To Study the Defensive Response of Kale against Cabbage Butterfly (*Pieris brassicae* Linn.)

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(2013-A-976-M)

Division of Entomology

Faculty of Agriculture

Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir

2015

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Thesis

Submitted to

The Faculty of Agriculture
Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir
in partial fulfilment of requirements for the award of the degree of

Master of Science in Agriculture
(Entomology)

2015

Dedicated

to my beloved Parents

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Agriculture, Division of Entomology

Certificate – I

This is to certify that the thesis entitled, **"To Study the Defensive Response of Kale against Cabbage Butterfly (*Pieris brassicae* Linn.)"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science in Agriculture (Entomology)**, to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Shahida Ibrahim (Regd. No. 2013-A-976-M)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or information received during the course of investigation has duly been acknowledged.

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We, the members of the Advisory Committee of **Ms. Shahida Ibrahim (Regd. No. 2013-A-976-M)**, a candidate for the degree of **Master of Science in Agriculture (Entomology)** have gone through the manuscript of the thesis entitled, **"To Study the Defensive Response of Kale against Cabbage Butterfly (*Pieris brassicae* Linn.)"** and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

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This is to certify that the thesis entitled, **"To Study the Defensive Response of Kale against Cabbage Butterfly (*Pieris brassicae* Linn.)"** submitted by **Ms. Shahida Ibrahim (Regd. No. 2013-A-976-M)** to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** in partial fulfilment of the requirements for the award of the degree of **Master of Science in Agriculture (Entomology)** was examined and approved by the Advisory Committee and External Examiner on …………….

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ABSTRACT

The present investigation entitled "To study the defensive response of Kale against Cabbage butterfly (*Pieris brassicae* Linn.)" was undertaken in net house and in laboratory of Research and Training Centre of Pollinizers, Pollinators and Pollination Management (RTCPPPM), SKUAST-Kashmir, Shalimar. There were four treatments (01+03) and the design of experiment was Randomized complete block design. Defensive response to *P. brassicae* through exogenous supply of jasmonic acid and salicylic acid was studied on Kale varieties (Khanyari and Kawdari) with different levels of resistance and susceptibility under net house conditions. Amounts of secondary metabolites and proteins were quantified at 6 days after jasmonic acid (1 mM) and salicylic acid (1 mM) application/insect infestation. Data were also recorded on plant damage and *P. brassicae* larval weight. The highest amount of secondary metabolites (Phenol, (196.07 μg/g) condensed tannin (301.57 μg/g) and flavonoids (170.37 μg/g)) and total protein (0.69 mg/g) were observed in plants treated with jasmonic acid and then infested with *P. brassicae* larvae as compared to plants treated with Salicylic acid + insect infestation. Among varieties the maximum amount of secondary metabolites and total protein was observed in variety Khanyari. Plants sprayed with jasmonic acid showed lower weight (100.17 mg) of *P. brassicae* larvae as compared to plants sprayed with salicylic acid, on the other hand plants which were not sprayed by any chemical showed highest larval weight (180.79 mg). Per cent damage was lowest in jasmonic acid treated plants (9.73%) and highest in plants that were not treated by phytohormone (23.61%). Among different concentrations of jasmonic acid the highest amount of secondary metabolites i.e., phenolic content (223.30 µg/g), condensed tannin (349.31 µg/g), total flavonoids (243.00 µg/g) and total protein (0.829 mg/g) was found in 1.5 mM concentration of jasmonic acid.

Key words: Jasmonic acid, salicylic acid, insect infestation, phytohormones

Signature of Student   Signature of Major Advisor

Dated_____________   Dated __________________

ACKNOWLEDGEMENT

ALL PRAISES AND THANKS TO 'ALLAH' THE ALMIGHTY, THE MOST BENEFICIENT, MERCIFUL AND COMPASSIONATE AND ENDLESS BLESSINGS BE UPON BELOVED PROPHET MOHAMMAD (S.A.W) THE GREAT BENEFACTOR OF MANKIND

It gives me immense pleasure to express my sincere gratitude to Chairman of my Advisory Committee, Dr. Barkat Hussain, Assistant Professor ,Division of Entomology, SKUAST-K for his able guidance, valuable suggestions, constructive criticism and constant encouragement during the investigations and preparation of the manuscript.

I extend my sincere thanks to the members of my Advisory Committee – Dr. G.M.Mir, Professor and Head (Entomology), Dr. S.A Mir, Associate Professor and Head (Agri-Statistics), Dr. M.D.Shah, Assistant Professor (Plant Pathology) and Dr. M.K.Sharma, Associate Professor, Division of Pomology SKUAST-K for their precious suggestions and comments in the present work which greatly improved its presentation.

During my studies in SKUAST-Kashmir I have found a person, who reciprocates my sincerity and loyalty, props my toppling faith, extends a selfless smile. He is my best teacher- Dr. F.A. Zaki, Dean Horticulture, SKUAST-K, Shalimar. It is my proud claim to be his taught.

I am highly thankful to all members of teaching/research and supporting staff of Division of Entomology, especially to Dr. R.K.Nehru, Dr. R.A.Wani, Dr. A. A. Khan, Dr. Jamal, Dr. Asma Sherwani, Dr. M.A. Malik, Dr. M.A. Parray, Dr. Munazah, Dr. Rizwana, Dr. Askaray, Dr. M.A.Sofi, Dr.M.A.Lone, Dr. Shaheena .Gul, Dr..Pathiania,Dr.Ishtiyaq, Mr. Nasir Ahmad, Mr. Yaqoob Ahmad, Mr. Mohammad Ramzan, Mr. Bashir Ahmad, Ms. Mehboooba and Ms. Jameela for their constant help, encouragement and suggestions as and when needed.

My sincere thanks to worthy Vice Chancellor, Director Resident Instructions, Director Research, Director Extension Education and Registrar SKUAST-K for their kind patronage.

I also acknowledge staff of Central Library, SKUAST-K for their cooperation and generosity.

I owe my sincere thanks to my friends and colleagues particularly Ms Varsha Rajput, Ms. Rahila Shameem, Ms. Shifa Muneer, Ms.Deelak Amin, Ms. Fozia Bari, Ms Rehana Mohiuddin, Ms. Rehana Javeed, Ms. Irham Rasool, Ms. Mehnaz Nisar, Ms. Parveen Akhtar, Ms.Rafiza,Ms.Sumaira Majeed, Ms. Anjum Aara, Ms. Irshad Anjum, Ms Gulshan, Ms.Asmat Aara, Ms Shahida

Nisar, Ms Gazala Nazir, Ms Rehana Jan, Ms Arifa, Ms.Nighat Mushtaq, Ms Mubeena , Ms. Qurat, Ms.Insha, Ms. Ujwala, Mr. Asad Ahmad,Mr.Ishtiyaq Ahmad, Mr. Sayed Ishtiyaq, Mr.John Shahid, Mr.Amin Mr. Nadeem Ahmad and Mr.Shafeeq for their cordiality, exchange of views on various topics, support and joyous company.

I unfeignedly owe my parents (Papa and Mummy) a tremendous filial respect and gratitude for their unending love and affection. They have been instrumental in every success that I have achieved hitherto. They have really endured every sort of hardship and privation for my sake only to reassure that I am not put to the trials and tribulations of life. I cannot afford to forget the times when my parents used to share my worries during my quarterly life crisis and never let me lose hope. The essence of their pristine love is inexplicably unforgettable.

The contribution of my mother, Mrs.Gulzar Begum, in my upbringing is immense and unfathomable. I can hardly imagine a moment of my life when her love did not play along. The paradise of my dreams is assuredly beneath her feet.

The moral support and confidence-build up provided by my father, Mr. Mohd Ibrahim, in my meek times has been pivotal during my studies. Whenever anything fascinated my eyes I never received a "No" from him. He always wanted me to excel in my academic and research endeavours and never let me fall victim to the misgivings and premonitions. He is the orchestrator of the chapters of the book of my life.

I am colossally grateful to the unconditional love and support of my loving brothers and sister namely, Mohammad Rafi, Qamar Ibrahim ,Khalid Ibrahim and Zahida Ibrahim. Their presence in my life has been a solace of my eyes bestowed on me by my Lord, Allah, the Almighty. My love increases for them with every passing day.

I also avail this opportunity to pay my hearty thanks to my Dadi, Nana, Nani, my uncles and all relatives and nears and dears for their constant unending prayers blended with pious, blessings and scarifies which enabled me to complete this programme successfully.

Shahida Ibrahim

Place : Shalimar, Srinagar

Dated :

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INTRODUCTION

Vegetables play an important role in agricultural economy and are an essential component of the human diet. Vegetables occupy 12 per cent of the total area under cultivation in India (Butani and Jotwani, 1984). In Kashmir, the area under vegetables is 30.25 thousand hectare with an annual production of 7.70 lakh tonnes (Kamili *et al.*, 2012). Vegetables constitute many groups and among these, cole crops form an important place comprising of a wide range of leafy cole crops of family *Brassicacea*. All cole crops are originated from Europe and cultivated all over the world from Arctic to subtropical region and also at higher altitude of tropics (Hill, 1975). In the valley, the commonly grown cole crops are Kale, Knol-khol, Cabbage and Cauliflower and area under Kale is 3600 ha with an annual production of 1, 06,780 tonnes (Anonymous, 2012).

Kale, Knol-khol, Cabbage and Cauliflower are widely grown vegetable crops in Kashmir Valley. These crops are attacked by a number of insect pests; the cabbage butterfly (*Pieris brassicae)* is one of the limiting factor for the successful cultivation of cole crops. *P. brassicae* (Linn.) (Lepidoptera: Pieridae), is a pest distributed all over the world (Feltwell, 1978). In Indian subcontinent, it is distributed along Himalayan region including Pakistan, Nepal and throughout the plains except southern states of India (Lal and Ram, 2004; Younas *et al.*, 2004). The losses caused by *P. brassicae* can be more than 50 per cent in Kashmir (Bhat, 2008). Host plant resistance to pests is the result of co-evolution between the plants and the insects for millions of years. Plants and insects have been living together for more than 350 million years. In co-evolution, both have evolved strategies to counter each other's defence system. This evolutionary arms race between plants and insects has resulted in the development of an elegant defence system in plants that has the ability to recognize the non-self-molecules or signals from damaged cells due to feeding by the herbivores thus, activates the plant immune response against them. Plants respond to herbivore through various morphological, biochemical and molecular mechanism to counter the effects of herbivore attack. To counter this type of herbivore attack, plants produce specialized morphological structures or secondary metabolites and proteins that have toxic, repellent and anti- nutritional effects on the insects.

Induced biochemical defences in plants are manifested through the production of anti-digestive proteins and toxic secondary metabolites (Kessler and Baldwin, 2002; Howe and Jander, 2008; Usha and Jyothsna, 2010). Secondary metabolites have diverse roles in plants and are involved in plant defence against biotic and abiotic stress responses and in hormonal regulation. Some of the secondary metabolites are phenolics, flavonoids, tannins etc.

Phenolic compounds are widely produced in plants and have been implicated in many important "secondary" ecological roles, including plant interaction with insects and microbes (Matsuki, 1996; Cooper-Driver and Bhattacharya, 1998; Taiz and Zeiger, 2006). Plant phenols play an important part in biochemical defence of plants against insect pests (Sharma *et al.,* 2009; Ushaand Jyothsna, 2010; Ballhorn *et al.,* 2011). Qualitative and quantitative alterations in phenols and elevation in activities of oxidative enzymes in response to insect attack is a general phenomenon (Usha and Jyothsna, 2010; Barakat *et al.,* 2010; He *et al.,* 2011).

Flavonoids are the major plant defensive phenolic compounds utilized against a variety of stresses (Treutter, 2006). More than 5,000 flavonoids have been reported in plants, which play a central role in various facets of plant life especially in plant-environment interactions (Treutter, 2006). Flavonoids and isoflavonoids protect the plant against insect pests by influencing the behaviour, growth and development of insects (Simmonds, 2003).

