Detailed Project Report

Project Title: *In vitro* defense biochemistry of *Cicer arietinum* L.

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Introduction:

The Chickpea (*Cicer arietinum* L.) is an ancient crop that has been grown in India, also known as a garbanzo bean, ceci bean, sanagalu, chana, hummus and Bengal gram. It has an interesting fact behind its name; it's called Chickpea because it looks like baby chick. This pulse is estimated to be at least 7,500 years old and was originally cultivated at south-eastern Turkey. Recently this crop grown throughout world including India, Pakistan, Turkey, Iran, Myanmar, Ethiopia, Mexico, Australia, Syria, Spain, Canada, United States, Bangladesh, Algeria, Ethiopia, Malawi, Sudan, Tanzania, Tunisia, and Portugal (NMCEI, India, 2009, Food and Agricultural Organization, 2009). This is a highly nutritious pulse, ranked third in the important list of the food legumes that are cultivated throughout the world. Chickpeas have mainly two types i.e. *Desi* and *Kabuli*. The white-seeded, bigger in size having a thinner seed coat known as "*Kabuli*" and other one with relatively smaller seed size having a thicker seed coat and the brown colored, small in size known as "*Desi*" types. Chickpea is mostly cultivated for its seeds being used in daily meals. Seeds are rich source of protein and form an important part of vegetarian diet. Chickpea seeds of *Desi* cultivars usually have about 17-20% of protein while that of *Kabuli* ranges upto 26%. Chickpea is an important rabi crop mainly sown in September-November and harvested in February. Crop duration is 90-120 days, depending on the variety.

India, as a major Chickpea producing country, highest production has been received from Madhya Pradesh by 39%, and followed by Maharashtra (14%), Rajasthan (14%), Andhra Pradesh (10%), Uttar Pradesh (7%), Karnataka (6%) and other remaining states and UT's of India (10%) (Production share calculation has been done for the 10 years average i.e. 2005-2014-DES, MoA, Govt of India). About 2-3 million tons of pulses are imported annually to meet the domestic consumption requirement. Thus, there is need to increase production and productivity of pulses in the country. The population is increasing

dramatically in the modern era and there is emerging problem of food supply and food security. Diseases of agricultural food plants added extra burden leading huge loss in productivity. So in the case of Chickpea. Therefore, it becomes necessary to develop some high yielding varieties having multiple disease resistance to meet the need.

Chickpea (*Cicer arietinum* L.) (Diploid, 2n = 16) is considered as one of the most important crops of modern agriculture. (Zohary and Hopf, 2000). Chickpea belong to the **class**- Equisetopsida **Subclass**-Magnoliidae **Superorder**-Rosanae. **Order**- fabales and **family**- Leguminosae/ Fabaceae and botanically recognized as *Cicer arientinum* L. *Cice arietinum* is one of 43 species in the genus *Cicer*. *Most of the cultivars of chickpea*, cultivated worldwide and specially in India are susceptible to various fungal, bacterial, viral and nematodal diseases. So far 67 different fungal pathogens have been reported on chickpea (Nene *et al*., 1989).

A Series of biotic and abiotic stresses on Chickpea reduces its yield and yield stability. This affects development of widely adapted cultivars/ variety and susceptibility to several biotic and abiotic stresses. Generally the crop produces excessive vegetative growth under high input conditions and is unable to translate the biomass into high seed yields. The major abiotic constraints to productivity include drought, heat, cold and salinity. The major biotic constraints are *Ascochyta* blight caused by the Ascomycete *Ascochyta rabiei* (Singh *et al*. 1984; Saxena and Singh 1985: Singh *et al*.1984; Jiminez diaz *et al*. 1993), *Fusarium* wilt caused by *Fusarium oxysporum f. sp. ciceris* (Matuo and Sato, 1962), Dry root rot (*Rhizoctonia bataticola*), *Botrytis* grey mould (*Botrytis cinerea*), Collar rot (*Sclerotium rolfsii*), Root-knot nematode (*Meloydogyne incognita* and *M. javanica*), Stunt-virus, Pod borer (*Helicoverpa armigera*), and Cutworm (*Agrotis ipsilon*). Amongst the causal agents of biotic stresses, about 67 fungi, six fungal diseases have been reported to be important and causing considerable damage to the crop (Haware *et al*., 1986; Nene *et al*., 1996); 3 bacteria, 22 viruses and 80 nematodes have been reported on chickpea (Nene *et al*., 1996) but only few of these cause economically important diseases (Haware, 1998). There has been considerable increase in different chickpea pathogens like fungi, bacteria and viruses over a period of two decades. Among the economically important fungal diseases of chickpea are root diseases like *Fusarium* wilt and root rots caused by a complex of soil borne fungi (*Fusarium oxysporum* f.sp. ciceris), foliar diseases like *Ascochyta* blight and *Botrytis* gray mould, of which wilt and blight are the most devastating diseases affecting chickpea in Maharashtra, India regions, respectively.

The most important disease is Chickpea wilt caused by *Fusarium oxysporum* f. sp. *Ciceris*, was first reported by Butler in 1918. Prasad and Pad wick (1939), identified *Fusarium* spp. to be the causal agent of chickpea wilt. The current scenario of chickpea production indicates that, wilt have resulted in huge yield loss (Trapero-Casas and Jimenez-Diaz, 1985; Jalali and Chand, 1992), and at times under specific suitable conditions for pathogen growth it is capable of complete destruction of the crop (Halila and Strange, 1996). In Indian context this loss ranges from 24 -65% (Haware and Nene, 1980). Therefore, it becomes necessary to develop some high yielding varieties having multiple disease resistance including wilt pathogen is one of major breeding objective. To achieve this goal, especially in case of fusarium wilt, it is essential to understand the Chickpea- *Fusarium* pathosystem and mechanism controlling effective resistance at genetic and biochemical level.

The synthesis of biochemical compounds and their level in plant offer a particular characteristic to it. Presence of various biochemical compounds in plant during its different developmental stages indicates their importance for plants survival. Production and level of plant hormones and some enzymes determine the percentage of germination, growth rate, development and reproduction (Abeles et al. l97l; Jones, 200l and Dangl, 200l). The resistance of plants to various pathogens depends on synthesis and level of various defense enzymes like hydrolases, peroxidases and antimicrobial compounds like phytoalexins (Kuc, *et al*., l991; Kauffmann *et al*., l987; Boiler, l987; Mauch *et al*., l988; Kale and Choudhary, 200l, Koche and Choudhary, 2005). Plants use an intricate defense system against pests and pathogens, including the production of low molecular mass secondary metabolites with antimicrobial activity, which are synthesized *de novo* after stress and are collectively known as phytoalexins. A range of phytoalexins is produced by different crop plants.

In addition to a variety of inducible secondary metabolic compounds viz. hydrolases such as chitinase and β,1-3 glucanases are two predominant proteins and those are induced in response to several abiotic and biotic stresses. These are also known as Pathogenesis-related proteins (PR- proteins). There are many direct and indirect evidences to support the antifungal activity of these two and their role in the plant defense (Abeles *et al*., 1971; Wessels and Sietsma, 1981; Young and Peg, 1982; Keen and Yoshikawa,1983; Boller, 1985, 1987; Mauch *et al*., 1988). Synthesis of these hydrolases in plants in response to invading pathogen is very important. Plant pathogenesis-related proteins are implicated in plant defense responses against pathogen infection (Saboki, 2011).

Thus all these reports suggest that the synthesis and level of biochemical compounds in plants play vital role in shaping various traits of all plant species including the resistance. Most of the workers isolate and quantified various biochemicals prominently from different parts of mature plants (Abeles *et al*., l97l; Bowels, l985 and Koche and Choudhary, 2005). However, there are very few reports on isolation and quantification of these biochemicals in seeds and seedlings. Isolating and characterizing the biochemicals at early stage or before germination, will help us to know the exact status of the developing plant and will reduce the labour and time. In such case we don't have to wait for entire season to know the characters. Similar strategy could be apply in case of deciding resistance status or defense behavior of the plant at early in seed or seedling stage.

The Defense mechanism of plants is an inbuilt system and plant uses it whenever necessary. It is now a known fact that, each plant species has the genes responsible for the defense mechanism. However, the individuals/ species which have active defense genes those further establish resistance against particular stress conditions (Biotic/ abiotic stress) and those where the defense genes are inactive are called as susceptible to particular pathogen or stress condition. Most of the crops cultivated in our region especially grains are susceptible to various pathogenic attack leading to huge yield loss. Therefore, it is necessary to understand the plant pathogen interaction and develop strategies against the pathogen.

The proposed topic is basically deals with the stress biology, which has the combination of' botany, microbiology and biochemistry. In true sense, it is an interdisciplinary approach. It leads to analyze the biochemical changes due to microbial pathogenic attack on the specific plants species of crop. Thus, it could be relate with agriculture also.

The present work is planned to analyze the resistance status and defense mechanism in vitro in the *Cicer* varieties to *Fusarium* wilt and other abiotic elicitors. *Cicer arientinum* L. is commonly called as Chickpea. This crop plant is selected because; it is one of the main crop in the Vidharbha region of Maharashtra and also been cultivated throughout India. However, most of the varieties used by the farmers for cultivation are susceptible to *Fusarium* causing a disease *Fusarium* wilt. This is a major soil-borne disease to Chickpea which lead to huge loss in the Chickpea productivity. Therefore, this aspect is selected for the proposed study with following

Objectives:

- Investigating *Cicer arientinum- Fusariurn oxisporium* pathosystem.
- Preparation and standardization of Elicitors/ inducers.
- Analysis of PR- proteins, PAL and phytoalexin medicarpin in susceptible and resistant cultivars of *Cicer arientinum* L.
- *In vitro* induction of defense system in *Cicer arientinum* L. using-
    - Pathogen Derived elicitors
    - Abiotic elicitors
- Comparative analysis of Defense gene induction and expression in *Cicer* cultivars at biochemical level.

Materials and Methods:

The present study was planned to investigate the biochemical aspects of defense response of Chickpea (*Cicer arientinum* L.) cultivars in response to pathogen derived elicitor and few abiotic elicitors like Copper sulphate and Zinc sulphate. For the present study, four cultivars of *Cicer arientinum* (L.) were selected namely Vijay, Digvijay, Jaki and JG-62.

Germplasm collection and Preliminary field evaluation:

In the present investigation, germplasm of four cultivars were procured from Pulse Research Center, Dr. Panjabrao Deshmukh Krishi Vidyapith, Akola and Mahatma Phule Krishi Vidyapith, Rahuri (MS). Seeds of all these cultivars were sown in the field for multiplication and the status of disease resistance was assessed under field conditions. The resistant status was analyzed by measuring percent infection per leaflet. All the varieties were found to perform true to their recommended properties.

