Brassica* for aphid resistance.
- Based on the data, several crosses have been made by the host laboratory.
- Transcriptome data was generated for one of the resistant species *Eruca sativa*. The generated genomics data will be useful in developing markers to assist in gene introgression process.

ABSTRACT

Title: Molecular and biochemical basis of differential aphid infestation in wild species of *Brassica.*

Rapeseed-mustard accounts for approximately 13% of the total global oilseed production. The global productivity of rapeseed-mustard is estimated to be 2244kg/ha. In the National scenario the productivity of rapeseed-mustard is 1183kg/ha, which is much lower than the global average. Indian mustard (*Brassica juncea*) is the chief oil yielding crop in this group. The productivity of mustard is severely affected by various biotic and abiotic stresses among which most notable is the biotic stress imposed by aphids. Aphids are light yellow to dark grey in colour, they feed by extracting the sugary sap from the phloem through their hardened stylet. Aphids poses a very high potential of yield reduction ranging from 9 to 91%. The available control measures such as physical, chemical and biological control measures are seemed to be very ineffective. Though the chemical control measures are the most predominantly used control measure but its detrimental effects towards environment and animal health has urged the scientific community to look for more eco-friendly and sustainable approaches for aphid control. In this respect the natural resistance present among the host plants as genetic resistance forms the best alternative. But unfortunately, due to non-availability of crossable germplasms no resistant cultivar is so far produced for cultivable germplasms.

In the present study screening for aphid resistance in 22 wild relatives of *Brassica* was carried out, out of which 4 found to be resistant and 2 are moderately resistant to aphid infestation. Further Biochemical and gene-expression analysis of defence related genes and enzymes in selected wild germplasms showing resistance to mustard aphids was carried out. The biochemical study revealed more activation of redox signalling related genes and other defence genes in resistant species compared to their activation in susceptible species following aphid infestation as a greater number of enzymes showed higher activity in resistant species compared to moderate and susceptible types. Also, transcriptome data was generated for one of the resistant species *Eruca sativa*. The generated genomics data will be useful in developing markers to assist in gene introgression process. The data of this research led to the foundation of gene introgression from wild relatives of *Brassica* for aphid resistance which can be utilised in distant hybridisation programs for development of resistant cultivars in *Brassica* cultivated species.

Keywords :- Rapeseed Mustard, Germplasms, Transcriptome, *Eruca sativa,* Aphid Resistance

सार

शीर्षक: ब्रासिका की जंगली प्रजातियों में अंतर एफिड्स संक्रमण के आणविक और जैव रासायनिक आधार।