Tannins are astringent (mouth puckering) bitter polyphenols. They are divided into two groups; condensed and hydrolysable tannins. Condensed tannins are the important and highly effective plant secondary metabolites involved in defence against herbivory (Forkner*et al.,* 2004; Sharma *et al.,* 2009).

The nutritional requirements of insects are similar to other animals and any imbalance in digestion and utilization of plant proteins by insects results in drastic effects on insect physiology.

Jasmonic acid (JA) is the most important phytohormone linked to plant defence against herbivores and activates the expression of both direct and indirect defences (Shivaji *et al.,* 2010). Jasmonic acid, its precursors and derivatives, collectively called jasmonates, represent a family of oxylipins that play an important role in a variety of plant processes including defence against insects, abiotic stresses, growth and development, fertility and senescence (Kumari *et al.,* 2006; Wasternack, 2007; Pauwele *et al.,*2008; Shivaji *et al.,* 2010; Kanno *et al.,* 2011).

Salicylic acid (SA), a benzoic acid derivative, is an important phytohormone involved in regulation of plant defence. It is important endogenous plant growth regulator that generates a wide range of metabolic and physiological responses in plants involved in plant defence (Zhao *et al.,* 2009; Vicent and Plasencia, 2011; Kanno *et al.,* 2011). The exogenous spray of these two phytohormones induces the resistance in host plant by activating the secondary metabolites in plants.

Plant resistance against insect pests can be constitutive or induced. Constitutive resistance is always present in plants irrespective of the external stimuli, while as the induced resistance is produced in response to the wounding, infestation by insects or by elicitor application. To counter attack the attacking insect, plants produce myriad specialized metabolites and proteins that have toxic, repellent, or anti-nutritional effects on the attacker (Duffey and Stout, 1996; Ryan, 2000; Wittstock and Gershenzon, 2002; Zhu-Salzman *et al.,* 2008; Usha and Jyothsna, 2010; He *et al.,* 2011). Induced resistance is very rapid and highly dynamic and plays an important role in protecting plants from further damage

(Karban and Baldwin, 1997; Kessler and Baldwin, 2002; Howe and Jander, 2008). Host plant resistance is the most economic and eco-friendly method of controlling insect pests (Sharma and Ortiz, 2002).

Keeping the above mentioned facts in consideration, the present study entitled, “To study the defensive response of Kale against Cabbage butterfly (*Pieris brassicae* Linn.)” was undertaken with following objective:

1. To study the induced resistance against cabbage butterfly on Kale.

Chapter - 2

REVIEW OF LITERATURE

2.1 Induced resistance

Induction of trichomes in plants in response to herbivory was considered an important defensive strategy to minimize subsequent damage by the herbivores (Agrawal, 1999a; Traw and Dawson, 2002). Trichomes and their exudates influenced both larval feeding and oviposition by insects (Handley *et al.,* 2005).

Numerous agricultural and wild plants demonstrate induced responses to insect damage. Induced responses are any change that occurs after herbivore attack. Many studies had documented negative effects of induced responses on herbivore preference or performance (Tallamy and Raupp, 1991; Stout and Duffey, 1996; Karban and Baldwin, 1997).

Resistance or tolerance of plants to insect herbivores was mediated via constitutive or induced defence mechanisms (Mauricio *et al.*, 1997; Buell, 1998). Inducible defences were thought to compromise plant fitness less and might be more durable, than constitutive defence mechanisms (Agrawal, 1998). Inducible defences played a major role in conferring insect resistance and their effects on phytophagous insects which included increased toxicity, delay of larval development, or increased attack by insect parasitoids (Baldwin and Preston, 1999).

Induced resistance had been reported from over 100 plant-herbivore systems and appeared to be a common feature of anti-herbivore defences in all walks of plant life. Induced resistance can have long lasting negative impacts on populations of herbivores and positive effects on plant performance (Agrawal, 1998; Agrawal, 1999a; Agrawal, 1999b).

Induced plant resistance to herbivores were a general phenomenon found in many plants and was characterized by reduced preference or performance of herbivores on previously damaged plants compared to that on controls (Karban and Baldwin, 1997). Such plasticity in plant resistance had been shown to be adaptive in several instances: induced plants had relatively higher fitness than un-induced controls in the presence of herbivores and relatively lower fitness than controls in the absence of herbivores (Agarwal, 1998; Agarwal, 1999a; Agarwal, 1999 b; Agarwal *et al.,* 1999a).

Inducible defences played a major role in conferring disease resistance against plant pathogens (Maleck and Dietrich, 1999) and their effects on phytophagous insects can include increased toxicity, delay of larval development or increased attack by insect parasitoids (Baldwin and Preston, 1999).

Induced resistance can be direct or indirect. Direct resistance aimed at the accumulation of substantial amounts of defence proteins and/or production of noxious chemical in damaged plants that reduced feeding, oviposition, growth and development of herbivores (Heil, 2004). The structural defence formed the first line of defence against insects and constituted the morphological and anatomical traits that were advantageous to the plants and directly deterred the insect herbivores (Hanley *et al.,* 2007; Agrawal *et al.,* 2009). Indirect resistance was mediated by the emission of volatile blend that specifically attracted natural enemies of herbivores (Arimura *et al.,* 2009). Plants were always under the biotic stress due to herbivores, which caused severe damage to crops worldwide. In order to defend themselves against these herbivores, plants had developed a wide range of physical and chemical mechanisms (Rasman and Agarwal, 2004; Sharma *et al.,* 2009 and War *et al.,* 2011).

Host plant selection for oviposition was crucial as the suitability of the host plant determined the survival and development of the progeny. Surface chemicals, plant volatiles and trichomes had a major influence on the oviposition behaviour of insects (Hilker *et al.,* 2002; Chamarthi *et al.,* 2011).

Plants respond to herbivory not only through biochemical mechanisms, but also through the induction of morphological features, such as trichome density in subsequent plant growth (Traw and Dawson, 2002). Herbivorous insects used diverse feeding strategies to obtain nutrients from their host plants. Rather than acting as passive victims in these interactions, plants responded to herbivory with the production of toxins and defensive proteins that targetted physiological processes in the insect (Zhao *et al.,* 2009; Kawazu *et al.,* 2012).

2.2 Secondary metabolites

Plants with high variability in defensive chemicals exhibited a better defence compared to those with moderate variability (Walling, 2000; Kessler and Baldwin, 2002; Sharma *et al.,* 2009; He *et al.,* 2011). The defensive secondary metabolites can be either constitutively stored as inactive forms or induced in response to the insect or microbe attack and/or elicitor application. Secondary metabolites and defensive proteins accumulated in plant tissues as a result of insect damage or pathogen infestation and defend the plants against further damage by insects and/or pathogens (Torres *et al.,* 2006).

The secondary metabolites did not affect the normal growth and development of plants, but reduced the palatability of the plant tissues in which they are produced (Stenvenson *et al.,* 1993; Kessler and Baldwin, 2002; Boerjan *et al.,* 2003; Bernards and Bastrup-Spohr, 2008). Secondary metabolites also acted as signal compounds for attracting the pollinating or seed dispersing animals and also protected the plants from ultraviolet radiation and oxidants (Boerjan *et al.,* 2003). Reduced damage, lower larval survival and lower larval weights might be due to the greater production of toxic secondary metabolites in the insect-resistant genotypes by insect damage and jasmonic acid application (Felton *et al.,* 1994; Mao *et al.,* 2007; Chen *et al.,* 2009 and Bhongwong *et al.,* 2009).

2.3 Tannins

Bernays (1998) reported that condensed tannins were oligomeric or polymeric flavonoids, also known as pro-anthocyanidins. They had diverse structures and functions. They acted as feeding deterrents against some insects such as, *Lymantria dispar* (L.), *Euproctis chrysorrhoea* (L.) and *Operophtera brumata*.

Induction of tannins in plants in response to insect infestation had been well documented. The increase in tannins in insect infested by *Pinus sylvestris* L. and accumulation of tannins in *Populus* spp. infested by several insects had been reported (Stevens and Lindroth, 2005). Tannins mediated the formation of semiquinone radicals and quinones and other reactive oxygen species by oxidation in insect gut due to high pH, thereby inhibiting insect growth and development (Stevens and Lindroth, 2005).

Tannins had a strong deleterious effect on phytophagous insects and affected the insect growth and development by binding to the proteins, reduced nutrient absorption efficiency and cause midgut lesions. Tannins are astringent (mouth puckering) bitter polyphenols and act as feeding deterrents to many insect pests. They precipitated proteins non-specifically (including the digestive enzymes of herbivores), by hydrogen bonding or covalent bonding of protein – NH_2 groups. In addition, tannins also chelate the metal ions, thereby reducing their bioavailability to herbivores. When ingested, tannins reduced the digestibility of the proteins thereby decreased the nutritive value of plants and plant parts to herbivores (Sharma and Agarwal, 1983; Sharma *et al.,* 2009; Barbehenn and Peter, 2011).

2.4 Proteins

Exogenous application of JA or methyl jasmonate (MeJA) intensified plant resistance to various herbivores (Avdiushko *et al.,* 1997; Omer *et al.,* 2001) and induced the expression of defensive proteins in tomato (Stout *et al.,* 1998; Cipollini and Redman, 1999; Boughton *et al.,* 2005). War *et al.* (2011) found ground nut plants infested with *Spodoptera litura* (Fab.) had greater protein content in ICGV 86699 at 24, 48 and 72 h after infestation as compared to control plants.

Alteration in protein content or sequence influenced the function of that protein. Likewise, anti-insect activity of a proteolysis-susceptible toxic protein can be improved by administration of protease inhibitors (PIs), which prevent degradation of the toxic proteins and allows them to exert their defensive function. Due to diverse feeding habits of arthropods, multiple signaling pathways including jasmonic acid (JA), salicylic acid (SA) and/or ethylene regulated arthropod-inducible proteins (Arimura *et al.,* 2009).

Better understanding of protein structure and post-translational modifications contributing to stability in the herbivore gut would assist in predicting toxicity and mechanism of plant resistance proteins (PRPs). Recent advances in microarray and proteomic approaches had revealed that a wide spectrum of PRPs was involved in plant defence against herbivores (Chen *et al.,* 2005 and Chen *et al.,* 2009). On account of biotic stresses, plants undergo an alteration in levels of various proteins and also produce new entities **(**Usha and Jyothsna, 2010**)**. Many plant proteins ingested by insects were stable and remain intact in midgut and also moved across the gut wall into the haemolymph.

2.5 Phenols

Lignin synthesis had been found to be induced by herbivory attack and its rapid deposition reduced further growth and herbivore fecundity (Johnson *et al.,* 2009). It limited the herbivores by blocking physically or increasing the leaf toughness that reduced the feeding and also decreased the nutritional content of the leaf (Johnson *et al.*, 2009). Phenol increased the leaf toughness that reduced the feeding by herbivores and also decreased the nutritional content of the leaf (Johnson *et al.*, 2009). Lignin, a phenolic hetero polymer acted as a central component of physical plant defence against insects (Barakat *et al.,* 2010).

Wu *et al.* (2008) reported that in *Nicotina attenuate,* application of MeJA

(Methyl jasmonate) induced greater accumulation of jasmonic acid, which in turn activated the production of phenols against *Menduca sexta.* Plant phenols comprised a structurally diverse and ubiquitous group of plant compounds that had been suggested to play variety of roles in plant defence (Usha and Jyothsna, 2010).