Analysis of Defense Response:

The defense response in four cultivars of chickpea to pathogens, pathogen-derived elicitors and various abiotic elicitors was investigated in terms of expression of defense-related genes, viz., Phenylalanine ammonia-lyase (PAL), Chitinase and β-1,3 glucanase. The level of phytoalexin Genestein, an antimicrobial compound, was also analyzed. The response was analyzed in cotyledons, seedling parts and in field grown plants (*in vivo*).

Analysis of Defense Response in field grown plants:

The level of phytoalexin, and expression of chitinase and β,1-3 glucanase genes were analyzed in the field grown plants. As the chickpea plants starts developing symptoms of leaf spot disease, by 40 days onwards, leaves of each cultivar were harvested after every 15 day from the day of germination to the age of 60 days. The harvested leaves were frozen in liquid nitrogen and then stored at -20^{o}C, until use. The enzyme assays and quantification of phytoalexin are described elsewhere in this chapter (The methods and techniques used).

Analysis of Defense Response in Seedlings:

The induced expression of chitinase, β-1,3 glucanase and phenylalanine ammonia-lyase (PAL) along with the level of genestein has been analyzed at the seedling stage. For this, uniform seeds of all four chickpea cultivars were surface sterilized with 0.1% aqueous $HgCl_2$ for 10 min and then with 70% alcohol for 10 min. The sterilized seeds were washed

thoroughly (3X) with sterile distilled water. All the operations were carried out aseptically in a laminar air flow bench. The surface sterilized seeds of each cultivar were inoculated separately in sterilized culture tubes (35 x 250mm) containing agar. The culture tubes then plugged and incubated at 25^{o}C in dark.

The nine-day-old, etiolated seedlings were used for the induction of defense related gene expressions. These seedlings were aseptically sprayed with filter sterilized biotic and abiotic elicitors, using sterile glass atomizer. The biotic elicitors used were, *Fusarium oxysporium* cell wall elicitor (50 μg/l). The abiotic elicitor used were Copper sulphate (0.125mg/l), Zinc sulphate (0.100mg/l) and Ultra violate light (wavelength between 100-290nm).

The elicited seedlings were then frozen in liquid nitrogen, periodically. For PAL assay, the seedlings were frozen every 4 hours after elicitation upto 16 hours. For the chitinase and glucanase assays, the seedlings were frozen every 24 hours (one day) after elicitation upto 7 days. For the analysis of phytoalexin content, the seedlings were frozen every 24 hours after elicitation upto 120 hours (four days). The frozen material was stored at -20^{o}C until use. The PAL, Chitinase, Glucanase and the procedure of phytoalexin analysis were done as described later in this chapter, under the heading, "The methods and techniques used".

Analysis of Defense Response in Cotyledons:

The induced expression of defense related genes and accumulation of phytoalexin, were also studied in the cotyledons. In this case, the seeds were sterilized with 0.1% HgCl2, 70% alcohol and distilled water, for 10 minutes each in laminar air flow bench. Sterilized seeds then incised vertically by sterile scalpel aseptically and then the cotyledons without embryo axes were inoculated on the wet filter paper in sterile petriplates (with lids). After nine days these cotyledons were sprayed with the abiotic as well as biotic elicitors, and then frozen in liquid nitrogen, periodically for the analysis of PAL, chitinases, glucanases and for phytoalexin, medicarpin, as described in “The method and techniques used”.

METHODS AND TECHNIQUES USED

Different methods and techniques used in this investigation are described below.

ENZYME ASSAY:

Activities of the enzymes, β-1, 3- glucanase, Chitinase and Phenylalanine ammonia-lyase (PAL) were determined by employing the methods described below.

Assay for Beta-1,3- glucanase assay:

The glucanase assay was performed according to procedure followed by Kauffmann et al., (1987).

a) Reagents-

i. **Extraction buffer (0.1M, pH 5):** Sodium citrate buffer was used in this investigation for extraction of the enzyme glucanase. It was prepared by mixing stock A and B.

Stock solution A was prepared by dissolving 21.01gm of citric acid in distilled water and the volume was adjusted to 1000 ml.

Stock solution B was made by dissolving 29.41gm of Sodium citrate dihydrate in distilled water and the volume was adjusted to 1000 ml.

The extraction buffer was prepared by mixing 180 ml of solution A, 320 ml of solution B and 500 ml distilled water. The pH was adjusted to 5.

ii. **Sodium acetate buffer (0.1M, pH 5.2):** It was prepared by mixing the stock solutions A and B.

Stock solution A (0.1M) was prepared by dissolving 13.6gm of sodium acetate in 100 ml distilled water and the volume was adjusted to 1000ml by distilled water.

Stock solution B (0.1M) was prepared by mixing 11.6ml of acetic acid with 988.5 ml distilled water.

Buffer was prepared by mixing 52 ml stock solution A with 197.5 ml of stock solution B. To this 1.05 ml β-mercaptoethanol was added and pH was adjusted to 5.2 with the addition of sufficient amount of distilled water to make the volume 1000 ml.

iii. Alkaline copper tartarate reagent:

This reagent was prepared by using following stock solutions-

Stock solution A was prepared by dissolving 2.5gm of sodium carbonate, 2gm sodium bicarbonate, 2.5gm potassium sodium tartarate and 20gm of anhydrous sodium sulphate, one by one in 80ml distilled water and the volume was adjusted to 100ml by distilled water.

Stock solution B was prepared by dissolving 15gm of copper sulphate in 50ml of distilled water and one drop of concentrated sulphuric acid was added to it. The volume was adjusted to 100ml by distilled water.

The reagent was prepared by mixing 96ml of stock solution *A* and 4ml of stock solution *B.*

iv. Arseno-molybdate reagent:

The reagent was prepared with the help of following two stock solutions.

Stock solution A was prepared by dissolving 2.5gm of ammonium molybdate in 45ml of distilled water. 2.5ml of concentrated sulphuric acid was also added to it.

Stock solution B was prepared by dissolving 0.3gm of disodiun hydrogen arsenate, in 25 ml of distilled water.

The reagent was prepared by mixing both stock solutions A and B. The mixture was then incubated at 37^{o}C for 48 hours and then used as arseno-molybdate reagent.

v. Laminarin *(Substrate)* stock solution:

Stock solution of laminarin was prepared by dissolving 50mg of laminarin in 10ml of distilled water. The 200 µl of this solution, containing 1mg of laminarin, was used as substrate for β-1, 3-glucanase assay.

b) Procedure:

I) Extraction of Enzyme:

Glucanase and chitinase were extracted by homogenizing 1gm of frozen tissue in 1ml ice cold extraction buffer (0.1M sodium citrate buffer, pH 5), in pre-chilled mortar and pestle.

The extract was centrifuged at 10,000 rpm for 15 minutes. The 1ml of supernatant was taken in 1.5ml of centrifuge tube and proteins were precipitated by adding ammonium sulphate to saturation. Precipitated proteins were centrifuged at 10,000 rpm for 15 minutes and supernatant was discarded. The protein pallet was resuspended in 1ml extraction buffer and used as enzyme source for glucanase and chitinase assay.

II) Assay:

The β-1, 3-glucanase was extracted from frozen tissue as described above. The assay mixture was prepared by mixing 0.48 ml of 0.1M sodium acetate buffer (pH 5.2), 100 µl enzyme extract and 200 µl laminarin solution. The mixture was incubated at 37^{o}C for 3 hours. Then, 0.5ml alkaline copper tartarate was added to it and mixture was heated at 100^{o}C, in the boiling water bath for 5 minutes. The mixture was cooled to room temperature and 0.5ml of arseno-molybdate reagent was added to it. After the development of blue color, 3 ml of distilled water was added to each sample and absorbance was recorded at 660 nm against the blank containing the enzyme and all other reagent, except laminarin.

III) Preparation of standard curve:

To prepare the standard curve, 100 µl aliquots of different concentrations of aqueous glucose (10-100 µg) were mixed with 400 µl alkaline copper reagent. The mixture was heated to 100^{o}C for 5 minutes. The mixture was then cooled and 500 µl of arseno-molybdate reagent was added to it. After the development of blue color, 3 ml distilled water was added and absorbance was recorded at 660 nm. The standard curve was prepared by plotting the absorbance against concentration.

Glucose residue released by glucanase activity in the sample was quantified by comparing the absorbance of the samples at 660 nm with the standard curve. Total protein content of the sample was determined by protein-dye binding method. The enzyme activity was calculated in ηKats/ mg protein. Glucanase activity was calculated as-

$$Glucanase\ activity\ (\mu Kats/mg\ protein) = \frac{486.9 \text{ X Amount of glucose residue released}}{\text{Amount of protein /gm of sample}}$$

Assay for Chitinase assay: -

The enzyme activity was analyzed according to the method of Reissig et al. (1955) and Boller et al. (1983)

a) Reagents:

i. Extraction buffer (0.1M, pH 5): Sodium citrate buffer was used in this investigation for extraction of the enzyme Chitinase. It was prepared as describe earlier.

ii. Sodium azide (0.3 μM): Sodium azide stock solution was prepared by dissolving 9.75 gm of sodium azide in 100 ml of distilled water. The working solution (0.3 μM) was prepared by diluting 100 μl of this stock solution to 500 ml of distilled water.

iii. Sodium acetate buffer (0.5 μM, pH 4.5): Stock solution of this buffer was prepared by dissolving 5 mg of sodium acetate in 10 ml of distilled water and 136 μl of it was diluted to 100 ml by distilled water. Then the pH was adjusted to 4.5 with acetic acid and stored at 2^{o}C. The working buffer was prepared by diluting 1 ml of this stock to 10 ml.

iv. Borate buffer (0.8M, pH 9.1): This buffer was prepared by mixing stock A with stock B.

Stock solution A was prepared by dissolving 7.636 gm of disodium tetraborate in sufficient volume to make 100 ml solution.

Stock solution B was prepared by dissolving 4.96 gm of boric acid in distilled water to make 100 ml solution.

The borate buffer was prepared by mixing 83 ml of stock solution A and 50 ml of stock B and the volume was adjusted to 200 ml with distilled water. The pH of this buffer was adjusted to 9.1 and stored at 2^{o}C until use.

v. DMAB reagent: The reagent was prepared by dissolving 10gm of p-dimethylamino benzaldehyde in 100 ml of glacial acetic acid (AR) containing 12.5 ml of HCl (AR). From this stock solution the working reagent was prepared by diluting the stock ten times with the glacial acetic acid.

vi. Stock solution of colloidal chitin (Substrate solution): This stock solution was prepared by homogenizing 500 mg of crab shell chitin (HiMedia make) with 50 ml of distilled water. 200 μl of this stock solution containing 1mg colloidal chitin was used for the enzyme assay.

b) Procedure:

I) Extraction of Enzyme:

Chitinase was extracted from frozen tissue as described in glucanase assay, using the same extraction buffer.

