Exploring Gene Indicators to Describe Susceptibility of Chickpea to Ascochyta Blight

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A dissertation submitted in the partial fulfillment of the requirements for the degree of Master of Philosophy

in

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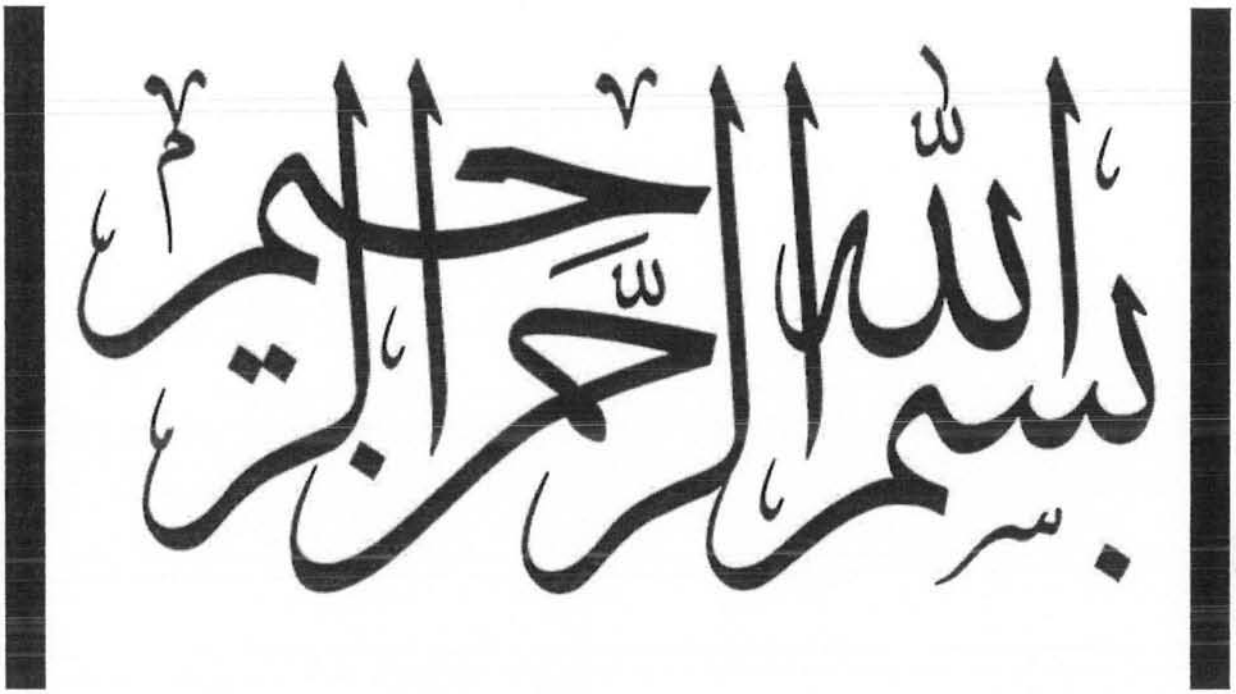

IN THE NAME OF ALLAH,

THE MOST BENEFICENT,

THE MOST MERCIFUL.

Allah in the name of The Most Affectionate, the Merciful.

Recite with the name of your Lord Who created,

He made man from the clot of blood,

Recite, for your Lord is the Most Generous,

Who taught writing by the pen.

Taught man what not.

Yes, undoubtedly, man transgresses.

Because, he thought himself self-sufficient.

Undoubtedly, unto your Lord is the return. Taught man what he knew

Well, you see him who forbids

A bondman of Ours when he offers prayer.

well, you see if he would have been on guidance,

Or he would have commanded piety, what a good thing it had been.

Well you see, if he belied and turned back, then what would be his condition.

Did he not know that Allah is seeing?

Yes, if he desisted not, We will assuredly drag him by catching his forelock hairs.

forelock of what type, lying, sinful.

Now let him call his association

Just now We call Our guards.

Yes, hear him not and prostrate and draw near to Us.

Surah Al-Alaq

DEDICATED TO

My Most Beloved Grand Parents (late)

Whose selfless love and prayers will always be cherished but can never be repaid.