2.6 Flavonoids

A number of flavonoids such as flavonols, flavones, proanthocyanidins, flavan 3-ols, flavonones, flavans and isoflavonoids had been investigated as feeding deterrents against many insect pests (Harborne, 1993), which included genistein, wighteone, 2-hydroxygenistein, luteone, licoisoflavone. Flavones 5-hydroxyisoderricin,7-methoxy-8-(3-methylbutadienyl) - flavanone and 5-methoxyisoronchocarpin isolated from *Tephrosia villosa* (L.), *Tephrosia purpurea* (L.) *and T. vogelii* Hook, respectively, had been found as feeding deterrents against *Spodoptera exempta* and *S. Littoralis* Bios. (Simmonds, 2003). Cyanopropenyl glycoside and alliarinoside strongly inhibited feeding by the native American butterfly, *Pieris napioleracea* L., while isovitexin-6”-D-β-glucopyranoside acted as feeding deterrent to the late instars (Renwick *et al.*, 2001). Isoflavonoids (judaicin, judaicin-7-*O*-glucoside, 2-methoxyjudaicin and maackiain) isolated from the wild relatives of chickpea acted as antifeedant against the pod borer, *Helicoverpa armigera* (Hubner) at 100 ppm. Judaicin and maackiain were also found deterrent to *Spodoptera littoralis* and *S. frugiperda*, respectively (Simmonds and Stevenson, 2001).

Flavonoids were the major plant defensive phenolic compounds utilized against a variety of stresses. More than 5,000 flavonoids had been reported in plants, which play a central role in various facets of plant life especially in plant-environment interactions (Treutter, 2006).

Piubelli *et al.* (2003) reported that higher level of flavonoids, such as daidezin and genistin, had been observed in soybean plants infested with *Nezara* *viridula*. Johnson and Dowd, 2004 reported that flavonoid conferred resistance against *Spodoptera frugiperda* in *Arabidopsis thaliana.* Flavonoids were divided into various classes that include anthocyanins, flavones, flavonols, flavanones, dihydroflavonols, chalcones, aurones, flavan and pro-anthocyanidins (Simmonds, 2003 and Treutter, 2006).

Phytoalexins included isoflavonoids, terpenoids, alkaloids, etc., that influenced the performance and survival of the herbivores (Walling, 2000). It had been reported that maize host plant resistance to corn earworm, *Helicoverpa zea* is mainly due to the presence of the secondary metabolites C-glycosyl flavone maysin[2”-*O*-a-L-rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl) luteolin] and the phenyl propanoid product chlorogenic acid (Nuessly *et al.*, 2007). Compound, 4, 4-dimethyl cyclo-octene had been found to be responsible for shoot fly, *Atherigona soccata* resistance in *Sorghum bicolor* (Chamarthi *et al.*, 2010).

Both flavonoids and isoflavonoids protected the plant against insect pests by influencing the behaviour and growth and development of insects (Simmonds, 2003).

2.7 Jasmonic acid

They induced toxic secondary metabolites and anti-nutritive compounds in plants, which reduced larval growth and development and deter adult moths from oviposition (Bruinsma *et al.*, 2007).

Caterpillars (*Manduca sexta* and *Spodoptera exigua*) fed on mutant tomato deficient in jasmonic acid production showed higher survivorship and greater weight gain than those fed on wild-type tomato (Thaler *et al.*, 2002). Jasmonic acid was the most important phytohormone linked to plant defence against herbivores and activated the expression of both direct and indirect defences (Shivaji *et al.*, 2010).

Moreover, insect damage had been reported to trigger the induction of jasmonic acid and salicylic acid, which in turn signal the expression of induced defensive enzymes and secondary metabolites and also the emission of plant volatiles (Farmer and Ryan, 1990a; Steppuhn and Baldwin, 2007; Dicke *et al.,* 1998; Ament *et al.,* 2004; van Schie *et al,.* 2007; Zhao *et al.,* 2009; Pieterse *et al.,* 2009; Shivaji *et al.,* 2010).

Jasmonic acid (JA), its precursors and derivatives, collectively called jasmonates, represented a family of oxylipins that played an important role in a variety of plant processes including defence against insects and pathogens, abiotic stresses, growth and development, fertility and senescence (Kumari *et al.,* 2006; Wasternack, 2007; Pauwele *et al.,* 2008; Shivaji *et al.,* 2010; Kanno *et al.,* 2011). Jasmonic acid activated the expression of both direct and indirect defences in plants (Shivaji *et al.,* 2010). Induction of endogenous jasmonic acid leads to the modulation of resistance related gene expression and the defensive metabolites involved in plant defence against herbivory (Korth and Thompson, 2006; Bruinsma and Dicke, 2008). Jasmonic acid gets quickly accumulated in plant tissues surrounding the site of damage (Kanno *et al.,* 2011).

Furthermore, exogenous application of jasmonic acid, salicylic acid and their precursors and derivatives induced the defensive proteins and other non-protein compounds in plants (Orozco-Cardenas *et al.,* 1993; Thaler *et al.,*1996; Thaler *et al.,* 2001; Cipollini and Sipe, 2001; Zhao *et al.,* 2009; Pieterse *et al.,* 2009; Shivaji *et al.,* 2010), besides increasing plant fitness, increased parasitism and reduced growth and development of insect pests (Baldwin, 1998; Thaler, 2001; Kessler and Baldwin, 2001; Verhage *et al.,* 2010).

Exogenous application of JA in tomato induced the volatile compounds which attract aphid parasitoids (Du *et al.,* 1998; Thaler, 2002), thereby mediating the indirect plant defence. A large numbers of genes involved in defence against herbivores are regulated by JA (Cipollini *et al.,* 2004; Shivaji *et al.,* 2010). Role of exogenous application of JA against insect herbivory had been well established.

Jasmonic acid (JA) and salicylic acid (SA) were the important phytohormones involved in plant defence against insect herbivory (Stotz *et al.,* 2002; Traw and Bergelson, 2003; Bruinsma *et al.*, 2007; Zhao *et al.,* 2009). They induced toxic plant secondary metabolites and antinutritive compounds in plants, which reduced larval growth and development and deter adult moths from oviposition (Bruinsma *et al.,* 2007). Plants responded to insect oviposition through direct and indirect defences, which aimed to get rid of the insect eggs and/or to kill them, thus avoiding the damage by the larvae (Hilker and Meiners, 2010).

Jasmonic acid was considered to be one of the most important elicitors of plant defences against herbivores, mediating the expression of both direct and indirect defences (Shivaji *et al.,* 2010) and accumulating rapidly in plant tissues near the site of herbivore attack (Moreira *et al.,* 2009).

Jasmonic acid affected plant development and physiology which in turn affected plant yield irrespective of effects on herbivory (Fan *et al.,* 1997, Koda, 1997). Jasmonic acid (JA) and salicylic acid (SA) were the important phytohormones involved in plant defence against insect herbivory (Stotz *et al.,* 2002; Traw and Bergelson, 2003; Bruinsma *et al.*, 2007; Zhao *et al.,* 2009).

Jasmonic acid, a ubiquitous regulator of the wound-induced response in plants, was an essential component of the signaling pathway in crop plants and when applied exogenously, it induced several defence related responses and production of volatile compounds (Wasternack, 2007). Induction of endogenous jasmonic acid leads to the modulation of resistance related gene expression and the defensive metabolites involved in plant defence against herbivory (Korth and Thompson, 2006; Bruinsma and Dicke, 2008). Jasmonates were derived from linolenic acid through octadecanoid pathway (Wasternack, 2007) and accumulated upon wounding and herbivory in plant tissues (Pauwele *et al.,* 2008; Shivaji *et al.,* 2010; Kanno *et al.,* 2011). JA gets quickly accumulated in plant tissues surrounding the site of damage (Kanno *et al.*, 2011).

2.8 Salicylic acid

As a single compound it can attract predatory mites (Dicke *et al.*, 1993). A volatile analogue of salicylic acid, methyl salicylic acid, was a common component of the blend emitted by herbivore infested plants (Van Poecke *et al.*, 2001).

Benzoic acid, the direct substrate for salicylic acid biosynthesis, was derived from phenylpropanoid pathways in plants with PAL as key regulatory enzyme (Dixon, 2001). Fluctuation in the quantity of specified metabolite might be involved in the pathway flux controlled by the activity of its rate control enzyme and substrate available (Kolosova *et al.*, 2001).

Recently, accumulating reports indicated that the salicylic acid pathway is involved in a wide range of plant defence responses. For example, plant defence in response to microbial attack was regulated through a complex network of signalling pathways that involve three signalling molecules: salicylic acid, jasmonic acid and ethylene (Kunkel and Brooks, 2002).

Salicylic acid played a key role in the signal transduction pathway leading to systemic acquired resistance (Enyedi *et al.,* 1992). The SA and JA signalling pathways were mutually antagonistic (Kunkel and Brooks, 2002). Plant defences against insects were regulated differentially by cross-communicating signal transduction pathways in which salicylic acid and jasmonic acid played key roles (Spoel *et al.,* 2003). A few available reports mentioned the function of SA in defence responses to insects (Stotz *et al.,* 2002; Martinez *et al.,* 2003). Branch *et al.,* 2004 found that salicylic acid was an important component of the signalling that leads to root-knot nematode resistance and the associated hypersensitive response.

The transcriptional responses of plants to herbivores were regulated by the coordination of salicylic acid and jasmonic acid pathways and can have both synergistic and antagonistic interactions (Rojo *et al.,* 2003). The JA and SA were regarded as the most important plant defence signalling molecules that induced different antioxidative enzymes and secondary metabolites (Farmer and Ryan, 1990a; Steppuhn and Baldwin, 2007; Westernack, 2007; Zhao *et al.*, 2009; Shivaji *et al.,* 2010), thereby, enhanced the host plant resistance against insect herbivores and other stresses (Koornneef and Pieterse, 2008; van Dam and Oomen, 2008; Zheng and Dicke, 2008).

Salicylic acid (SA), a benzoic acid derivative, was an important phytohormone involved in regulation of plant defence. It is important endogenous plant growth regulator that generated a wide range of metabolic and physiological responses in plants involved in plant defence (Zhao *et al.,* 2009; Vicente and Plascencia, 2011; Kanno *et al.,* 2011). SA induced greater defence against piercing and sucking type of insect pests and also against the chewing pests (Zhao *et al.*, 2009; Peng *et al.*, 2004; Thaler *et al.,* 2010; Kanno *et al.*, 2011).

Chapter - 3

MATERIALS AND METHODS

The study was conducted at Division of Entomology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar Campus, on the Kale plants which were grown under iron mesh net house and the larvae of insect *Pieris brassicae*, were reared in the laboratory on natural food Kale.

Two commercial varieties of Kale (Kawdari and Khanyari) were raised in plastic pots (30×30cm). Seeds of the varieties were raised in Green House of Division of Agricultural Engineering, SKUAST-Kashmir and when seedling, became 30-40 days old, seedlings of the two varieties namely Kawdari and Khanyari were transplanted in plastic pots (30×30cm). Fifteen days after transplanting, Kale plants of the two varieties were pre-sprayed with two phytohormones i.e., jasmonic acid and salicylic acid @ 1 mM. Plants presprayed with two signalling molecules (phytohormones) at 1 mM concentrations were quickly infested with the second instar larvae of *P. brassicae*. The another treatment was run by only infesting with the second in star larvae on two test varieties (without spraying of jasmonic acid and salicylic acid) and a separate control (no spray, no insect infestation) was run for both the varieties. The treatment details were as follows:

3.1 Treatment detail

Design of Experiment: RCBD (Randomized Complete Block Design)

Crop = Kale

No. of varieties: 02

V_1=Khanyari

V_2=Kawdari

No. of chemical treatments: 04 (01+03)

01=control

03=treatments

Insect reared on natural food (Kale)

Plants sprayed with phytohormones

Plants infested with larvae of *Pieris brassicae*

Crushing of leaf sample

Plate-1: Procedure for extraction of secondary metabolites

C_1 = Pre infestation application of Jasmonic acid (JA) + infestation by *Pieris brassicae* larvae (PBL).

C_2 = Pre infestation application of Salicylic acid (JA) + infestation by *Pieris brassicae* larvae (PBL).