II) Assay:

Chitinase enzyme assay mixture contained 100 µl of Sodium acetate buffer (pH 4.5), 100 µl of Sodium azide solution, 200 µl of colloidal Chitin and 100 µl of enzyme extract. The volume of this enzyme mixture was adjusted to 1ml by extraction buffer and was incubated at 37^{o}C, for 3 hours. After the incubation, 100 µl of sodium borate buffer (pH 9.1) was added to the reaction mixture and heated to 100^{o}C in water bath for 3 minutes. The mixture was then cooled in tap water and centrifuged at 1000 rpm for 5 minutes. The clear supernatant was collected and to it 3 ml of DMAB reagent was added. The mixture was then incubated for 20 minutes at 37^{o}C. The absorbance was recorded immediately at 585 nm containing enzyme and all reagents, except chitin.

III) Preparation of standard curve:

For the preparation of standard curve, different concentrations of N-acetyl d-glucosamine, ranging from 1mg to 100mg were prepared and then incubated with DMAB reagent at 37^{o}C for 20 minutes and absorbance was recorded at 585 nm. The absorbance was plotted against concentration to prepare the standard curve. From this standard curve, the quantity of N-acetyl d-glucosamine formed by the Chitinase activity, in the different samples was calculated. Total protein content of the extract was quantified by protein-dye binding method.

The Chitinase activity was expressed in µKats/ mg protein. The Chitinase activity was calculated as,

$$Chitinase\ activity\ \left(\frac{\mu Kats}{mg}protein\right) = \frac{407.3\ \text{X Quantity of N} - \text{acetyl d} - \text{glucosamine}}{\text{Amount of protein /gm of sample}}$$

Protein Estimation by Protein-Dye Binding Method: -

The amount of protein in each sample was estimated by protein-dye binding method of Bradford (1976).

a) Reagents:

I) Sodium chloride solution (0.15N):

This solution was prepared by dissolving 0.877 gm of sodium chloride in 100 ml of distilled water.

II) Dye solution:

Dye used, in the protein estimation, was Coomassie Brilliant blue G-250 (Sigma make). The stock dye solution was prepared by dissolving 100 mg of Coomassie brilliant blue G-250 in 50 ml of 95% ethanol (AR grade) and 100 ml of o-Phosphoric acid was added to it. The stock dye solution was stored in amber colored bottle at 10^{o}C. Just before use, the dye was diluted 6.66 times with distilled water. The final concentrations of the reagents were 0.01% (w/v) coomassie brilliant blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) o-Phosphoric acid.

b) Procedure:

The 100 µl of enzyme extract was taken in approximately 8 ml test tubes and the volume was adjusted to 1 ml by distilled water. To this 4.0 ml of dye solution was added. Blue color developed after 3-5 minutes at room temperature. Then the absorbance was recorded at 595 nm against the blank containing all the reagents except enzyme extract.

c) Preparation of Standard curve:

To prepare the standard curve of protein, the aliquots of different concentrations, ranging from 10 µl to 80 µl of bovine serum albumin were made and the volume was adjusted to 100 µl with Sodium chloride (0.15N) solution. To it 900 µl of distilled water and finally 4 ml diluted dye was added. The absorbance was recorded at 595 nm, 5 minutes, after the development of blue color. The absorbance was plotted against the concentrations, from this curve the protein content of sample was determined.

Assay for Phenylalanine Ammonia-Lyase (PAL):

The enzyme Phenylalanine ammonia-lyase (PAL) was assayed according to the procedure given by Lamb et al. (1980).

a) Reagents:

i. **Tris buffer (Extraction buffer):** This buffer was prepared by dissolving 0.605 gm Tris in 1000ml of distilled water (50mM) and to it 226 µl β-mercaptoethanol was added (14mM). The pH was adjusted to 8.5 by 1N hydrochloric acid.

ii. **L-Phenylalanine *(Substrate*) solution (12.1mM):** This solution was prepared by dissolving 149.9 mg of L-Phenylalanine in 75 ml of 50mM tris buffer. The pH was adjusted to 8.5.

iii. **D-Phenylalanine (*Substrate check*) solution (12.1mM):** This solution was prepared by dissolving 149.9 mg of D-Phenylalanine in 75 ml of 50mM tris buffer. The pH was adjusted to 8.5

b) Procedure:

I) Extraction of enzyme:

Phenylalanine ammonia lyase was extracted from frozen tissue by homogenizing 1 gm tissue in 1 ml ice-cold extraction buffer, in pre chilled mortar and pestle. To avoid the interference in Spectrophotometric readings, due to the presence of phenolics particularly cinnamic acid, 0.5% (w/v) Polyvenylpyrroledone (PVP) was added at the time of extraction to remove (adsorb) these phenolics. The extract was centrifuged at 10,000 rpm and clear supernatant was used as enzyme source.

II) Assay:

For assaying PAL, two different sets of each sample were prepared. The vials of first set contained 100 µl enzyme extract and 2.9 ml of L-Phenylalanine (substrate) and second set contained 100 µl of enzyme extract and 2.9 ml of D-Phenylalanine solution (substrate check). The blank was prepared for each set separately, The blank of first set contained 100 µl L-Phenylalanine and 2.9 ml extraction buffer and of second set contained 100 µl D-Phenylalanine and 2.9 ml extraction buffer. These vials were then incubated in a water bath, at 40^{o}C for 1 hour.

Then absorbance was recorded at every 30 minutes interval upto 2 hours at 290 nm. The assay was done in triplicate. The enzyme activity of PAL was calculated using following formula.

$$PAL\ activity\ (\mu Kats\ /\ protein\ ) = \frac{27780\ \text{X}\ (\square\ \text{in absorbace of L} - \text{Phe}\ - -\ \square\ \text{in absorbance of D} - \text{Phe})}{\text{Amount of protein /gm of sample}}$$

Phytoalexin analysis:

In this investigation the accumulation of phytoalexin medicarpin, in response to natural infection by *Fusarium oxysporium,* was analyzed, by high performance liquid chromatography (HPLC) following the method adopted by Edward and Strange (1991). The phytoalexin was also analyzed in cotyledons as well as in seedlings of different cultivars.

One gm of sample was extracted with 5 ml of 80 % methanol. The methanol extract was reduced to 1/4th of initial volume, under vacuum and extracted (3x) with ethyl acetate. The pooled ethyl acetate extract was reduced to dryness and contents were dissolved in one-ml acetonitrile. The 20 µl acetonitrile extract was injected for quantitative analysis. The samples were chromatographed on Shimadzu HPLC system with ODS C_{18} (Spherosphere) column (4 x 250 size) maintained at 35^{o}C temperature. The flow rate of mobile phase (50 % aqueous acetonitrile) was 1.5ml/ minute. The medicarpin was detected at 290 nm using a PDA detector with retention time of 23 minutes. The retention time was determined by co-chromatography of standard obtained from Sigma.

Fungal culture:

In this investigation, biotic elicitor was prepared from *Fusarium oxysporium.* The culture of this fungus was obtained from infected roots of chickpea and soil around plant root, respectively. The infected roots were collected from the field. The root discs of about 1 cm in diameter, containing the infected area were cut and surface sterilized with mercuric chloride (0.1%), alcohol (70%) and distilled water for 2-3 minute each. The discs were washed thoroughly with distilled water and inoculated on modified Coo's medium added Chickpea leaf extract (2gm/l).

Elicitor Preparation:

Preparation of Fungal cell wall elicitor:

The 3-week-old *Fusarium oxysporium* liquid cultures was used to prepare cell wall elicitor. Mycelia were harvested from the medium by vacuum filtration through four layers of muslin cloth. The starch of muslin cloth was removed prior to use. Mycelia were washed

thoroughly, with distilled water to remove the traces of medium. It was then extracted with 100ml chloroform- methanol (1:1) mixture and organic solvents were evaporated by vacuum. The mycelia were then extracted with 50ml methanol, followed by extraction with 50ml acetone. All organic fractions were discarded by vacuum evaporation. The residual wall debris were air dried overnight, weighted and homogenized in distilled water (40 times w/v). Mycelial debris with water extract were autoclaved at 15psi pressure for 20 minutes. Autoclaved extract was centrifuged at 5000 rpm to remove cell wall debris. The clear supernatant was used as crude cell wall elicitor, after estimating its carbohydrate content in terms of glucose equivalents by standard anthrone reagent method. The elicitor containing 50 μg glucose equivalent/ml was used to elicit the defense response.

Preparation of Copper sulphate solution:

Copper sulphate solution was prepared by dissolving 12.5mg compound in 100 ml distilled water. This solution was sterilized by autoclaving and used as elicitor.

Preparation of Zinc Sulhate solution:

Zinc sulphate solution was prepared by dissolving 10.0mg compound in 100 ml distilled water. This solution was sterilized by autoclaving and used as elicitor.

Use of UV- C:

UV-C (wavelength between 100- 290nm) light was used to irradiate the seedlings/ calli. The material was kept in a laminar airflow chamber at the distance of 30 cm from light source for 30 minutes. The source emitted UV rays with the wavelength below 290nm.

Determination of total carbohydrate by Anthrone method:

The carbohydrate content in crude cell wall elicitor was estimated in terms of glucose equivalents by standard anthrone reagent method (Sadasivam and Manikam, 1992) as described below.

a) Reagents:

I. HCl solution (2.5N): This solution was prepared by diluting 21.5 ml concentrated HCl (11.6N) to the total volume of 100 ml by distilled water.

II. Anthrone Reagent: This was prepared by dissolving 200 mg anthrone in 100 ml of ice cold 95% Sulphuric acid. This solution was prepared fresh every time.

III. Standard Glucose Solution: Glucose stock solution was prepared by dissolving 100 mg glucose in 100 ml distilled water. Working standard was prepared by diluting 10 ml of stock solution to 100 ml distilled water.

b) Procedure:

Crude cell wall elicitor, 100ml was hydrolyzed with 5ml 2.5N HCl, in a boiling tube at 100^{o}C in water bath for 3hours. The hydrolyzed solution was cooled to room temperature and then neutralized by adding solid sodium carbonate until effervescence ceased. Volume of this solution was made to 100 ml by distilled water. From this solution 0.5 ml was used to analyze carbohydrate content.

Glucose standards were prepared by pipetting out 0.2-1ml of working standard solution and adjusting the volume in each tube to 1ml with distilled water. Then 4 ml anthrone reagent was added. All the above solutions including samples, were heated in boiling water bath for 8 minutes, cooled immediately in ice cold water and absorbance was recorded at 630 nm. Blank included 1 ml distilled water and 4 ml Anthrone reagent. A standard curve of different glucose concentrations was plotted against the absorbances.

$$Amount\ of\ carbohydrate\ in\ the\ sample = \frac{mg\ glucose\ x\ 100}{Volume\ of\ test\ sample}$$

Modified Coon's medium used for culture maintenance of *Fusarium oxysporium*.