My Most Beloved Parents

Who opened up avenues for me to learn and their loving support in every aspect enabled me to achieve my goals and without whom I am nothing.

My Loving, Caring and Supportive Brothers, Sisters and other Family Members

My all Supportive teachers and loving friends

Who always make me to believe in me.

DECLARATION

I hereby declare that work accomplished in this thesis is the result of my own research carried out in Molecular Plant Pathology Laboratory, Department of Plant Sciences, Quaid-i-Azam University, Islamabad. This thesis has not been published previously nor it contains material from the published resources that can be considered as violation of international copy right law. Furthermore, I also declare that I am aware of the terms "copyright" and "plagiarism". If any copyright violation is found in this research work, I will be responsible for the consequences of any such violation.

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APPROVAL CERTIFICATE

This is certified that the dissertation entitled **"Exploring Gene Indicators to Describe Susceptibility of Chickpea to Ascochyta Blight"** submitted by **Muhammad Nawaz** is accepted in its present form by the Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the dissertation requirement for degree of **Master of Philosophy in Plant Sciences.**

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Dated - May 10, 2019

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LIST OF ABBREVIATIONS

$AlCl_3$	Aluminum chloride
^oC	Centigrade
Cdna	Complementary DNA
Conc	Concentrated
Cu	Copper
$CuSO_4$	Copper sulphate
dNTP	Deoxyribo Nucleoside triphosphate
DAO	Diamine oxidase
Fe	Ferric
GST	glutathione S-transferases
G	Gram
H	Hour
HCL	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
Mg	Magnesium
M	Meter
MT	Million ton
M	Molar
R-QTL	Multiple quantitative trait loci
NARC	National Agricultural Research Centre
N	Nitrogen
NWFP	North-West Frontier Province (Khyber Pakhtunkhwa)

NIAB	Nuclear Institute for Agriculture and Biology
PR	Pathogenesis related
%	Percent
POD	Peroxidase
PAL	Phenylalanine ammonia lyase
P	Phosphorus
PCR	Polymerase chain reaction
K	Potassium
RDA	recommended dietary allowance
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
Rpm	Revolution per minute
SDA	Sabouraud Dextrose Agar
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
S	Sulphur
H_2SO_4	Sulphuric acid
SOD	Superoxide dismutase
SAR	Systemic acquired resistance
TBE	Tris Borate EDTA
WB	wash buffer
H_2O	Water

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ABSTRACT

Chickpea is an important legume crop of dryland in many countries of Africa, America and Asia. It is an important grain crop in Pakistan and represent 70% of total pulses. It is an inexpensive source of protein. The Chickpea crop is affected by many abiotic and biotic stresses. Among these, Ascochyta blight is measured to be one of the most destructive disease of chickpea. In this study, ten different desi and kabuli varities are used for the virulence assay of *Ascochytarabiei*. These varities showed different levels of resistance against *A. rabiei*. Prior to inoculation, different biochemicals, physiological and genetic indicators were observed in chickpea lines. Few of them helped us to understand defense mechanism of this crop. The study revealed that the protein content was low in susceptible cultivars as compared to resistant varieties. It was also observed that the susceptible cultivars were having higher concentration of sugar as well as flavonoid contents. It was also observed that the resistant and moderate resistant varieties were having higher level of POD and PAL activity as compared to susceptible. The susceptible plants were also having low weight of shoot as compared to resistant ones. Before the inoculation, it was observed that the biochemicals and physiological indicators such as proline, chlorophyll pigments, phenol, amino nitrogen, catalase, root/ shoot length varied in all treatments and they did not give any clue to describe the resistance and susceptibility of plants. Expression profiling of chitinase gene in chickpea plants showed that it is highly expressed in the resistant cultivars as compared to susceptible. RT-PCR results also show that the resistant cultivars were having higher expression of SOD gene. Expression of SOD gene was very low is highly infected line. Expression of β-glucosidase could not be observed in any plant which is might be due to reason that β-glucosidase is typically involved in hypersensitive response (HR) and systemic acquired response (SAR) and it is expressed after infection. The prresent results helped us to understand defense mechanism of chickpea against *A. rabiei*.