C_3 = only infestation by *Pieris brassicae* larvae (PBL).

C_0 = Control (no spray, no insect infestation).

Total No. of treatment combination = 08

3.2 Treatment combination detail

Code	Symbol	Detail
T_1	V_1C_0	Khanyari + Control
T_2	V_1C_1	Khanyari + pre infestation application of Jasmonic acid + infestation by *Pieris brassicae* larvae
T_3	V_1C_2	Khanyari + pre infestation application of Salicylic acid acid + infestation by *Pieris brassicae* larvae
T_4	V_1C_3	Khanyari + only infestation by *Pieris brassicae* larvae
T_5	V_2C_0	Kawdari + control
T_6	V_2C_1	Kawdari + pre infestation application of Jasmonic acid + infestation by *Pieris brassicae* larvae
T_7	V_2C_2	Kawdari + pre infestation application of Salicylic acid acid + infestation by *Pieris brassicae* larvae
T_8	V_2C_3	Kawdari + only infestation by *Pieris brassicae* larvae

No. of replications : 04 (12 plants/treatment)

No. of plants required: 96

In addition, five larvae each from all the treatments were collected and brought to the laboratory to determine their weight (mg) on basis to see the effect of phytohormones on *P. brassicae* larvae which were introduced in the treatments.

After 6 days of infestation, leaves were excised and collected from all the treatments including control. The leaf samples were collected from all the treatments and brought to the laboratory. The leaf samples were then stored for two days in Deep freezer to study the following components as detailed below:

3.3 Phenolic content

3.4 Condensed tannins

3.5 Total flavonoids

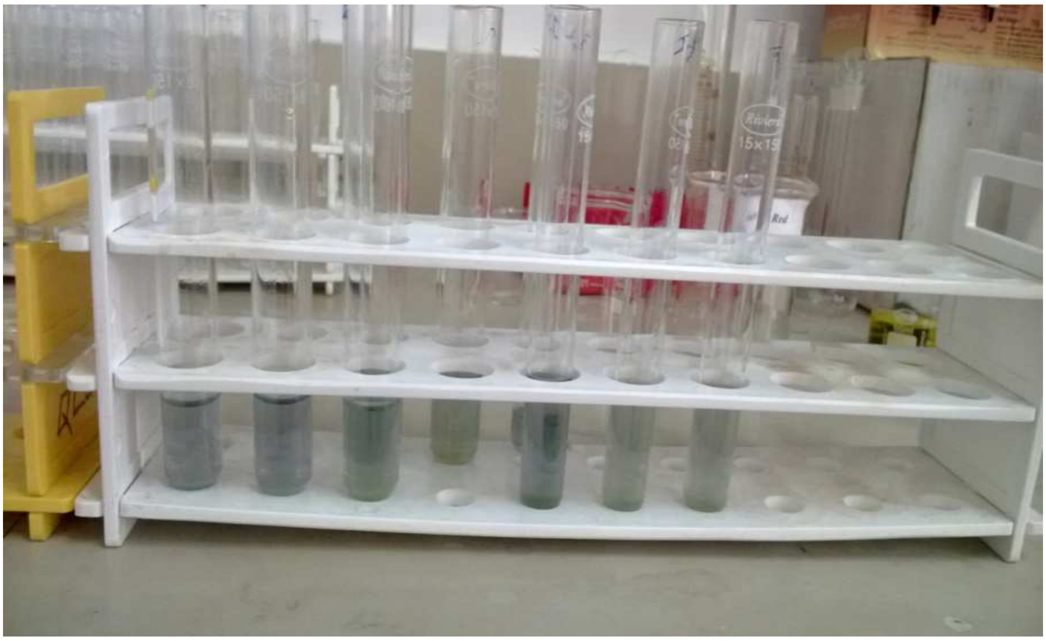
3.6 Total proteins

3.3 Phenolic content

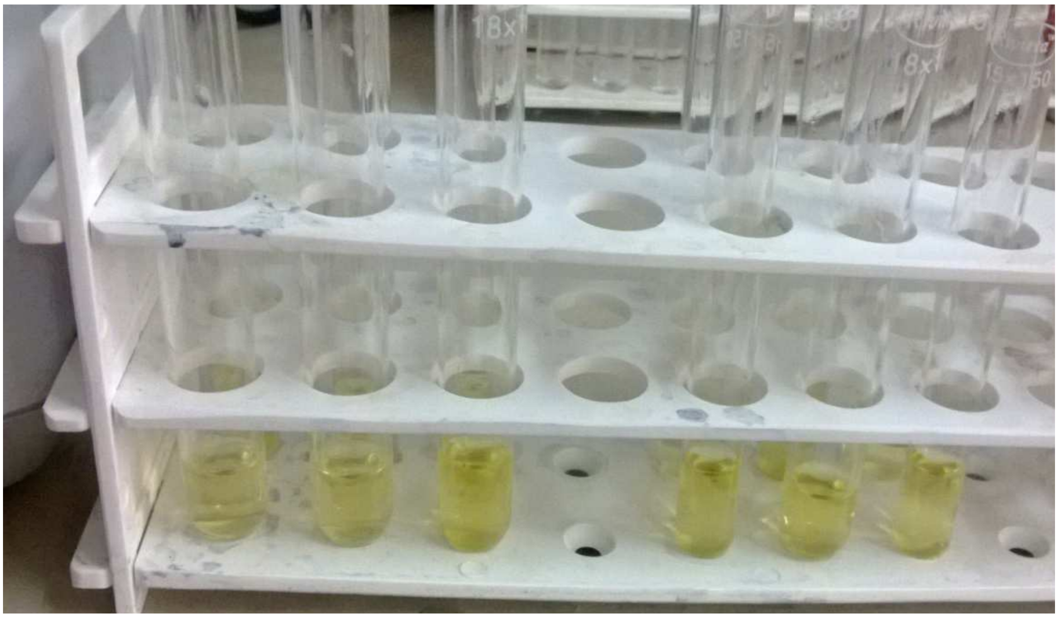
Procedure : Fresh leaves (0.5 g) were homogenized in 3 ml of 80 per cent methanol and agitated for 15 min at 70 ºC. The solution was centrifuged at 10, 000 rpm (revolution per minute) for 10 min. and the supernatant collected. The supernatant was used for the estimation of total phenolic content. The Phenolic content was estimated as per Zieslin and Ben-Zaken (1993) method with some modifications. To 2 ml of 2% sodium carbonate (Na_2CO_3), 1 ml of methanol extract was added. The solution was incubated for 5 min at room temperature after which 0.1 ml of 1 N Folin-Ciocalteau reagent was added. The solution was incubated again for 10 min and absorbance of the blue colour was measured at 760 nm. Phenolic concentration was determined from standard curve prepared with gallic acid and was expressed as µg Gallic acid equivalents g^{-1} FW (µg GAE g^{-1} FW).

3.4 Condensed tannins

Procedure : Condensed tannins content was estimated by using vanillin-hydrochloride method as described by Robert (1971), with some modifications. The 0.5 ml of supernatant was added to 2.5 ml of vanillin-HCl reagent [equal volumes of 8 per cent HCl (in methanol) and 4 per cent vanillin (in methanol) and the solutions mixed just before use]. The reaction mixture was incubated at room temperature for 20 min and the absorbance read at 500 nm against a blank



Phenol



Total flavonoids

Plate-2: Secondary metabolites (Phenol and Total flavonoids)

containing the reagent alone. Tannic acid was used as the standard. The total amount of condensed tannins was expressed as µg tannic acid equivalents g^{-1} FW (µg TAE g^{-1} FW).

3.5 Total flavonoids

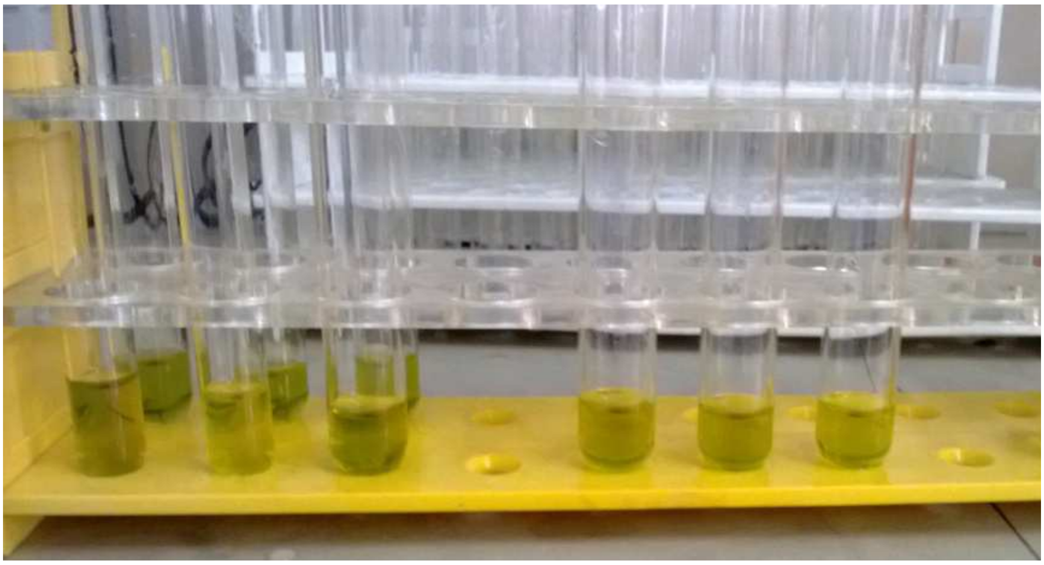
Procedure : Total flavonoid content was determined by the modified aluminium chloride method as described by Woisky and Salatino (1998). Leaf extract (0.2 ml) was added to 0.8 ml of distilled water in a test tube. To the above solution, 0.06 ml of 5 per cent $NaNO_2$ was added. The solution was allowed to stand for 5 min. To the solution, 0.06 ml of 10 per cent $AlCl_3$ and 0.4 ml of 1 M NaOH was added and mixed well. The absorbance was read at 510 nm. The total amount of flavonoids was expressed as µg catechol equivalents g^{-1} FW (µg C g^{-1} FW).

3.6 Total protein estimation by Lowry's method

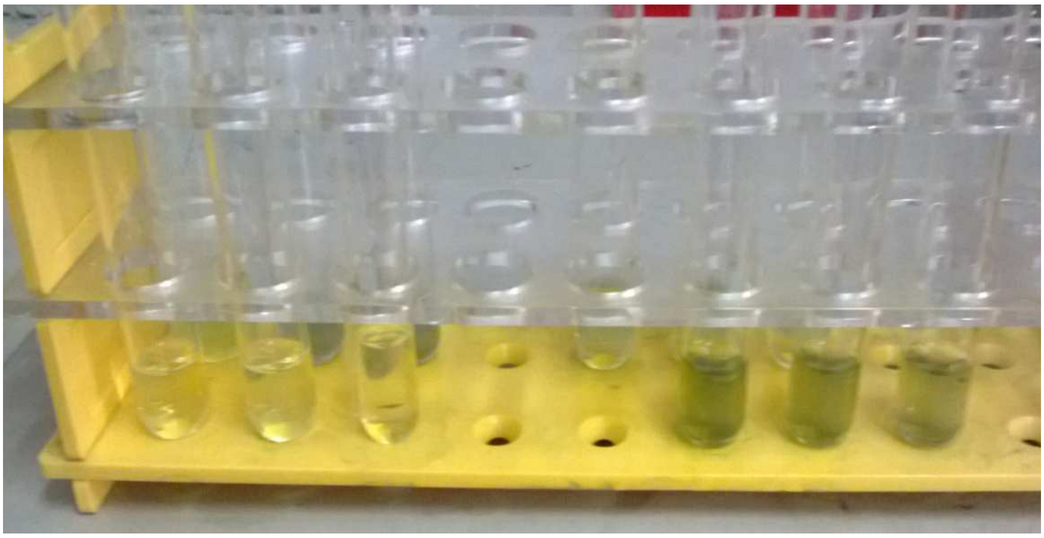
3.6.1 Materials

- 2 per cent Sodium Carbonate in 0.1 N Sodium Hydroxide (Reagent A)
- 0.5 per cent Copper Sulphate ($CuSO_4.5H_2O$) in Potassium Sodium tartarate (Reagent B)
- Alkaline copper solution Mix 50 ml of A and 1ml of B prior to use (Reagent C)
- Folin Ciocalteau Reagent (reagent D)
- Protein solution (stock standard): Weigh accurately 50 mg of bovine serum albumin and dissolve in distilled water and make up to 50 ml in a standard flask.
- Working standard: Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask.