Sr. No.	Chemical Content	Quantity in gm
1	Sachharose	7.2 gm
2	Dextrose	3.6 gm
3	Mangnesium Sulphate	1.23 gm
4	Potassium dihydrogen Phosphate	2.72 gm
5	Potassium Nitrate	2.02 gm
6	Distilled Water	1000 ml
7	Agar Agar	20.0 gm
8	Host Leaf extract	2.00 gm

Results:

Symptoms, Disease Incidence and Resistance Status:

Fusarium wilt is one of the most dangerous diseases of chickpea resulting huge loss in yield. During present investigation, the symptoms of *Fusarium* wilt, disease incidence and severity was studied in four Chickpea cultivars (Digvijay, Vijay, Jaki and JG-62). All four cultivars were grown in different plots as per the standard protocol. The progeny was observed for rate of germination, growth, environmental stress, disease incidence and death thereby. The prominent symptoms of *Fusarium* wilt on chickpea include- stunted growth; yellowing of lower leaves and wilting of plants; desiccation and death of leaves and eventually the plants (Plate-1). The disease incidence percentage was measured as number of plants infected by *Fusarium* wilt per 100 plants of progeny. The disease incidence index (DII) was measured on four point scale. The data of incidence of *Fusarium* wilt is presented in table- 1. It was observed that, out of selected cultivars Digvijay is highly resistant to *Fusarium* wilt. It showed about 07% of disease incidence and death thereby. Cultivar Vijay showed about 12% disease incidence and considered as resistant followed by cultivar Jaki with 26% disease incidence (moderate resistant). As per the four point disease incidence scale, JG-62 was found to be susceptible with 38% disease incidence *Fusarium* wilt (table-1).

Table-1: Disease incidence and disease severity of Fusarium wilt in four selected cultivars of *Cicer* under naturally grown condition:

Cultivar	Progeny size (No. of plants)	Disease incidence	Resistant status
Digvijay	2380	07%	Highly Resistant
Vijay	2562	12%	Resistant
Jaki	1980	26%	Moderate Resistant
JG-62	2286	38%	Susceptible

Note: Four point scale of DII : 0-10% = Highly resistance, 11-20% = Resistance, 21-30%= Moderate resistance, Above 30% = Susceptible ; The values are mean of triplicate analysis

Plate- 1

Fusarium **infected plants in field and Root discoloration due to infection**

Table- 2: Glucanase, chitinase activity (μkats/mg protein) and phytoalexin (μg/g fresh tissue weight) content in leaves of naturally grown plants of chickpea cultivars (60 days after germination):

Cultivar	Resistant status	Chitinase activity	Glucanase activity	Phytoalexin content
Digvijay	Highly Resistant	36.55	15.39	81.5
Vijay	Resistant	22.65	12.60	75.3
Jaki	Moderate Resistant	15.54	12.26	17.2
JG-62	Susceptible	10.63	08.33	13.8

Note: The values are mean of triplicate analysis

In vivo Analysis of Defense Response in *Cicer* Cultivars:

The defense response of Chickpea cultivars was also tested biochemically in leaves of field grown plants. The activity of PR- proteins like glucose and chitinase along with phytoalexin medicarpin content in leaves of 60 days old plants were tested. Among the four cultivars, the plants of cultivar- Digvijay with least disease incidence showed highest chitinase and glucanase activities (36.55 and 15.39 μkats/mg protein) respectively. It is followed by activities in the leaves of cultivar Vijay and Jaki. JG-62 showed the least chitinase and glucanase activities. The phytoalexin content of the leaves after 60 days of germination, Digvijay showed highest value followed by leaves of cultivar –Vijay (Table-2). however, the medicarpin content in Jaki and JG-62 was very less. This indicates that, these two cultivars are susceptible to *Fusarium* wilt, however the degree of susceptibility varies with other parameters.

Time course analysis of Medicaarpin in Cheakpea leaves.

The HPLC analysis of Chickpea cultivars showed specific trend in the accumulation of phytoalexin. Phytoalexin medicarpin in leaves of naturally grown chickpea cultivar was analyzed periodically. The time course analysis showed that, the content of medicarpin in leaves increases from 10^{th} day after germination upto 60 day and then start to decline. The highest accumulation of medicarpin is showed by the cultivar Digvijay after 60 days of

germination (81.5 mg/g fresh leaf tissue) followed by in cultivar Vijay and Jaki. The least content was observed in JG-62 where the peak accumulation was 22.9 mg/g fresh leaf tissue after 40 days of germination. Further it could be stated that, the peak content of phytoalexin medicarpin of Jaki and JG-62 was achieved nearly 40 days i.e. 20 days before that of Digvigajy and Vijay. However the content is very less and also not persists steadily towards the maturity (Table- 3).

Table-3: Phytoalexin Medicarpin content (μg/g fresh tissue weight) in naturally grown chickpea cultivars

Cultivar	Days after germination				
	After 10 days	After 20 days	After 40 days	After 60 days	After 80 days
Digvijay	10.6	24.7	45.6	81.5	52.3
Vijay	09.2	21.6	42.9	75.3	48.7
Jaki	09.9	16.3	29.5	17.2	15.6
JG-62	08.5	18.5	22.9	13.8	15.4

Note: The values are mean of triplicate analysis

In vitro Defense Response Analysis:

β-1,3-Glucanase activity in response to FCWE:

The time course analysis of β-1,3 glucanase was done in cotyledons and seedlings of Chickpea cultivars after elicitation with *Fusarium* cell wall elicitor (FCWE). It was observed that, FCWE could induce the level of β-1,3 glucanase. After elicitation, FCWE induces response within a day and peak response in the form of β-1,3-glucanase was recorded after 3rd day of elicitation. Among all the cultivars, elicited cotyledons of Digvijay showed highest peak glucanase activity (9.28 μkats/mg protein) followed by that in cultivar Vijay (8.50 μkats/mg protein). The elicited cotyledons of Jaki showed (6.83 μkats/mg protein) and least in JG-62 (3.25 μkats/mg protein). After reaching the peak, the glucanase activity starts to decline slowly (Table- 4). The peak glucanase activity in resistant cultivars was found to 6.5-7.5 fold higher than in unelicited control; while this raise in case of susceptible cultivar was 3 fold and moderate resistant cultivar 6-6.5 folds.

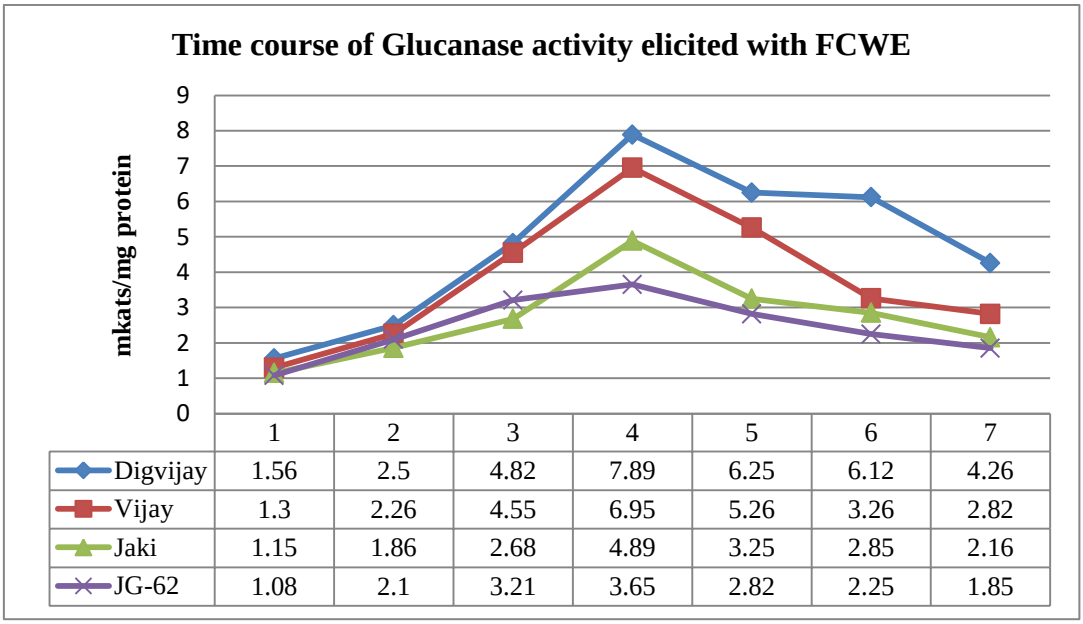
The similar trend of β-1,3- glucanase induction after elicitation with FCWE, showed similar pattern. The details of time course of FCWE induced glucanase activity is presented in fig. 1.

Table- 4: β-1,3-Glucanase activity in Cotyledons of chickpea cultivars (µkats/mg protein) elicited by *Fusarium* cell wall elicitor (FCWE)

Cultivar	Control	Days after elicitation					
		1st day	2nd day	3rd day	4th day	5th day	6th day
Digvijay	1.24	3.68	6.26	9.28	7.22	5.36	3.25
Vijay	1.30	2.25	4.58	8.50	6.29	4.66	2.95
Jaki	1.05	1.85	2.68	6.83	4.26	2.25	1.76
JG-62	1.08	1.85	2.16	3.25	2.86	2.50	1.95

Note: The values are mean of triplicate analysis

Fig. 1: Time course analysis of β-1,3 Glucanase activity (µkats/mg protein) in Seedlings of chickpea cultivars elicited by *Fusarium* cell wall elicitor (FCWE).



	1	2	3	4	5	6	7
Digvijay	1.56	2.5	4.82	7.89	6.25	6.12	4.26
Vijay	1.3	2.26	4.55	6.95	5.26	3.26	2.82
Jaki	1.15	1.86	2.68	4.89	3.25	2.85	2.16
JG-62	1.08	2.1	3.21	3.65	2.82	2.25	1.85

Chitinase activity in response to FCWE:

Similar to that of β-1,3- glucanase, chitinase enzyme was also analyzed in FCWE elicited cotyledons and seedlings. The time course analysis showed that, chitinase was induced early after elicitation and reaches its peak activity after 3rd day of elicitation and then starts declining. In present study, the FCWE induced cotyledons of cultivar Digvijay showed highest chitinase activitity (8.29 μkats/mg protein); followed by that in Vijay (7.52 μkats/mg protein). The peak chitinase activity in cotyledons of Jaki and JG-62 after elicitation with FCWE was 5.25 μkats/mg protein and 3.26 μkats/mg protein respectively (Table-4.5). The peak chitinase activity in cotyledons was found to be 3.5 times more in moderately resistant and resistant cultivars while this raise in less than 3 folds as compare to control in fusarium wilt susceptible cultivars.