रेपसीड-सरसों कुल वैश्विक तिलहन उत्पादन का लगभग 13% है। रेपसीड-सरसों की वैश्विक उत्पादकता 2244 किलोग्राम / हेक्टेयर आंकी गई है। राष्ट्रीय परिदृश्य में रेपसीड-सरसों की उत्पादकता 1183 किग्रा / हेक्टेयर है, जो वैश्विक औसत से काफी कम है। भारतीय सरसों (ब्रैसिका जंकिया) इस समूह में मुख्य तेल उपज वाली फसल है। सरसों की उत्पादकता विभिन्न जैविक और अजैविक तनावों से गंभीर रूप से प्रभावित होती है, जिनमें से सबसे उल्लेखनीय एफिड्स द्वारा लगाया गया जैविक तनाव है। एफिड्स हल्के पीले से गहरे भूरे रंग के होते हैं, वे अपने कठोर स्टाइललेट के माध्यम से फ्लोएम से शर्करा के सैप को निकालकर खाते हैं। एफिड्स से उपज में 9 से 91% तक की कमी की संभावना है। उपलब्ध नियंत्रण उपाय जैसे कि भौतिक, रासायनिक और जैविक नियंत्रण उपाय बहुत अप्रभावी प्रतीत होते हैं। यद्यपि रासायनिक नियंत्रण उपाय मुख्य रूप से उपयोग किए जाने वाले नियंत्रण उपाय हैं, लेकिन पर्यावरण और पशु स्वास्थ्य के लिए इसके हानिकारक प्रभावों ने वैज्ञानिक समुदाय से एफिड नियंत्रण के लिए अधिक पर्यावरण-अनुकूल और टिकाऊ दृष्टिकोण की तलाश करने का आग्रह किया है। इस संबंध में आनुवंशिक प्रतिरोध के रूप में मेजबान पौधों के बीच मौजूद प्राकृतिक प्रतिरोध सबसे अच्छा विकल्प है। लेकिन दुर्भाग्य से, क्रॉस्बल जर्मप्लाज्म्स की अनुपलब्धता के कारण कोई प्रतिरोधी प्रजातियां अभी तक खेती योग्य उत्पन्न नहीं हुआ है।वर्तमान अध्ययन में ब्रैसिका के 22 जंगली रिश्तेदारों में एफिड प्रतिरोध के लिए स्क्रीनिंग की गई थी, जिसमें से 4 प्रतिरोधी पाए गए और 2 एफिड संक्रमण के लिए मामूली प्रतिरोधी हैं। सरसों एफिड्स के प्रतिरोध को दिखाने वाले चयनित जंगली जर्मप्लाज्म्स में रक्षा संबंधी जीनों और एंजाइमों के आगे जैव रासायनिक और जीन-अभिव्यक्ति विश्लेषण किया गया था। जैव रासायनिक अध्ययन में रेडॉक्स सिग्नलिंग संबंधित जीनों और प्रतिरोधी प्रजातियों में अन्य रक्षा जीनों की अधिक सक्रियता का पता चला, जो कि एफिड इन्फेक्शन के बाद अतिसंवेदनशील प्रजातियों में उनके सक्रियण की तुलना में अधिक संख्या में एंजाइमों में मध्यम और अतिसंवेदनशील प्रकारों की तुलना में प्रतिरोधी प्रजातियों में उच्च गतिविधि दर्शाते हैं। इसके अलावा, प्रतिरोधी प्रजातियों एरुका सतीवा में से एक के लिए ट्रांसक्रिप् टोम डेटा उत्पन्न किया गया था। उत्पन्न जीनोमिक्स डेटा जीन अंतर्मुखी प्रक्रिया में सहायता करने के लिए मार्करों को विकसित करने में उपयोगी होगा। इस शोध के आंकड़ों ने एफिड प्रतिरोध के लिए ब्रैसिका के जंगली रिश्तेदारों से जीन अंतर्मुखता की नींव को जन्म दिया, जिसका उपयोग ब्रैसिका की खेती की प्रजातियों में प्रतिरोधी खेती के विकास के लिए दूर संकरण कार्यक्रमों में किया जा सकता है।

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Appendix1

Brassica chinensis

Brassica fruticulosa

Brassica fruticulosa
(Spain)

Brassica spiniscens

Brassica tournefortii
(RBT 2003)

Camelina sativa

Diplotaxis cretacia

Diplotaxis erucoides

Diplotaxis gomezcampoi

Diplotaxis enuisilique

Erucastrum canariense

Erucastrum cardaminoides

Erucastrum gallicum

Orychophragmus violaceous

Oxycampus sp.

WDKY

Crambe abyssinica
(EC 145)

Crambe abyssinica
(EC 159)

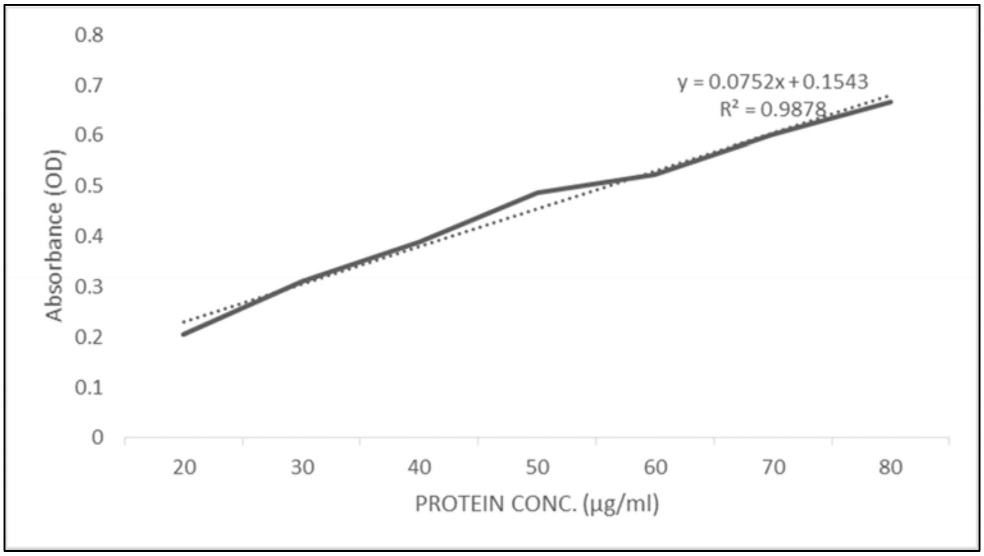
Brassica cysimbrium

Eruca sativa

Lepidium sativum

Crambe abyssinica
(EC071)

Appendix 2



Protein standard curve