Procedure : This method was given by Lowry (1951). Leaf sample of about 500 mg grinded with mortar and pestle in 5 to 10 ml of the sodium phosphate buffer. The sample was centrifuged and the supernatant used for protein estimation. Bovine Serum Albumin (BSA) was used as a standard. The solution was



Condensed tannin



Total protein

Plate-3: Secondary metabolite (condensed tannin) and total protein

incubated at room temperature in the dark for 30 min. Blue colour was read at 660 nm. The amount of protein was calculated from the standard graph and expressed as mg/g on FW.

3.7 Per cent damage

Per cent damage of Kale plants was calculated 3 DAT (days after treatment), 6 DAT and 9 DAT. Per cent damage was calculated based on foliage damage index as given below :

Ratings boundary	Scale (% damage)	Damaging
1.	0-3	Occasional holes in leaves
2.	4-10	Few leaves with holes
3.	11-25	Moderate number of leaves
4.	26-50	Most leaves with holes
5.	51-100	All leaves with holes

Per cent damage/intensity was calculated by following formula:

$$\text{Per cent damage intensity} = \frac{\Sigma\text{ (No. of obs. in particular damage score)}}{\text{Total No. observations} \times \text{Maximum grade}} \times 100$$

[George *et al.*, 1986]

Chapter – 4

EXPERIMENTAL FINDINGS

The experimental findings of the studies entitled "To study the defensive response of kale against cabbage butterfly (*Pieris brassicae* Linn.)" are presented under the following headings:

Experiment 1: To study the induced resistance against Cabbage butterfly on Kale

4.1 Phenolic content

4.2 Condensed Tannins

4.3 Total Flavonoids

4.4 Total Proteins

4.1 Phenolic content

The phenolic content was estimated as per Zielson and Benson (1993) method. In this method only 0.5g leaves were used. The Kale varieties pre-sprayed with signaling molecules (jasmonic acid and salicylic acid) and then infested with *P. brassicae* larvae showed variation in their phenolic content.

Estimation of phenolic content was observed on the basis of standard curve as depicted in the Fig.1 as a strong co-efficient of (R=0.998) with a regression equation Y = 0.017 + 0.004X.

The Kale variety Khanyari showed significant maximum phenolic content (145.05µg/g) as compared to Kale variety Kawdari (108.59 µg/g). The highest amount of phenolic content in both the varieties was due to phytohormone, jasmonic acid (Table-1).

The significantly maximum phenol among the treatments was obtained by jasmonic acid + insect infestation (T_1) (196.07 µg/g) and the total minimum phenol (68.72 µg/g) was observed in control (T_4). Among interactions, the interaction jasmonic acid + insect infestation and Khanyari (208.23 µ/ml) showed maximum amount of phenolic content.

4.2 Condensed tannins

The amount of condensed tannins was significantly affected by the spray of both the signaling molecules i.e., jasmonic acid and Salicylic acid**.** Significantly maximum condensed tannins (301.57µg/g) recorded in both the varieties i.e., Khanyari and Kawdari which may be due to effect of phytohormone i.e., jasmonic acid (Table-2). Similarly the significantly minimum condensed tannin (97.91µg/g) in both the varieties was found in control.

Estimation of condensed tannins was observed on the basis of standard curve as depicted in Fig. 2 as a strong regression coefficient of (R= 0.999) with a regression equation of Y= 0.010 + 0.002X.

The maximum condensed tannins was observed in plants treated with jasmonic acid followed by infestation with *P. brassicae* larvae in both Kale varieties (T_1) as compared with the plants treated with salicylic acid followed by insect infestation (T_2), only insect infestation (T_3) and untreated control plants (T_4). Among the varieties, Khanyari and Kawdari, the Khanyari variety showed significantly higher (212.34 µg/g) condensed tannin content in plants pre-treated with jasmonic acid and salicylic acid followed by insect infestation as compared to Kawdari. Among interactions, the interaction jasmonic acid + insect infestation and Khanyari showed maximum (347.76 µg/g) condensed tannins.

The plants sprayed with jasmonic acid showed more resistance than the plants sprayed with Salicylic acid. Similarly the Kale variety, Khanyari showed more resistance as compared to Kawdari.

4.3 Total flavonoids

In total flavonoid estimation the significantly highest amount of total flavonoids (170.37 µg/g) was due to phytohormone i.e., jasmonic acid in both the varieties of Kale and the lowest (71.39 µg/g) content of flavonoid was found in plants that were untreated i.e., control of both the varieties of Kale (Table 3).

Estimation of total flavonoids was observed on the basis of standard curve as depicted in Fig. 3 as a strong regression coefficient of (R= 0.993) with a regression equation of Y=0.025 +0.002X. Both the kale varieties pre-sprayed with signalling molecules jasmonic acid and salicylic acid and then infested with *P.brassicae* larvae showed higher total flavonoids.

In Khanyari variety the significantly maximum amount (175.61 µg/g) of flavonoid content was found in plants that were pre-sprayed with jasmonic acid and then infested with *P. brassicae* larvae (T_1) and the minimum (98.89 µg/g) amount of flavonoid was found in plants of no treatment i.e., control (T_4). Similarly in Kawdari variety of Kale, the maximum (165.13µg/g) amount of flavonoid was due to effect of phytohormone jasmonic acid + insect infestation (T_1) and the lowest (43.90 µg/g) amount of flavonoid was found in control (T_4) in which the plants were untreated i.e., no spray, no insect infestation.

In both the varieties of Kale, the highest (145.56 µg/g) resistance which is due maximum amount of flavonoid was found in Khanyari and the lowest (110.35 µg/g) amount was found in Kawdari.

4.4 Total proteins

The total proteins was estimated by Lowry's method (1951). The significantly highest amount (0.69 mg/g) of total protein was observed in plants pre-sprayed with jasmonic acid in both the varieties of Kale and the lowest (0.20 mg/g) amount was observed in plants that were untreated i.e., control (Table 4).

Estimation of total proteins was observed on the basis of standard curve as depicted in Fig. 4 as a strong regression coefficient of (R= 0.994) with a regression equation of Y=0.032 +0.998X.

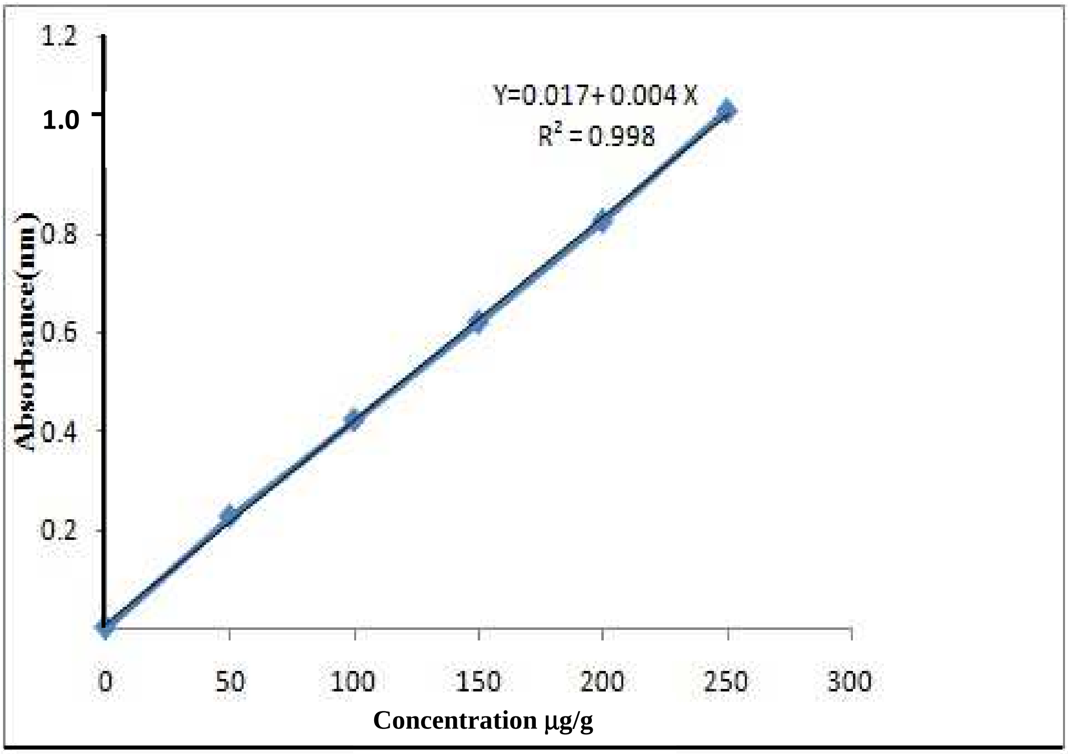


Fig. 1: **Standard curve of gallic acid was used to determine phenolic content in Kale**

Table-1: Effect of phytohormones (jasmonic acid and salicylic acid) on phenolic content of Kale varieties

Treatments	Phenolic content (µg/g) in different Kale varieties		Mean
	Khanyari	Kawdari	
T_1	208.23	183.91	196.07
T_2	191.14	118.60	154.87
T_3	106.22	69.01	87.61
T_4	74.60	62.85	68.72
Mean	**145.05**	**108.59**	**126.82**

C.D ($p \leq 0.05$)

Treatment : 10.31

Variety : 7.29

Treatment × Variety : 14.59

T_1 = Jasmonic acid + Insect infestation

T_2 = Salicylic acid + Insect infestation

T_3 = Only insect infestation

T_4 = Control (Neither spray of any signalling molecule nor insect infestation)

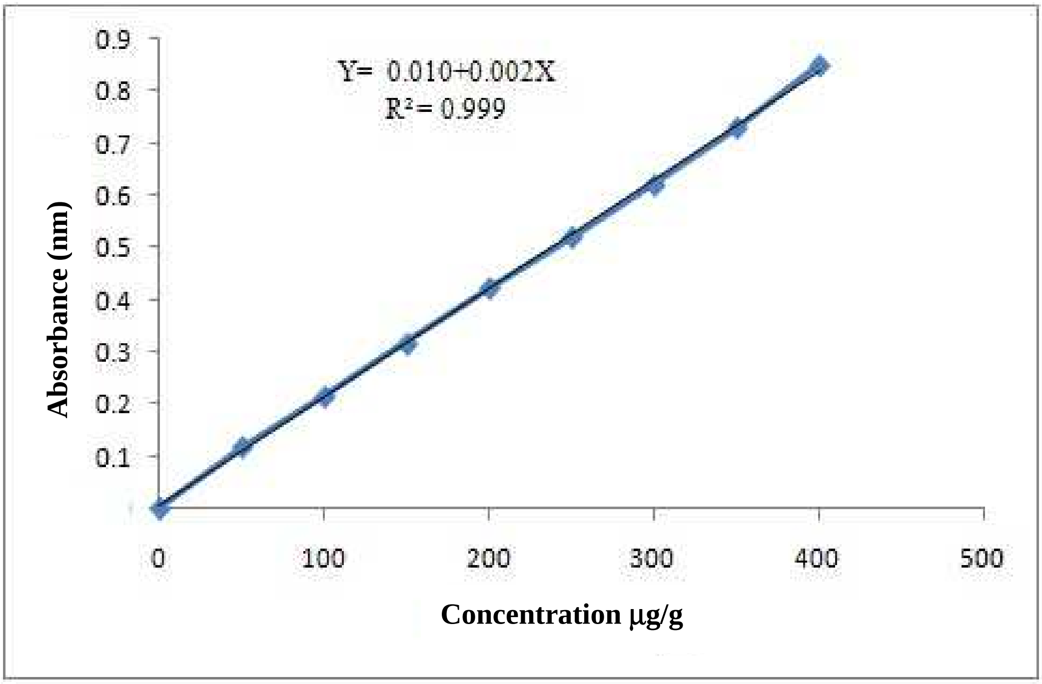


Fig. 2: Standard curve of tannic acid was used to determine condensed tannins in Kale