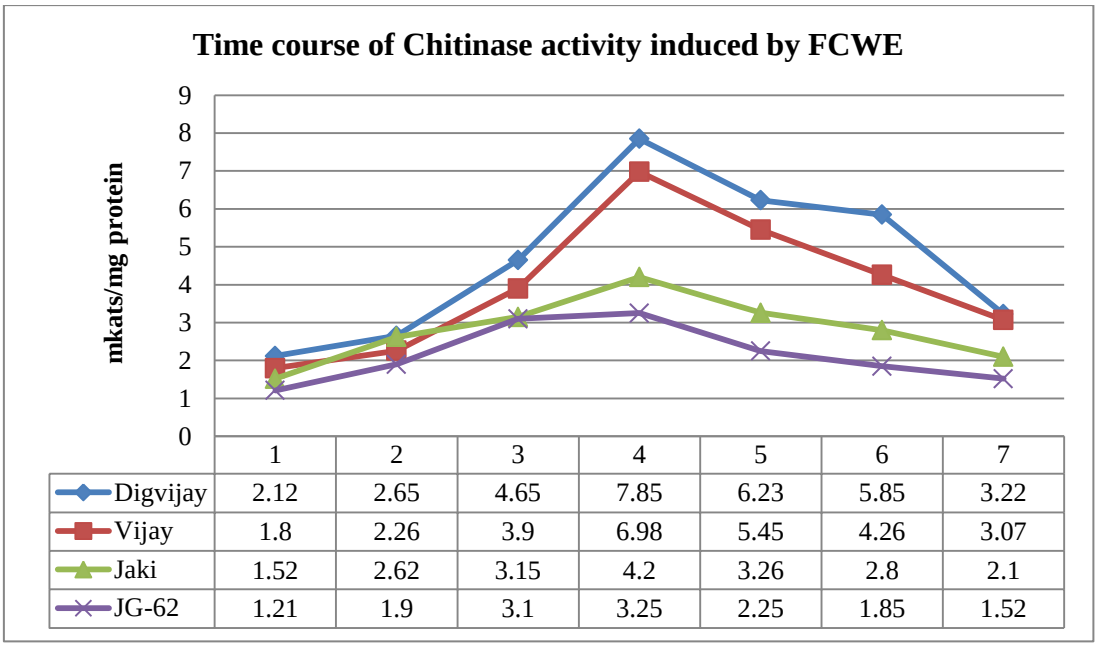
FCWE also induced chitinase activity in seedlings of chickpea. The induction could be seen by its peak activities on 3rd day after elicitation and then starts declining. The peak chitinase activity in seedlings of Digvijay elicited with FCWE was highest followed by in that of Vijay. The least FCWE induced chitinase activity was recorded in seedlings of JG-62. The time course study of chitinase activity induced by FCWE in seedlings of four chickpea cultivar is presented in fig. 4.2.

Table- 5 Chitinase activity (μkats/mg protein) in Cotyledons of chickpea cultivars elicited by *Fusarium* cell wall elicitor (FCWE):

Cultivar	Control	Days after elicitation					
		1st day	2nd day	3rd day	4th day	5th day	6th day
Digvijay	2.25	2.80	5.25	8.29	7.65	5.78	4.25
Vijay	2.02	2.85	4.86	7.52	7.12	4.36	3.25
Jaki	1.85	2.29	3.45	5.25	3.29	2.86	1.45
JG-62	1.05	1.85	2.25	3.26	2.85	2.15	1.76

Note: The values are mean of triplicate analysis

Fig. 2: Chitinase activity in Seedlings of chickpea cultivars (µkats/mg protein) Elicited by *Fusarium* cell wall elicitor (FCWE).



	1	2	3	4	5	6	7
Digvijay	2.12	2.65	4.65	7.85	6.23	5.85	3.22
Vijay	1.8	2.26	3.9	6.98	5.45	4.26	3.07
Jaki	1.52	2.62	3.15	4.2	3.26	2.8	2.1
JG-62	1.21	1.9	3.1	3.25	2.25	1.85	1.52

PAL activity in response to FCWE:

PAL was analyzed in the elicited cotyledons as well as seedlings, every 4 hrs after elicitation upto 24 hrs. It was observed that, after elicitation, the PAL was induced early reaches its peak activity by 8 hrs after elicitation and them starts declining. The highest peak activity after elicitation with FCWE was 38.25 µkats/mg protein, in cotyledons of Digvijay, it was followed by that of Vijay and Jaki cotyledons. The least activity of PAL was recorded in cotyledons of JG-62 after elicitation with FCWE. The peak PAL activity in the cotyledons was always more than 3 fold higher than that of control, while that in susceptible cultivars, it was always less than 3fold. The actual recorded PAL activity is presented in table 4.6. The activity in case of resistant cultivars, there is significantly high level in case of resistance or moderately resistant cultivars; but is becomes lower in case of susceptible cultivars and can be compare with control values (Table-6).

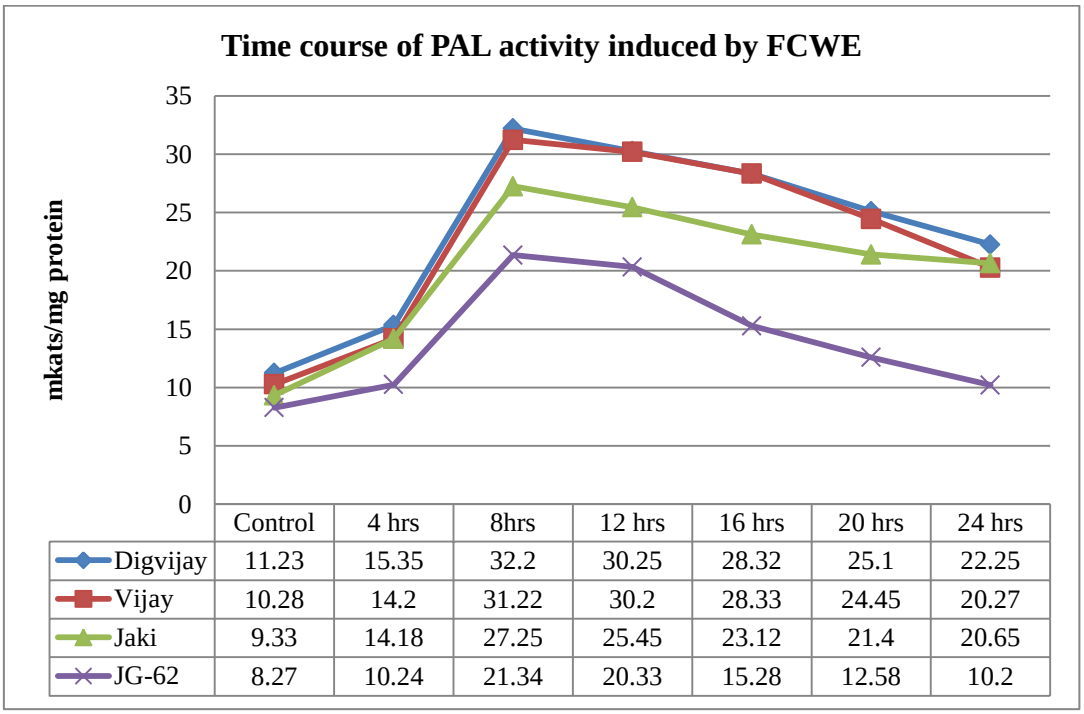
The similar kind of induction of PAL in response to FCWE in seedlings of chickpea cultivars was recorded. However, the raise upto the peak level was slight low in case of seedlings. The peak PAL activity is higher in Digvijay and Vijay and lowest in case of JG-62 seedlings elicited with FCWE (Fig. 3).

Table- 6: PAL activity (µkats/mg protein) in cotyledons of chickpea cultivars elicited by *Fusarium* cell wall elicitor (FCWE)-

Cultivar	Control	Days after elicitation					
		4hrs	8hrs	12hrs	16hrs	20 hrs	24 hrs
Digvijay	11.23	18.33	38.25	33.28	31.35	29.13	24.25
Vijay	10.28	17.25	32.33	32.24	30.45	29.15	23.27
Jaki	9.33	15.38	29.59	27.42	24.48	25.14	22.85
JG-62	9.27	12.30	27.65	25.36	18.45	16.52	12.28

Note: The values are mean of triplicate analysis

Fig- 3: PAL activity (µkats/mg protein) in seedlings of chickpea cultivars elicited by *Fusarium* cell wall elicitor (FCWE).

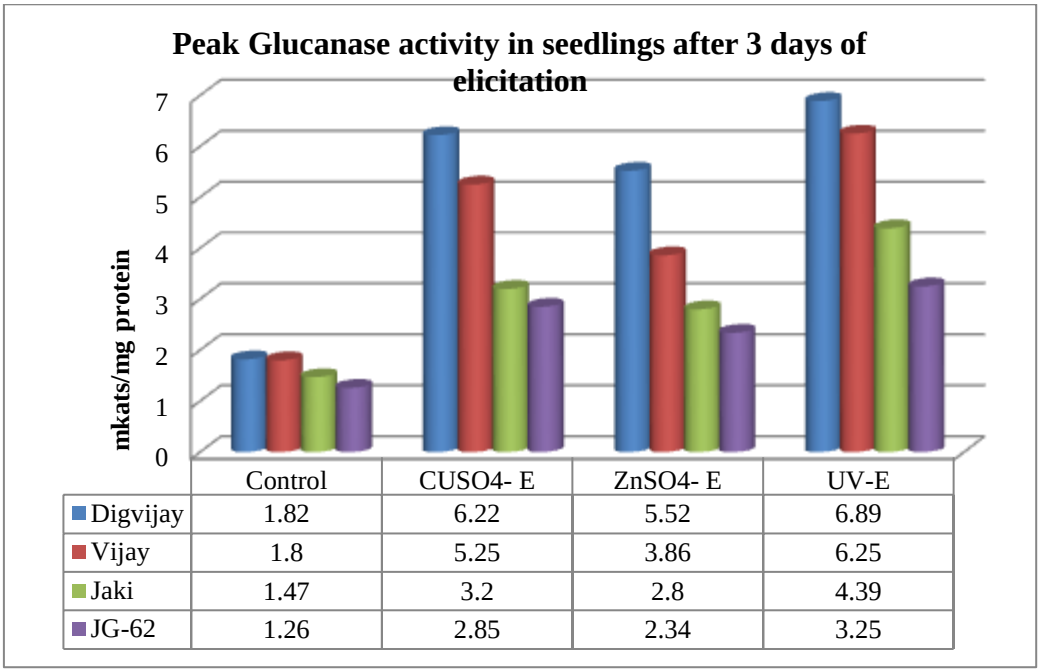


	Control	4 hrs	8hrs	12 hrs	16 hrs	20 hrs	24 hrs
Digvijay	11.23	15.35	32.2	30.25	28.32	25.1	22.25
Vijay	10.28	14.2	31.22	30.2	28.33	24.45	20.27
Jaki	9.33	14.18	27.25	25.45	23.12	21.4	20.65
JG-62	8.27	10.24	21.34	20.33	15.28	12.58	10.2

β-1,3- glucanase induction in response to abiotic elicitors:

Induced defense response in the form of β-1,3-glucanase, of Chickpea cultivars was analyzed in seedling stage after elicitation with different abiotic elicitors i.e. $CuSO_4$, $ZnSO_4$ and UV elicitor. The Peak glucanase activity after 3 days of elicitation is presented in fig. 4. It was found that the glucanase activity in unelicited control was in the range of 1.26 to 1.82 µkats/ mg protein; however, the peak activity after induction was found to be 2 to 4 fold. The increase in glucanase activity was more in case of resistant cultivars (Digvijay followed by Vijay) as compare to the susceptible and moderate resistant cultivars (Fig. 4). In general, UV elicitor was found to be more effective than copper sulphate and zinc sulphate.

Fig- 4: Peak glucanase activity in seedlings of chickpea cultivars elicited by different abiotic elicitors.



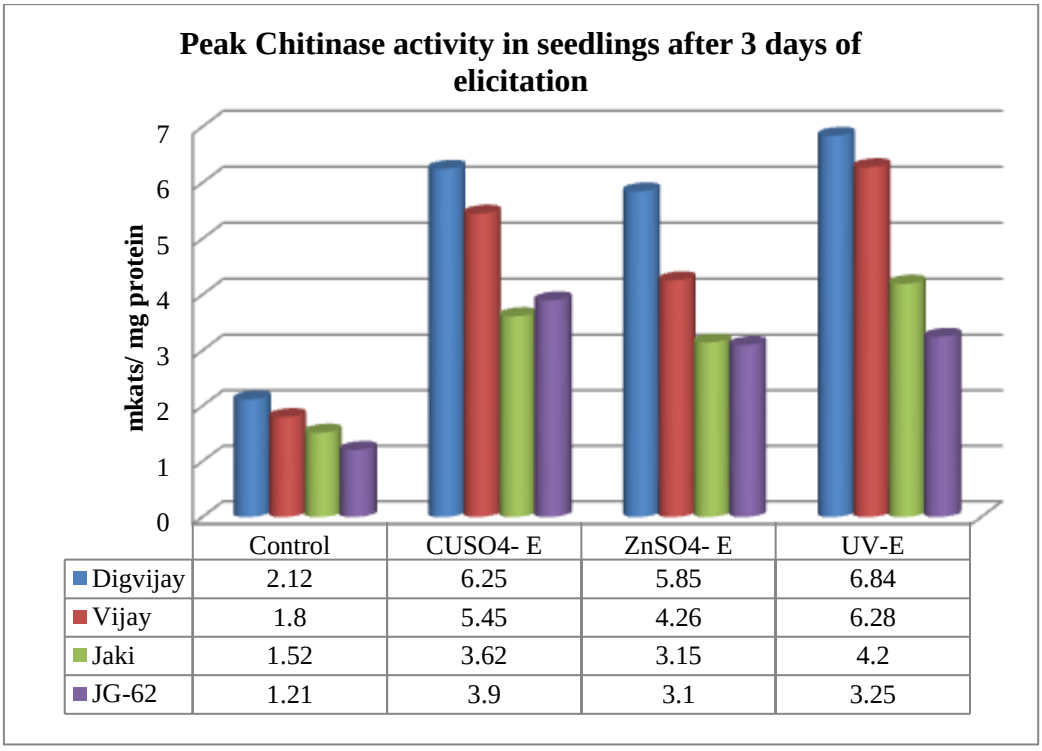
	Control	CUSO4- E	ZnSO4- E	UV-E
Digvijay	1.82	6.22	5.52	6.89
Vijay	1.8	5.25	3.86	6.25
Jaki	1.47	3.2	2.8	4.39
JG-62	1.26	2.85	2.34	3.25

Chitinase induction in response to abiotic elicitors:

Chitinase activity was also recorded in seedlings of chickpea cultivars elicited with different abiotic elicitors. All cultivars showed significant induction of chitinase enzyme after elicitation. The peak activity in seedlings of chickpea cultivars recorded after elicitation with copper sulphate, zinc sulphate and UV light is presented in fig. 5. It was noted that, peak chitinase activity in seedlings of each cultivar was significantly higher than control. Among

the elicitors, UV light was most potent in induction of chitinase followed by copper sulphate and least was zinc sulphate (Fig. 5).

Fig. 5: Peak chitinase activity in seedlings of chickpea cultivars elicited by different abiotic elicitors.

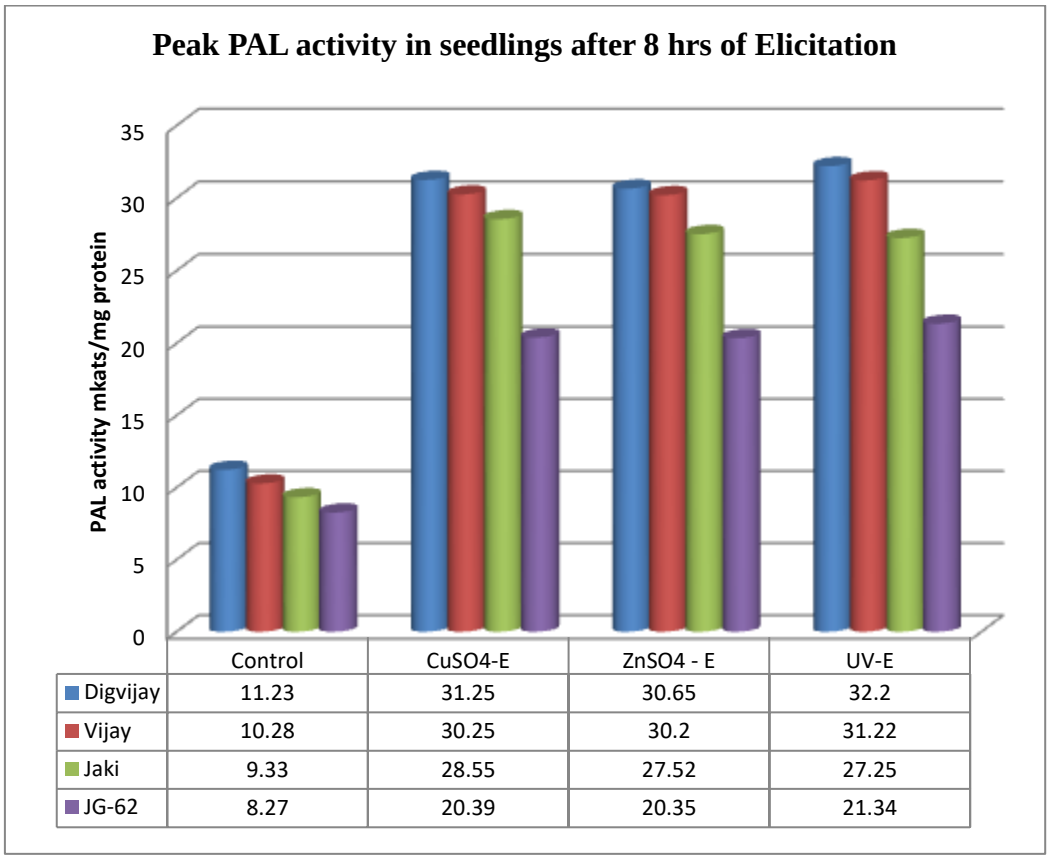


	Control	CUSO4- E	ZnSO4- E	UV-E
Digvijay	2.12	6.25	5.85	6.84
Vijay	1.8	5.45	4.26	6.28
Jaki	1.52	3.62	3.15	4.2
JG-62	1.21	3.9	3.1	3.25

PAL induction in response to abiotic elicitors:

Phenylalanine ammonia lyase (PAL) is the key enzyme of phenylpropanoid pathway. It play vital role in synthesis of phytoalexins. Induced PAL activity was analyzed in four chickpea cultivars in repose to abiotic elicitors, $CuSO_4$, $ZnSO_4$ and VU as an elicitor. In all cases, the peak PAL activity in seedlings of all cultivars were more than double. The highest is found in Digvijay followed by Vijay and then Jaki and JG-62. However, the defense response of each elicitor was found to be comparatively higher in resistant cultivars than susceptible one (fig.6). UV radiations as an elicitor observed to be more effective than other elicitors used in the study.

Fig. 6: Peak PAL activity in seedlings of Chickpea cultivars elicited by different abiotic elicitors.



	Control	CuSO4-E	ZnSO4 - E	UV-E
Digvijay	11.23	31.25	30.65	32.2
Vijay	10.28	30.25	30.2	31.22
Jaki	9.33	28.55	27.52	27.25
JG-62	8.27	20.39	20.35	21.34

Analysis of Phytoalexin medicarpin in response to biotic and abiotic elicitors.

The medicarpin content (µg/g fresh tissue weight) in FCWE, UV, $CuSO_4$ and $ZnSO_4$ induced cotyledons and seedlings were also analyzed. The Peak medicarpin value in each chickpea cultivar was analyzed 4 days after elicitation with different biotic and abiotic elicitors. It was noted that, induction of medicarpin in cotyledons was more expressive than in seedlings. In cultivar Digvijay FCWE induced about 7 fold more medicarpin in cotyledons as compare to control; while this induction in seedling was found to be about 3 fold (Table-7). If we compare the induction of medicarpin as defense compound by FCWE (pathogen derived) and abiotic elicitors, pathogen derived elicitors has more potential to induce the defense genes as shown in the level of medicarpin. In case of cotyledons of cultivar Digvijay, FCWE was observed to be most potent elicitor followed by CS-E, ZS-E and least one UV-E. Apart from this, we did not observed any specific trends except all peak values are higher

than that of control (Table-7). Further, the results also showed that, similar readings were obtained in case of induced seedlings.

Table- 7: Peak Medicarpin content (μg/g fresh tissue weight) in cotyledons and seedlings of chickpea cultivar (4 days after elicitation)

Cultivars	Medicarpin content (Peak value)								
	Control	In cotyledons				In seedlings			
		FCWE	UV-E	CS-E	ZS-E	FCWE	UV-E	CS-E	ZS-E
Digvijay	10.5	75.6	52.4	58.6	57.5	35.6	31.8	27.8	25.6
Vijay	08.7	72.8	46.5	48.5	46.2	52.9	31.5	29.6	27.5
Jaki	08.4	27.8	27.6	23.2	20.6	26.5	24.5	23.4	20.5
JG-62	08.4	19.5	14.8	18.2	18.5	16.5	15.2	15.4	16.2

Note: The values are mean of triplicate analysis

Discussion and Conclusion:

Chickpea (*Cicer arientinum* L.) is one of the most popular crops in India. As a major chickpea grower, India's agricultural economy depends on its production. Unfortunately, several chickpea cultivars grown in Indian subcontinent are susceptible to various pathogenic microbes life fungi, bacteria and viruses which leads huge yield loss. Among the most dangerous diseases of chickpea, *Fusarium* wilt caused by *Fusarium oxysporium* is most frequent.