Table-2: Effect of phytohormones (jasmonic acid and salicylic acid) on condensed tannins of Kale varieties

Treatments	Condensed tannins (µg/g) in different Kale varieties		Mean
	Khanyari	Kawdari	
T_1	347.76	255.38	301.57
T_2	241.93	194.08	218.00
T_3	156.88	141.57	149.23
T_4	102.80	93.08	97.91
Mean	**212.34**	**171.01**	**191.68**

C.D ($p \leq 0.05$)

Treatment : 4.59

Variety : 3.24

Treatment × Variety : 6.49

T_1 = Jasmonic acid + Insect infestation

T_2 = Salicylic acid + Insect infestation

T_3 = Only insect infestation

T_4 = Control (Neither spray of any signalling molecule nor insect infestation)

In Khanyari variety of Kale, the significantly maximum (0.71 mg/g) amount was observed due to effect of jasmonic acid + insect infestation (T_1) and lowest amount (0.23 mg/g) was observed in control (T_4), similarly in Kawdari variety, the highest amount (0.67 mg/g) of protein was due to effect of jasmonic acid + insect infestation (T_1) and the lowest amount (0.18 g/g) was found in control (T_4) i.e., untreated plants.

Among the varieties, Khanyari showed significantly higher (0.43mg/g) protein content as compared to Kawdari (0.37 mg/g).

4.5 Larval weight

Five larvae each from all the treatments (jasmonic acid, salicylic acid and insect infestation) were collected to determine their weight on mg basis. There was an overall effect of phytohormones, which reduced the weight gain of caterpillars. Only when the plants were damaged by *P. brassicae* larvae, there was no detectable effect of damage on subsequent growth of the caterpillars. The maximum larval weight (180.79 mg) was observed in larvae that were collected from plants which were infested by insects only (T_3) and minimum larval weight (100.17 mg) was observed in larvae collected from plants which were sprayed by phytohormone i.e., jasmonic acid and then infested with *P. brassicae* larvae (T_1) (Table-5). Among the varieties, Khanyari (130.62 mg) showed significantly lower larval weight (144.80 mg) as compared to variety Kawdari.

4.6 Per cent damage

The highest per cent damage (23.61%) was observed in plants that were infested by larvae of *P. brassicae* only (T_3) and the lowest damage (9.73%) was observed in plants that were sprayed with phytohormone jasmonic acid and then infested by larvae of *P. brassicae* (T_1). The per cent damage decreased when days after treatment increased in case of both i.e., plants sprayed with jasmonic acid and plants sprayed with salicylic acid but the per cent damage in case of plants that were only infested by *P. brassicae* larvae increased when days after treatment increased. Among varieties the highest per cent damage (17.31%) was observed in variety Kawdari and lowest (14.39%) was observed in variety Khanyari (Table-6).

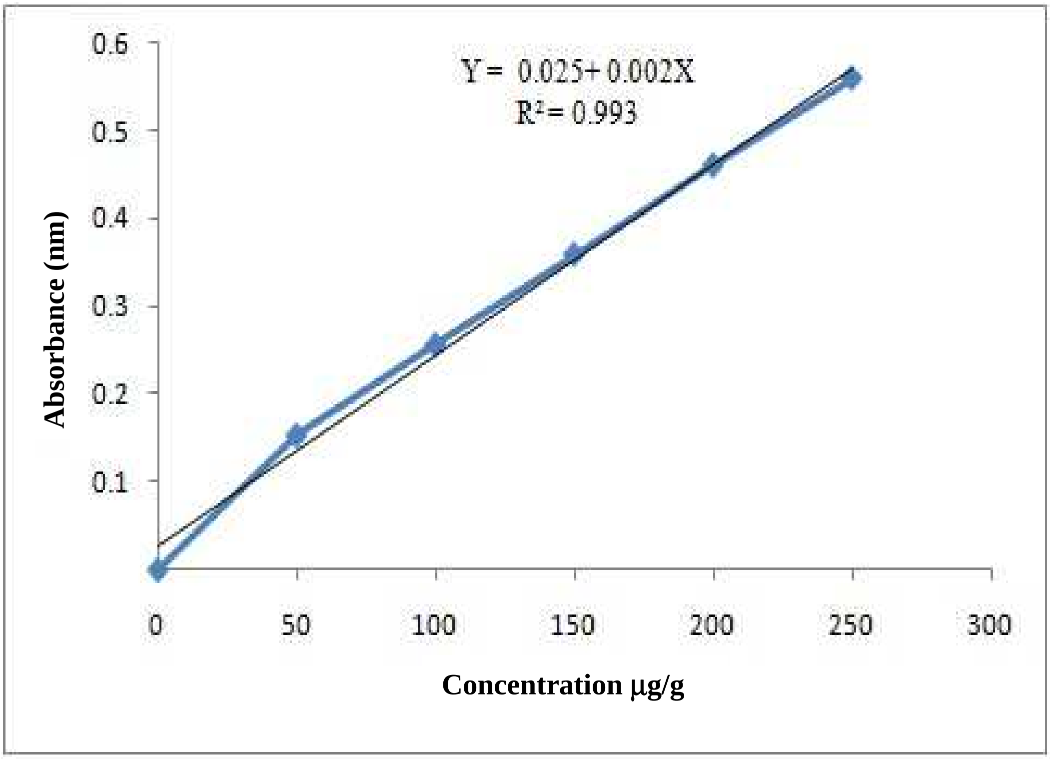


Fig. 3: Standard curve of catechol was used to determine total flavonoids in Kale

Table-3: Effect of phytohormones (jasmonic acid and salicylic acid) on total flavonoids of Kale varieties

Treatments	Total flavonoids (µg/g) in different Kale varieties		Mean
	Khanyari	Kawdari	
T_1	175.61	165.13	70.37
T_2	170.26	126.43	148.35
T_3	137.47	105.94	121.71
T_4	98.89	43.90	71.39
Mean	**145.56**	**110.35**	**127.95**

C.D ($p \leq 0.05$)

Treatment : 7.02

Variety : 4.96

Treatment × Variety : 9.93

T_1 = Jasmonic acid + Insect infestation

T_2 = Salicylic acid + Insect infestation

T_3 = Only insect infestation

T_4 = Control (Neither spray of any signalling molecule nor insect infestation)

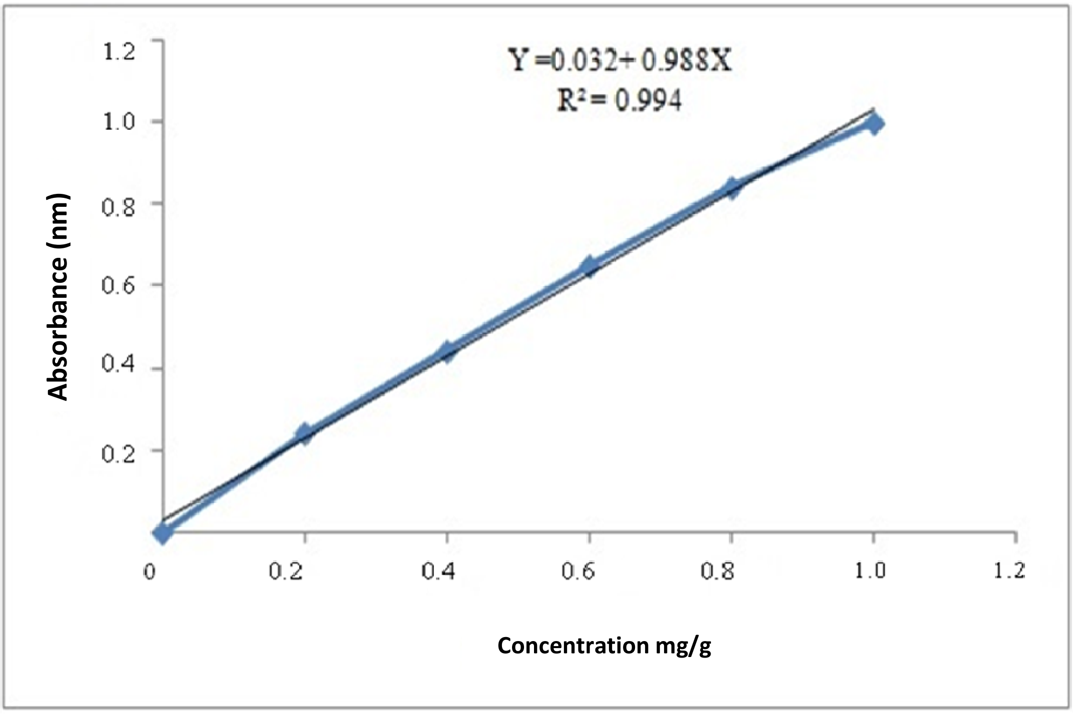


Fig. 4: Standard curve of bovine serum albumin was used to determine total proteins in Kale

Table-4: Effect of secondary metabolites (jasmonic acid and salicylic acid) on total proteins of Kale varieties

Treatments	Total proteins (mg/g) in different Kale varieties		Mean
	Khanyari	Kawdari	
T_1	0.71	0.67	0.69
T_2	0.50	0.40	0.45
T_3	0.29	0.23	0.26
T_4	0.23	0.18	0.20
Mean	**0.43**	**0.37**	**0.40**

C.D ($p \leq 0.05$)

Treatment : 0.01

Variety : 0.01

Treatment × Variety : 0.02

T_1 = Jasmonic acid + Insect infestation

T_2 = Salicylic acid + Insect infestation

T_3 = Only insect infestation

T_4 = Control (Neither spray of any signalling molecule nor insect infestation)

Table-5: Effect of different treatments (jasmonic acid and salicylic acid) on larval weight of insects

Treatments	Larval weight (mg) in different Kale varieties		Mean
	Khanyari	Kawdari	
T_1	97.88	102.46	100.17
T_2	123.38	141.00	132.19
T_3	170.62	190.96	180.79
Mean	**130.62**	**144.80**	**137.69**

C.D ($p \leq 0.05$)			**SE(m)**
Treatment	:	1.50	0.50
Variety	:	1.22	0.41
Treatment × Variety	:	2.12	0.72