Disease control by the use of chemicals (fungicides, pesticides, insecticides etc.) is not cost effective and it also pollute the soil. However, disease control by breeding resistant cultivar could be more effective. Moreover, developing a cultivar with multiple disease resistance would be appreciable solution for this problem. Secondly, developing the resistance in existing cultivars by enhancing its defense mechanism would be a proper step in this line. For this, it is most essential to understand the defense mechanism of that plant and the triggering mechanism underlying it.

When a pathogen comes in contact with the plant it has to break several lines of defense, set up by plants, before reaching the living cells. These includes mechanical barriers like cuticle, several chemical exudates (milk, latex, gum etc.) that inhibit the pathogen invasion and spread. These constituents are the preformed anti-pathogenic compounds produced by plant usually known as pre-infectional metabolites or prohibitins or phytoanticipins.

If all these plant weapons are not sufficient to stop the pathogen growth/ spread then plants usually respond by blocking or delaying the advancement of invader. Active oxygen species (AOS) are often generated as warning signals within the cells or in the neighboring cells, triggering off various reactions (Baker *et al.*, 1997; Lamb and Dixon, 1997; Mc Dowell and Dangl, 2000). This includes the structural reinforcement of the cell wall. Plants may respond hyper sensitively by programmed cell death (PCD), necrosis, development of systemic acquired resistance (SAR) and accumulation of newly formed antifungal compounds that are known as phytoalexins and PR- proteins. All these responses directly or indirectly play vital roles in restricting the growth of pathogen in the host cells (Greenberg *et al.*, 1994; Dixon *et al.*, 1996; Lamb and Dixon, 1997).

The present investigation was undertaken to understand various mechanisms involved in the resistant interaction of *Cicer arientinum* (Chickpea) against fungal pathogen *Fusarium oxysporium*.

The study involved the defense response of chickpea to *Fusarium oxysporium* and biotic inducer and few abiotic inducers like UV light, copper sulphate and zinc sulphate. It was analyzed in the form of-

1. Induction of two PR proteins namely b-1,3-glucanase and chitinase in response to pathogen derived biotic elicitor and abiotic elicitors.
2. Induction of defense genes encoding for PAL, a key enzyme in phytoalexin synthesis at seedling level.
3. Accumulation and level of phytoalexin (an antimicrobial compound) in vivo and in vitro.

For this investigation, four chickpea cultivars were used. Off which, Digvijay was highly resistant, Vijay was resistant, Jaki was moderately resistant and JG-62 was susceptible to Fusarium wilt disease.

Phytoalexin accumulation and Resistant Status:

The phytoalexins are antimicrobial in nature and serves for plant defense mechanism; several previous reports supports this fact. The presence of phytoalexins in plants exposed to any type of stress conditions indicates its role in plant defense. Phytoalexins, not only are accumulated at the site of infection but do so, following penetration by microbes, quickly enough and in sufficiently high concentration to inhibit the growth of fungi (Bailey 1974; Sato *et al.*, 1971; Rossall *et al.,* 1980 and Badere *et al.,* 2007) and bacteria (Lyon *et al.*, 1975). Perhaps, the best example of this type of evidences has been obtained by Mansfield *et al.,* (1974), who studied the rate of accumulation of phytoalexin at the site of infection in the epidermis of broad bean leaves by fungal pathogen *Botrytis fabae*. The similar observations were made by Strange (1992) in lettuce- *B. cinerea* interaction, Dollar and Gurcan (1993) in chickpea- *Ascochyta* interaction, Paiva *et al.,* (1994), in alfalfa- *Phoma* interaction, Mayama *et al.,* (1995) in Oat- *Puccinia* interaction. Kale (1998) reported the positive interaction between Groundnut- *Cercospora* sp. He showed that the level of **medicarpin** found to be high in the leaves of resistant cultivar Girnar-1 as compared to other susceptible cultivars

when both were allowed to grow in the field and natural infection occurs. Salles *et al.,* (2002) also reported similar observations in the alfalfa- *Colletotrichum* interaction. Badere *et al.*, (2007) showed that, there is increased accumulation phytoalexin genestein in naturally infected leaves of mungbean cultivars.

In field grown plants of different chickpea cultivars, phytoalexin medicarpin level was found to be increased steadily from 10 day of germination onwards. The disease symptoms starts to appear after 40 days of germination and on its severity by 60 days. Stunted growth, yellowing of leaves, wilting and eventual death are the most common symptoms of this disease. More than 38% disease incidence was reported in *Fusarium* wilt susceptible cultivar (JG-62), with highest number of plants dying due to infection. Cultivar Jaki showed 26% disease incidence (moderate resistance to wilt), however, Vijay was with 12% disease incidence (resistance) and Digvijay was with (07%) disease incidence (highly resistance to wilt). In correspondence with the disease incidence, the leaves of Digvijay and Vijay showed high content of medicarpin and high activity of PR protein; which is closely followed by Jaki and least content was found in JG-62 (Table-1, 2 and Plate-1).

These observations indicates that, resistant chickpea cultivars can be correlated with the rapid accumulation of phytoalexin and significantly high activity of PR- proteins.

Kale (1998) studied the *Arachis- Cercospora* interaction extensively and showed the positive relation between increasing level of **medicarpin** and resistance in *Arachis hypogaea*. Duranga *et al.,* (2002) have also reported the accumulation of a variety of phytoalexins in Columbian bean. They correlated the level of phytoalexin with resistant status of Columbian bean to *Colletotrichum lindmuthianum*. Badere *et al.*, (2007) also reported the positive correlation of phytoalexin accumulation, higher activity of PR proteins and resistance status of Mungbean cultivars.

Expression of Phenylalanine Ammonia- Lyase:

The phytoalexin biosynthesis involve several complex steps and each step is catalyzed by different enzymes (Dixon, 1995; Dixon and Paiva 1995; Zhao *et al.,* 1998; Mansfield, 1999; Grayer and Kokubun, 2001). Phenylalanine ammonia lyase (PAL) is the key enzyme in phytoalexin biosynthesis that catalyzes the first step in this process. The role and importance of PAL and other related enzymes were already explained by several workers. They observed the induction of this enzyme in several plant species challenged by pathogens and pathogen derived elicitors (Crammer *et al.,* 1995; Yamada *et al.,* 1995 and Koche and

Choudhary, 2012). This enzyme have also been shown to be induced by several other biotic as well as abiotic elicitors at seedling stage (*et al.*, 1994; Bahl *et al.*, 1998; Kale and Choudhary, 2001 and Koche, 2004).

In the present investigation, defense related genes coding for the enzyme phenylalanine ammonia lyase (PAL) were differentially expressed in resistant as well as in susceptible cultivars. In the resistant cultivar Digvijay, Vijay closely followed by Jaki; the PAL genes expressed at higher level in the seedling and cotyledons of these cultivars as compared to that of susceptible cultivars (JG-62). Similar type of responses have been observed in several plant species *Viz.,* tomato (De Wit 1977), potato (Fritzmeier *et al.*, 1987), alfalfa (Cooke *et al.,* 1994), Cotton (Gorski *et al.,* 1995) and Groundnut (Kale, 1998).

The resistant response shown by chickpea cultivars Digvijay and Vijay was characterized by early and rapid increase in the PAL activity. In these resistant cultivars the PAL activity rapidly increased and reached to its maximum level within 8 hours after elicitation. However, the maximum activity in susceptible cultivars reached far after with lower activity indicating the slow response. Hence, a clear correlation was observed in between resistance and rapid PAL gene expression (Fig. 3). Such a correlation between resistance and early/ rapid PAL induction in different seedling parts has been demonstrated in several species, *viz.,* Parsley (Johnen and Hahlbrock 1988), in Barley (Carver *et al.,* 1994) and Koche and Choudhary, (2012).

The early peak obtained for PAL activity in resistant mungbean cultivars indicates that in resistant interaction, the early peak activity might be due to early transcriptional activation of PAL gene. The increased PAL activity due to early transcriptional activation of PAL gene has been shown by Hahlbrock *et al.,* (1981), Lawton *et al.,* (1983) and Ebel *et al.,* (1984).

For present investigation, various elicitors have been used to induce the expression of PAL genes. Elicitors are characterized by their ability to induce the differential expression of defense related genes. The elicitor molecules derived from cell walls of different microorganisms, pathogens, toxins, peptides, enzymes and fatty acids constitute the biotic elicitors. Likewise there is an array of compounds that come under abiotic elicitors. The abiotic elicitors include, heavy metals, toxic compounds or gases, UV- radiation and mechanical injuries etc. to determine the resistant status in chickpea at biochemical level. Seedlings were challenged by different abiotic and biotic elicitors. It was observed that

cotyledons and whole seedlings of resistant cultivars showed hypersensitive response to elicitors in terms of expression of defense genes like PAL and PR- proteins.

The PAL gene expression was found to be induced effectively by biotic elicitors, in cotyledons and seedlings of resistant cultivars Digvijay and Vijay as compared to abiotic elicitors. The response of susceptible cultivars to PAL gene induction by biotic elicitors was found comparatively low. Similar responses of PAL gene induction was also reported by Lamb *et al.,* (1986) in bean- *Colletotrichum lindmuthianum* interaction.

The present study showed that amongst all the abiotic elicitors, the most effective was UV- C light that induced maximum PAL gene expression in the resistant cultivars Digvijay and Vijay. The expression of PAL genes induced by Zinc sulphate and Copper sulphate was comparatively less than that induced by UV-C light (Fig. 6).

Results obtained on the effectiveness of different elicitors for the induction of PAL genes, in this investigation showed that different elicitors induced the expression of PAL genes at various degrees, indicating the different mechanisms of induction. Genes encoding the enzyme PAL and Chalcone synthase (CHS) was found to rapidly induced. This indicates that signal transduction system is in place prior to elicitation and that there are few intervening steps between elicitor binding to a putative cell receptor (Darvill & Alberscheim, 1984) and specific transcriptional activation of these genes. Hence PAL and CHS gene activation might involve direct interaction of elicitor- receptor complex with cis- acting regulatory DNA sequences similar to that postulated in animal cells (Lawton and Lamb 1987).

The rapid induction of genes involved in phenylpropanoid pathways indicates that the signal pathways between the microbial recognition and activation of these defense genes may contain very few steps. Following infection, plant defense genes can be activated not only by microbial elicitors but also in response to endogenous factors. Intercellular signaling might be modified by endogenous factors (Dixon *et al.,* 1983) or by related wound signals and proteinase inhibiting factors (Ryan, 1984).