T_1 = Jasmonic acid + Insect infestation

T_2 = Salicylic acid + Insect infestation

T_3 = Only insect infestation

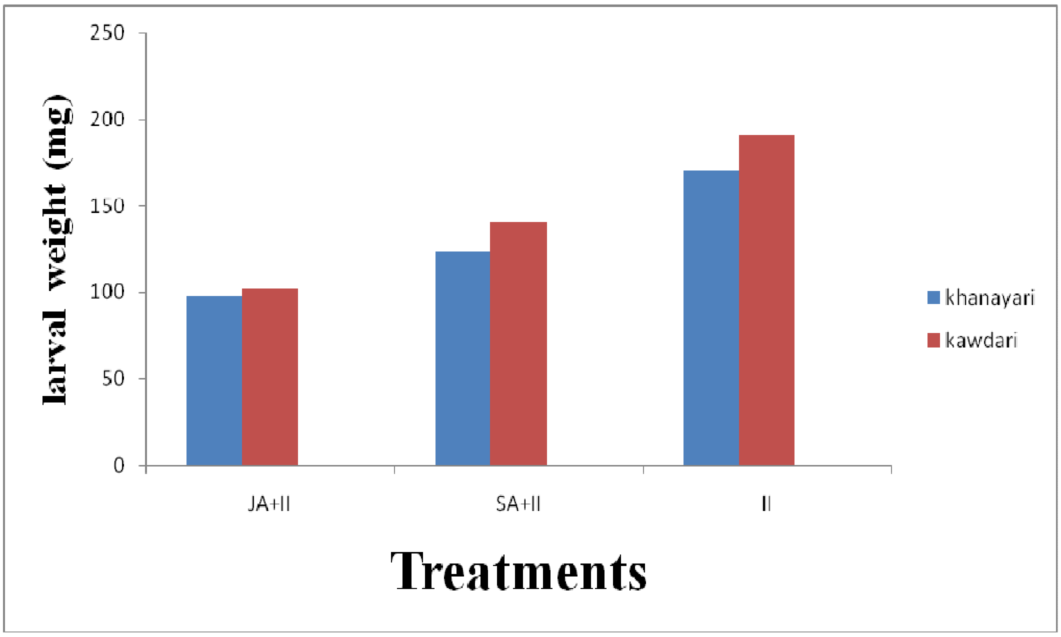


Fig. 5: Larval weight of insect

Table-6: Effect of jasmonic acid and salicylic acid on per cent damage of leaves caused by larvae of *Pieris brassicae* on two varieties of Kale

Treatments	Percent damage at different time intervals Days after treatment (DAT)									Mean	Factor mean
	3 DAT			6 DAT			9 DAT				
	V_1	**V_2**	**Mean**	**V_1**	**V_2**	**Mean**	**V_1**	**V_2**	**Mean**		
T_1	10.52 (3.39)	15.07 (4.00)	12.79 (3.69)*	8.52 (3.08)	10.55 (3.39)	9.53 (3.23)	5.30 (2.50)	8.42 (3.07)	6.86 (2.78)	9.73 (3.24)	V_1= 4.39 (3.79)
T_2	13.65 (3.82)	17.51 (4.30)	15.58 (4.01)	12.47 (3.67)	14.42 (3.79)	12.94 (3.73)	9.00 (3.38)	11.25 (3.50)	10.12 (3.44)	13.12 (3.74)	
T_3	20.21 (4.60)	23.42 (4.73)	21.81 (4.66)	22.37 (4.73)	24.50 (5.05)	22.43 (4.89)	25.50 (4.95)	28.70 (5.45)	27.10 (5.20)	23.61 (4.91)	V_2=17.31 (4.14)
Mean	14.79 (3.94)	18.66 (4.34)	16.72 (4.14)	14.45 (3.82)	16.49 (4.08)	15.13 (3.95)	13.26 (3.61)	16.12 (4.00)	14.60 (3.80)	15.48 (3.96)	

C.D ($p \leq 0.05$)

Treatment : 0.02

DAT : 0.02

Variety : 0.01

T_1 = Jasmonic acid + Insect infestation

T_2 = Salicylic acid + Insect infestation

T_3 = Only insect infestation

***** Values in parenthesis are square root transformed values

Experiment 2: Effect of different concentration of jasmonic acid on secondary metabolites and total protein

4.7 Phenolic content

4.8 Condensed tannins

4.9 Total flavonoids

4.9 Total proteins

4.7 Phenolic content

Different concentration of jasmonic acid significantly affected the phenolic content of both Khanyari and Kawdari varieties of Kale. With the increase in concentration of jasmonic acid in plants the amount of phenolic content also increased but at concentration 2 mM it starts decreasing because at this concentration it causes phyto-toxicity to plants. The significantly highest (223.30 µg/g) phenol content was observed in plants that were sprayed with 1.5 mM concentration of jasmonic acid (T_2) and lowest (186.63 µg/g) content was observed in plants pre-sprayed with 1 mM concentration of jasmonic acid (T_1). Among the two varieties, Khanyari showed maximum (208.53 µg/g) phenolic content as compared to Kawdari (202.08 µg/g) which showed lowest phenolic content (Table-7).

4.8 Condensed tannins

The amount of condensed tannin also varied with the change in concentration of jasmonic acid (Table-8). The significantly maximum (349.31 µg/g) amount of condensed tannin was observed in plants that were pre-sprayed with 1.5 mM concentration of jasmonic acid (T_2) and the significantly lowest (241.72 µg/g) amount was observed in plants pre-sprayed with 1mM concentration of jasmonic acid (T_1). This means that with the increased in concentration of phytohormone the amount of condensed tannins also increases but at concentration 2 mM it starts decreasing because it causes phyto-toxicity. The variety Khanyari (329.71 µg/g) showed highest amount of condensed tannin and Kawdari (248.57 µg/g) showed lowest as compared to Khanyari.

4.9 Total flavonoids

When we increased the concentration of jasmonic acid in Kale varieties the amount of total flavonoids also increased. The maximum amount (243.00 µg/g) of total flavonoid observed in Kale varieties was due to higher concentration 1.5 mM of jasmonic acid and minimum amount (161.65 µg/g) of total flavonoid was due to lower (1 mM) concentration of jasmonic acid. Among varieties, Khanyari showed highest (218.72 µg/g) amount of total flavonoid and Kawdari showed (190.61 µg/g) lowest condensed tannin (Table-9).

4.10 Total proteins

The total protein content significantly varied with the change in concentration of jasmonic acid, the signling molecule. The significant highest (0.829 mg/g) amount of total protein was observed in plants pre-sprayed with 1.5 mM concentration of jasmonic acid (T_2) and lowest (0.726 mg/g) amount was observed in plants which were pre-sprayed with lowest amount of jasmonic acid i.e., 1 mM concentration of jasmonic acid (T_1) (Table 10). Among the two varieties of Kale, Khanyari (0.804 mg/g) showed best result i.e., highest amount of total proteins as compared to Kawdari (0.735 mg/g) which showed lowest amount of total proteins.

Table-7: Effect of different concentration of jasmonic acid on phenolic content of Kale varieties

Concentrations	Phenolic content (µg/g) of two varieties of Kale		Mean
	Khanyari	**Kawdari**	
T_1	194.01	179.26	186.63
T_2	224.01	222.58	223.30
T_3	207.58	204.40	205.99
Mean	**208.53**	**202.08**	**205.30**

C.D ($p \leq 0.05$)

Treatment : 3.75

Variety : 3.06

Treatment × Variety : 5.30

T_1 = 1.0 mM

T_2 = 1.5 mM

T_3 = 2.0 mM

Table-8: Effect of different concentration of jasmonic acid on condensed tannins of Kale varieties

Concentrations	Condensed tannins (µg/ml) of two varieties of Kale		Mean
	Khanyari	Kawdari	
T_1	282.02	201.42	241.72
T_2	400.46	298.16	349.31
T_3	306.65	246.12	276.39
Mean	**329.71**	**248.57**	**289.14**

C.D ($p \leq 0.05$)

Treatment	:	6.34
Variety	:	5.18
Treatment × Variety	:	8.97

T_1 = 1.0 mM

T_2 = 1.5 mM

T_3 = 2.0 mM

Table-9: Effect of different concentration of jasmonic acid on total flavonoids of Kale varieties

Concentrations	Total flavonoids (µg/g) of two varieties of Kale		Mean
	Khanyari	Kawdari	
T_1	176.83	146.47	161.65
T_2	248.47	237.54	243.00
T_3	230.86	187.90	209.38
Mean	**218.72**	**190.61**	**204.66**

C.D($p \leq 0.05$)

Treatment : 6.56

Variety : 5.36

Treatment × Variety : 9.28

T_1 = 1.0 mM

T_2 = 1.5 mM

T_3 = 2.0 mM

Table 10: Effect of different concentration of jasmonic acid on total proteins of Kale varieties

Concentrations	Total proteins (mg/ml) of two varieties of Kale		Mean
	Khanyari	Kawdari	
T_1	0.755	0.698	0.726
T_2	0.879	0.779	0.829
T_3	0.779	0.729	0.754
Mean	0.804	0.735	0.769

C.D($p \leq 0.05$)

Treatment : 0.008

Variety : 0.006

Treatment × Variety : 0.012

T_1 = 1.0 mM

T_2 = 1.5 mM

T_3 = 2.0 mM

Chapter – 5

DISCUSSION

The present investigation was undertaken "To study the defensive response of kale against cabbage butterfly, *Pieris brassicae* Linn." The results obtained during the course of investigation are discussed as under in light of available literature:

In recent years increased emphasis has been laid on the development of effective, non-chemical strategies for managing insect pests attacking crops. Induced resistance to insects is viewed as a desirable crop protection strategy with relatively benign environmental impacts. It allows plants to be phenotypically hard in order to face different stresses. Utilization of plant's own defence mechanism is an attractive area of research practiced all over the world to manage plant insect pests and diseases. In this study, examination of the defensive biochemical response of two varieties of kale to *P. brassicae* larvae feeding was done.

5.1 Phenolic content

A high concentration of foliar phenolics generally provided plant resistance against insect herbivores. Treatment, pre-infestation application of jasmonic acid + infestation by *P. brassicae* larvae induced higher phenolic content (196.07 µg/g) as compared to other treatments because jasmonic acid is a central molecule in induced direct defence against insects in many plant species. The importance of jasmonic acid in wound-induced defence responses had been demonstrated by the fact that (i) exogenous application of JA or methyl jasmonate (MeJA) induced these defence responses, (ii) the increase of endogenous JA after wounding correlated with the induced defence responses and (iii) inhibition of the JA production pathway also inhibited the induction of the defence responses. Among all the varieties, Khanyari had maximum (145.05 µg/g) amount of phenol. This might be due to the strong induction of the octadecanoid and phenylpropanoid signaling pathways by JA (Scott *et al.,* 2010). Increased production of phenols as a result of phytohormones i.e., jasmonic acid and Salicylic acid treatment might be due to the signaling of defensive pathways by insect damage, JA and SA. Accumulation of phenols was a common reaction of plants to herbivory that affected insect feeding and development adversely (Shivaji *et al.,* 2010). Felton and Summers (1993), reported that quinones and ROS (Reactive Oxygen Species) such as superoxide (O^-), hydroxyl radicals (HO^-), hydrogen peroxide (H_2O_2) and singlet oxygen (O^{2-}) formed by the oxidation of phenols activated the defensive enzymes that induced resistance in plants against insects. The same result was obtained by Wu *et al.* (2008) who reported that in *Nicotiana attenuate,* application of MeJA induced greater accumulation of jasmonic acid, which in turn activated the production of phenols against *Menduca sexta.* A high concentration of foliar phenolics generally provided plant resistance against insect herbivores. Treatment with PJA + HIN induced higher phenols in ICGV 86699, NCAc 343 and TMV 2 as compared to the other treatments. Among all the genotypes tested, ICGV 86699 had high amounts of phenols and there was a progressive increase with time. Increased production of phenols as a result of PJA + HIN treatment might be due to the signaling of defensive pathways by insect damage and JA. Accumulation of phenols was a common reaction of plants to herbivory that affected insect feeding and development adversely (Kessler and Baldwin, 2002; Ramiro *et al.,* 2006; Sharma *et al.,* 2009; Usha and Jyothsna 2010).