Involved complexities in the mechanism of differential regulation of PAL and other defense related genes by various elicitors are further demonstrated by following evidences. Distinct subsets or individual members of PAL and CHS multigene families are induced by different environmental or developmental stimuli. Dangl *et al.,* (1987) reported that parsley

protoplasts synthesize a typical quantitative and qualitative pattern and amount of potentially protective flavonoids, glycosides and coumarin phytoalexins, upon elicitation with UV-C light and pathogen derived elicitor respectively. While Lois *et al.,* (1989) have shown in parsley cell culture that biotic elicitors induced rapid and higher PAL gene activity than induced by UV-C light.

It is evident from these reports that the effect of elicitors on the plant tissues, particularly for the induction of defense related genes probably is a result of more than one mechanism involved, not only in signal recognition but also signal perception and transduction. Moreover, a single signal molecule is also reported to show the different effects on the same promoter depending upon physiological and developmental state of tissue (Lawton and Lamb1987).

The existence of such possible mechanism is further strengthened by host- parasite interaction studies, which have conclusively proved that for every avirulent (*avr*) gene in parasite there exists a resistant gene in the host (Flor 1947; Keen 1990 and Lindsay *et al.,* 1993). Flor (1947) reported that corresponding genes in host and pathogen determined interaction of Flax with rust fungus *Melampsora lini*. This simple genetic relationship implies that in host- parasite interactions, there exist molecular recognition between the product of paired resistance and avirulence genes. During the physiological and genetical studies of *Cladosporium fulvum* - tomato interaction, De wit *et al.,* (1986), isolated *cf 9* resistant gene in tomato and *avr 9* virulent gene in fungus. They stated that *C. fulvum* carrying *avr 9* infect and cause necrosis to those plants carrying *Cf9* gene in their genotypes and not to the plants carrying *Cf 9* gene.

Keen (1991) had also reported that *avrD* gene from *Pseudomonas sy*ringae conditioned the production of glycolipid that cause host cell necrosis, only in genotypes carrying the corresponding soybean resistant gene *Psg4.* These studies clearly suggest the specificity conferred by a pathogen or pathogen derived elicitor for the induction of defense response in the host.

Resistant status and expression of PR- proteins:

Some hydrolase enzymes of plant origin are known to have antifungal activities and commonly known as Pathogenesis related proteins (PR- proteins). Thus they play very vital role in plant defense. β-1, 3 glucanase and chitinase are grouped in this category of

hydrolases. These enzymes have been shown to be involved in conferring resistance to plants (Van Den *et al.,* 1993 and Broglie and Broglie 1993). Therefore, these enzymes have been analyzed in both resistant as well as susceptible cultivars *in vitro* and *in vivo*.

In the present investigation, the activity of two PR- proteins i.e. β-1, 3 glucanase and chitinase have been analyzed in field grown plants as well as in aseptically grown seedlings and cotyledons by different elicitors. In all the three cases, the activity of induced glucanase and chitinase was found to be significantly high in resistant cultivars (Digvijay and Vijay) as compared to susceptible cultivars (Fig. 1, 2 and Table-4, 5). β-1, 3 glucanase are the polymers of β-1, 3 linked glucan. These polymers are the major components of the cell wall of higher fungi (Boller, 1985). Glucanase is constitutively expressed in most plant tissue (De Carvalho *et al.,* 1992), at both m-RNA and protein level. They are also induced upon infection or elicitation with biotic as well as abiotic elicitors (Maher *et al.,* 1993 and Wubben *et al.,* 1996). Glucanase genes expressed singly or in combination with other defense genes. Such combinations were reported in canola (Benhmau *et al.,* 1993), rice (Lin *et al.,* 1995), alfalfa (Masoud *et al.,* 1996) and tobacco (Loritom *et al.,* 1998). Glucanase genes are thought to play a direct role in plant defense by digesting fungal cell walls as suggested by their antifungal properties *in vitro* either alone or in combination with chitinase genes (Mauch *et al.,* 1988 and Ji *et al.,* 1996). However, it has also been suggested that they play an important role in defense by releasing elicitors of other defense responses, such as phytoalexin production. Yoshikawa *et al.,* (1993) transformed tobacco plants with a soybean β-1,3 glucanase gene that had previously been shown to release elicitor *in vitro* from *Phytophthora megasperma* cell wall (Keen *et al.,* 1983), and observed enhanced resistance against *P. parasitica* var. *nicotianae.* It was argued that the glucanase produced in transgenic tobacco plant was able to release the elicitors from the invading pathogen and thus leading to enhanced production of phytoalexin.

There are several evidences that relates the increased activity of glucanase and chitinase in naturally infected plants or elicitor treated plant parts with the resistance (Boller *et al.,* 1983; Roby *et al.,* 1986; Kellmann *et al.,* 1996). Schlumbaum *et al.,* (1986) reported that in bean –*Fusarium solani* interaction chitinase was the main proteinacious inhibitors of fungus. Roby *et al.,* (1987) showed that early and more rapid induction of chitinase can be correlated with increased resistance in melon plants infected with *Collitotrichum lagenarium*. Broglie *et al.,* (1991) reported that transgenic tobacco expressing bean chitinase showed enhanced resistance against *Rhizhoctonia solani*.

The demonstration of Mauch *et al.,* (1988) have valuable contribution in this regard. They demonstrated that chitinase and glucanase induced in pea pods inhibited the growth of 15 out of 18 fungus tested. Other important reports were that of De wit and Vander Meer (1986) and Joosten and De wit (1989). They found that in tomato leaves, inoculated with *Cladosporium fulvum*, chitinase and glucanse accumulated rapidly at high level in incompatible interaction as compared to compatible interaction. On the basis of these observations, they correlated the response with the disease resistance. In pepper- *Phytophthora capsici* compatible as well as incompatible interaction, Kim & Hwang (1994) noted that glucanase and chitinase activity induced rapidly by pathogen. However, chitinase activity was comparatively less than glucanase activity in compatible interaction. From this they inferred that the in such cases, chitinase and glucanase have synergistic effect. Simoni *et al.,* (1995) used callus and germinating seeds for screening of active plant defense products in *Glycine max- Dioporthe phaseorum* var. *caulivora* interaction. They observed that both, callus and seedling parts produced Peroxidase and β, 1-3 glucanase. The similar report was made by Xue *et al.,* (1998) in bean- *Rhizoctonia* interaction. Kale and Choudhary (2003) studied the induction of chitinase and glucanase in seedlings of susceptible and resistant cultivars of groundnut on infection with *Cercospora* cell wall elicitor and correlated the increased level of these PR- proteins with the resistance. Similar observations were made by Koche and Choudhary (2005) in mungbean- *Cercospora* interaction.

In the present investigation, resistant and susceptible cultivars responded differentially to the pathogen derived biotic and abiotic elicitors. The differential response of resistant and susceptible cultivars elicited with pathogen derived elicitors, in terms of rapidity of induction and level of accumulation of these enzymes indicates the probable role of chitinases and glucanases in resistance of chickpea.

The effectiveness of various biotic and abiotic elicitors for the induction of chitinase and glucanse genes was analyzed in seedlings and cotyledons of different chickpea cultivars. The elicitor response was found to be differential and biotic elicitors acted more effectively and induced chitinase and glucanse genes at higher level as compared to abiotic elicitors (Fig. 4.4).

The differential responses of different cultivars to various abiotic and biotic elicitors in terms of activity of PR- protein genes, particularly chitinase and glucanase is in agreement with the previous reports. Herget *et al.,* (1990) have identified four chitinase genes in

groundnut. They observed that these genes were differentially induced by different elicitors *e.g.* the chit-2 gene was selectively induced by cell wall components of *Phytophthora megasperma,* the chit-4 gene was induced by UV light, dilution of culture media and yeast extract. The chit-1 gene was induced only by dilution of culture medium and yeast extract. However, chit-3 gene was constitutively expressed at very low level. Connads -Strauch *et al.,* (1990) reported that chit-1 gene was induced selectively only in incompatible interaction of Turnip- *Xanthomonas compestris* that associated with hypersensitive response, while chit-2 gene was induced only by heat. Daugrois *et al.,* (1990) noticed that in compatible and incompatible interaction of bean- *Colletotrichum,* β-1-3 glucanase genes were expressed differentially. The induction of different chitinase isozymes were reported by Koga *et al.,* (1992) in Yam callus when treated with elicitor like *Fusarium oxysporium*, ethylene, chitin and chitosan oligosacahhrides. Pinheiro *et al.,* (1993) demonstrated that alfalfa mosaic alfamovirus induced *P3* chitinase gene more effectively in bean than mercuric chloride and UV. In contrast mercuric chloride induced *P4* chitinase gene more effectively than other two elicitors.

The present study showed that the resistant and susceptible cultivars respond differentially to different elicitors in terms of PR- protein gene expression. The differences in responses of cultivars to various elicitors might be due to differences in number and type of chitinase and glucanase genes activated by them.

Many workers have also reported the tissue specific expression of chitinases and glucanases. Lamb *et al.,* (1991) showed that *Phytophthora megasperma* cell wall elicitor induced higher chitinase activity in roots as compared to stem and leaves of *Glycine max.* The accumulation of glucanase and chitinase at varying level in different plant parts was also reported in Soybean and Potato (Kombrink *et al.,* 1988), Pumpkin (Esaka *et al.,* 1993), Maize (Cordero *et al.,* 1994); tomato (Wubben *et al.,* 1996) and Mungbean (Koche and Choudhary, 2005). Gupta et al., (2015) also supported the role of plant secreted proteins in plant defense mechanism.

After the detail analysis and interpretation of result obtained in this investigation, it is concluded that the *Fusarium* wilt resistant cultivars Digvijay and Vijay showed hypersensitive response after elicitation with biotic as well as abiotic elicitors. While in moderate resistant cultivar Jaki, it was slight less and least and very slow in susceptible cultivar- JG-62. The response of resistant cultivars was very rapid and can be determined in

terms of increased level of phytoalexin genestein and higher activity of PAL, glucanase and chitinases. In susceptible cultivars the defense response is much slow and the level of these defense gene products was significantly less.

On the basis of observations, made in this investigation, it is concluded that, defense responses induced by biotic and abiotic elicitors are different. Generally, the pathogen derived biotic elicitors induced defense genes more rapidly and at higher level. The effectiveness of the elicitors also varies from cultivar to cultivar.

Studies on phytoalexin accumulation and other defense related enzymes lead us to conclude that the phytoalexin medicarpin, PAL and PR- proteins are playing the vital roles, in resistant interaction between *Cicer arientinum* L. and its pathogens.

Publications out of the above Study:

1) Sopan Chavan, Prajwal Bogawar, Shivdas Aher and Deepak Koche (2017) Resistance analysis of four Chickpea cultivars against fusarium wilt using biochemical markers. *Bioscience Discovery*, 8(3): 307-310.

2) S. B. Chavan and D. K. Koche (2021) Correlating medicarpin content of chickpea cultivars as a key defense compound against Fusarium wilt. *Research on Crops*, 22(1): 178-182.

Signature of Principal Investigator

Signature of Principal