For different concentration of jasmonic acid, plants sprayed with 1.5 mM concentration of jasmonic acid had highest (223.30 µg/g) amount of phenolic content and among the varieties, Khanyari had maximum (208.53 µg/g) phenolic content. With the increase in concentration of jasmonic acid, the phenolic content also increased because with increase in concentration of phytohormone the activity of defensive enzymes becomes more faster but at concentration 2.0 mM it started causing phyto-toxicity to plant. Jasmonic acid induced plants were tested against control plants and all three parasitoid species (*Apantalese glomeratus*) preferred the volatiles from jasmonic acid induced plants. The preference of *C. glomerata* for the volatiles such as phenols from jasmonic acid treated or control plants was tested with different concentration of jasmonic acid (1 µm,10 µm,100 µm and 1 mM).The jasmonic acid treated plants were significantly more attractive than control plants. Only for the lowest concentration tested, the parasitoids not showed a preference for either of the plants. With the increase in concentration of jasmonic acid the preference of *C. glomerata* also increases which was due to increase in concentration of volatiles such as phenols which shows resistance to plant against herbivory (Dicke *et al.*, 1993).

5.2 Condensed tannins

An abrupt increase in condensed tannins content occurred in plants damaged by insects and/or treated with elicitors, including jasmonic acid and salicylic acid. Plants sprayed with phytohormones had significantly greater condensed tannin content as compared to control. Plants pre-sprayed with jasmonic acid and then infested with *P. brassicae* larvae had maximum condensed tannin (301.57 µg/g) as compared to other treatments. Among the varieties, Khanyari had maximum tannin content than Kawdari (212.34 µg/g). Our observations were in line with the findings of Stevens and Lindroth (2005). He observed that tannins mediated the formation of semiquinone radicals, quinines and other reactive oxygen species by oxidation in insect gut due to high pH, thereby inhibiting insect growth and development. Accumulation of tannins in *Populus* spp. infested by several insects had been reported (Stevens and Lindroth, 2005).

The condensed tannin increased with the increase in concentration of jasmonic acid. Plants treated with 1.5 mM concentration of jasmonic acid had maximum (349.31 µg/g) condensed tannin content as compared to plants treated with 1 and 2 mM. Among the varieties, Khanyari had maximum (329.71 µg/g) condensed tannins. With the increase in concentration of phytohormone, the activity of tannin production became faster but at concentration 2.0 mM the concentration of condensed tannins started decreasing because it started causing phyto-toxicity. Different concentrations of jasmonic acid induced increased in concentration of tannins. The total tannin concentration in soybean reached the highest concentration at 200µm of jasmonic acid concentration (Moreira *et al.*, 2009).

5.3 Total flavonoids

Khanyari had significantly higher total flavonoids (145.56 µg/g) content in *P. brassicae* infested plants at 6 days after infestation as compared to uninfested control plants. Significant differences were observed in total flavonoid content between control and infested plants of both the varieties. Significantly higher total flavonoid content (170.37 µg/g) were recorded in infested plants that were treated with jasmonic acid the signalling molecule than those treated with Salicylic acid and only infested with insects. Our findings were just like the mirror images of the findings taken by Johnson and Dowd (2004) who reported that flavonoid confer resistance against *Spodoptera frugiperda* in *Arabidopsis thaliana.* Piubelli *et al.* (2003) reported that higher level of flavonoids, such as daidezin and genistin, had been observed in soybean plants infested with *Nezara viridula.*

The total flavonoid content was higher in plants treated with 1.5 mM concentration (243.00 µg/g) of jasmonic acid and the varieties Khanyari (218.72 µg/g) had maximum total flavonoid content as compared to Kawdari.

Different concentration of jasmonic acid (25, 50, 100 and 200 µg/ml) increased the concentration of various classes of defensive compounds, including alkaloids, phenolics and flavonoids in tomato plant against *Meloidogyne incognita*. The highest amount was found at 200 µg/ml (Annigeri *et al.*, 2011).

5.4 Total proteins

Plant infested with *P. brassicae* larvae had significantly greater protein content in Khanyari (0.43 mg/g) at 6 days after infestation as compared to control.

Among the two signalling molecules i.e., jasmonic acid and salicylic acid, plants pre-sprayed with jasmonic acid and then infested with insects had greater protein (0.69 mg/g) content as compared to salicylic acid. Our findings were in agreement with the findings of Avdiushko *et al.* (1997), Omer *et al.* (2001), who found that the exogenous application of JA or methyl jasmonate (MeJA) intensified plant resistance to various herbivores and induced the expression of defensive proteins in tomato (Stout *et al*., 1998; Cipollini and Redman 1999; Boughton *et al.,* 2005). War *et al.* (2011) found Ground nut plants infested with *Spodoptera litura* (Fab.) had greater protein content in ICGV 86699 at 24, 48 and 72 h after infestation as compared to control plants. Significant difference was observed in protein content between control and infested plants in both the varieties at 6 days after treatment.

Among different concentrations of jasmonic acid sprayed on both the varieties of kale and then infested with *P. brassicae* larvae, Khanyari had significantly greater protein content (0.804 mg/g) at 6 days after treatment. Plants sprayed with 1.5 mM concentration of jasmonic acid had greater protein content as compared to other concentrations (0.829 mg/g).

In best induction conditions of exogenous application of methyl jasmonate, four time interval (3,6,12 and 24 hours) and four concentrations (50, 100, 225 and 450 µm) were tested .The total protein concentration in maize reached the highest concentration at 225 µm of methyl jasmonate concentration (Zheng *et al.*, 2015).

5.5 Larval weight

Plants pre-sprayed with phytohormones and then infested with second instar larvae of *P. brassicae* and the plants that were only infested with larvae of *P. brassicae* had significant difference in larval weight. Plants that were only infested with *P. brassicae* larvae had maximum (180.79 mg) larval weight as compared to plants that were pre-sprayed with phytohormones. Among the varieties, Kawdari had greater (144.80 mg) larval weight in plants that were only infested with *P. brassicae* larvae. Caterpillars (*Manduca sexta* and *Spodoptera exigua*) fed on mutant tomato deficient in JA production showed higher survivorship and greater weight gain than those fed on wild-type tomato (Thaler *et al.*, 2002). Significant difference was observed in larval weight in plants that were only infested with larvae and the plants that were firstly sprayed with phytohormones and then infested with the larvae of *P. brassicae.* Among treatments the lowest larval weight was found in plants treated with phytohormone jasmonic acid and then infested with larvae of *P. brassicae.* Reduction in larval weight is an important aspect of plant resistance to insect pests. The increase in tannins, proteins, flavonoids and phenol content of plants resulted in loss of larval weight of *P. brassicae.* Greater larval mortality was recorded for insects that fed on ICGV 86699 and NCAc 343 than for those that fed on TMV 2. Larval weights were also lower in insects that fed on ICGV 86699 and NCAc 343 than those that fed on TMV 2. There were significant differences in the larval weights of insects reared on plants treated with PJA + JA, HIN + JA and HIN. Reduced damage, decreased larval survival and low larval weights might be due to higher induction of secondary metabolites and other defensive compounds in the insect-resistant genotypes on account of insect damage and JA application (Lawrence and Koundal, 2002; Sharma *et al.*, 2005; Bhonwong *et al.*, 2009; Chen *et al.*, 2009).

5.6 Per cent damage

Plants sprayed with jasmonic acid showed lower damage (9.73%) due to *P. brassicae*. This is because jasmonic acid quickly activated the production of secondary metabolites than salicylic acid which in turn provided defensive response to the plant against *P. brassicae* larvae and therefore caused reduction in damage. The insect resistant variety i.e., Khanyari showed greater reduction in plant damage (14.39%) than other variety i.e., Kawdari because genetic nature of Khanyari was that it is more resistant to the attack of larvae of *P. brassicae*. As the days after treatment increased the percent damage decreased in case of both phytohormones and the lowest percent damage was observed in phytohormone jasmonic acid (5.30%) that was observed in variety Khanyari and 9 days after treatment (DAT). The reason for this decrease in per cent damage was that, when days after treatment increased the concentration of secondary metabolites also increased. Reduced damage, lower larval survival and lower larval weights might be due to the greater production of toxic secondary metabolites in the insect-resistant genotypes by insect damage and jasmonic acid application (Felton *et al.,* 1994; Mao *et al.,* 2007; Chen *et al.,* 2009 and Bhongwong *et al.,* 2009). Reduced damage and lower larval growth and development were correlated with increased activity of POD (peroxidase), PPO (polyphenoloxidase) and other defensive enzymes induced following insect attack and/or elicitor application. The PJA+HIN treats plants suffered lower damage due to *Helicoverpa armigera* across genotypes. The insect resistant showed greater reduction in plant damage than the susceptible check JL24 (Chen *et al.,* 2009).

Chapter - 6

SUMMARY AND CONCLUSION

The salient features of the present investigation entitled, “To study the defensive response of Kale against cabbage butterfly, *Pieris brassicae* Linn.” under Kashmir condition are summarized and concluded as under:

Two phytohormones were used i.e., jasmonic acid and salicylic acid and second instar larvae of *P. brassice* were also used to study the induced resistance. The concentration of secondary metabolites i.e., phenolic content, condensed tannin, total flavonoids, total protein, larval weight and per cent damage were evaluated on the other hand different concentration of jasmonic acid (1.0, 1.5 and 2.0 mM) were used to evaluate the concentration of secondary metabolites and total protein content in kale varieties.

- The highest phenolic content (196.07 µg/g) was observed in treatment jasmonic acid + insect infestation and among varieties, Khanyari showed highest amount (145.05 µg/g) of phenolic content.
- Plants treated with phytohormone jasmonic acid and then infested with larvae of *P. brassicae* showed highest amount of condensed tannin (301.57 µg/g) and variety Khanyari had maximum amount (212.34 µg/g) of condensed tannin.
- Total flavonoid was found highest in plants sprayed with jasmonic acid (170.37 µg/g) and then infested with *P. brassicae* larvae and among varieties Khanyari showed maximum amount (145.56 µg/g) of total flavonoid.
- Total protein content was also found highest (0.69 mg/g) in plants sprayed with jasmonic acid - the phytohormone and then infested with larvae of *P. brassicae* variety Khanyari showed highest (0.43 mg/g) concentration of total protein.
- Lowest larval weight (100.17 mg) was observed in plants that were

sprayed with jasmonic acid and highest weight was observed in plants (180.79 mg) that were not sprayed with any phytohormone but only infested with larvae of *P. brassicae.*

- Highest per cent damage (23.61 %) was observed in plants that were only infested with larvae of *P. brassicae* and lowest was (9.73%) observed in plants that were sprayed with phytohormone jasmonic acid, among varieties lowest damage (14.39%) was observed in variety Khanyari and also the lowest damage (5.30%) was observed in 9 DAT in variety Khanyari.
- Among different concentration of jasmonic acid the highest concentration of phenolic content (223.30 µg/ml), condensed tannin (349.31µg/ml), total flavonoids (243.00 µg/ml) and total protein (0.829 µg/ml) were found at concentration 1.5mM.

CONCLUSION

Jasmonic acid and salicylic acid induced the activity of secondary metabolites in kale against cabbage butterfly. Higher amount of secondary metabolites were observed in variety Khanyari. Induced response to application of jasmonic acid was greater than salicylic acid. Plants treated with jasmonic acid showed lowest larval weight as compared to salicylic acid, while as only insect infestation showed highest larval weight. Per cent damage caused by *P. brassicae* larvae was highest in 3 DAT in both jasmonic acid and salicylic acid treated plants and lowest was observed in 9 DAT. Among varieties Khanyari showed lowest per cent damage. Regarding chemicals, lowest per cent damage was observed by use of jasmonic acid. The maximum amount of secondary metabolites was observed in 1.5 mM jasmonic acid. Thus it is concluded from the present investigation that induced resistance can be used as a component of integrated pest management. It is also concluded that induced resistance is not only used for Kale but we can use it for all cole crops, pulses such as soybean, groundnut and many other crops.

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner Dr. Sheikh Abdul Gaffar, Former Associate Dean, Faculty of Agriculture, Wadura during Viva-Voce examination held on September 14, 2015 have been incorporated in the manuscript entitled **"To Study the Defensive Response of Kale against Cabbage Butterfly (*Pieris brassicae* Linn.)"** submitted by **Ms. Shahida Ibrahim (Regd. No. 2013-A-976-M)**.

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