INTRODUCTION

1. INTRODUCTION

Crops seed are the main staple food which is directly or indirectly consumed by the human being (Baird *et al.*, 2008). About 70% of human diet is from legumes (soybean, pea, cowpea, chickpea, soybean, etc.) and grains (rice, wheat, maize, sorghum, millet, etc.). Half of the world consumption of nutritional proteins in the human is directly provided by seed proteins. Over the last couple of years, globally seed production has been doubled due to the demand of the seeds in the human and animal food (www.worldseed.org/isf/agriculture).

In the plants, legume family are the rich source of protein for feed, food and fodder. They can be used to manage increasing food demand. The Leguminoseae (Fabaceae) consist of about 650 genera and over 18,000 species distributed in three subfamilies (Graham and Vance, 2003). It is considered to be the third largest family of the flowering plants and second to cereals crops in worldwide production and agricultural importance (Young *et al.*, 2003).

Cicer arietinum L. (Chickpea) is a member of Fabaceae (Leguminosae) family andPapilionaceaesubfamily (Nasir and Ali, 1972). This genus comprises only one cultivated species i.e., *Cicer arietinum* L. and 42 wild species (Vavilov, 1951). It is a vital grain legume of Pakistan and adominant pulse crop in the western Asia, South-East,Central America, South America, northern Africa and eastern Africa. Over the world, chickpea is known by many names, e.g. Bengal gram, garbanzo bean and Spanish pea (Abdul *et al.*, 2014).

1.1. Taxonomic Characteristics of Chickpea

The taxonomic characteristics of the species include shrubby stem, erect branches, and dark green or bluish green, glandular pubescent and 0.3 to 1m tall stem. They have tap-root system with three to four well defined branches of lateral roots. The roots (primary and secondary) mainly produce large nodules containing rhizobia for fixing atmospheric nitrogen. The main stem may be semi erect, erect or prostate. The main stem comprises of ribbed, quadrangular and sometimes copiously branches at different levels (Duke, 1981). Leaves generally contain 3-10 pairs of leaflets, which are elliptic or ovate, leaf margin serrate, 0.7-3.0cm wide, however sometimes with a high separated compound leaf or with simple leaf lamina also occur. Stipules (if present) are 2-5 and toothed, leaf apex acuminate or aristate with cuneate base. Flower are pink, purplish or white: stamens are diadelphous (9-1)

surrounding sessile ovary(Duke, 1981). Pod is usually 1.5-2.5cm long; generally with 3 seeds, surface is glandular-pubescent. Seed coat is smooth or wrinkled with a frontal beak and a middle furrow. Germination is crypto-cotylar (Cubero, 1987), self-pollinated and sometimes cross pollinated. It is dry climate, rain fed and cool weather crop (Rao *et al.*, 2010). In Pakistan, it is sown in the middle of September to mid-November or sometimes later and is matured in three to six months.

1.2. Types of Chickpea

Chickpea have two major kinds;Desi and Kabuli. The Kabuli type is also known as the macrosperma types. This type havebeige-colored or white seeds, a thin and flat seed surfacewith a ram's head shape, white flowers, with no anthocyanin pigmentation on stems. Desi type is also known as microsperma, have colored and copious seed coat, anthocyanin pigmentation on stems and pink flowers (Moreno and Cubero, 1978).

Another in-between type with peashaped seedsis also known in India. The seed weight ranges from 0.2-0.6 gram and 0.1-0.3 gram in the Kabuli and Desi types, respectively (Frimpong *at al.*, 2009). The Desi types account for 80 to 85% of the entire chickpea area and are mostly cultivated in Africa and Asia (Pande, e*t al.*, 2005). The Kabuli varieties are largelycultivated in North Africa, Europe,West Asia and North America(Jukanti *et al.*, 2012).

Based on a review of producing zones, 90% of the chickpea cultivated in Pakistan is of Desi and remaining 10% is of kabuli varieties. NWFPand Punjab provinces of Pakistan are the main producers of chickpea, comprising 7% and 87% of the area for chickpea cultivation, correspondingly (Ali, *et al.*,2003).

1.3. Worldwide Production of Chickpea

Production of the chickpea increased regularly in the last 20 years. In 2003, chickpea production about 7.1 million tons (MT), position 3rd after the bean (19.0 MT) and pea (10.3 MT) (FAO, 2004). The average yield of chickpea seed range from 390-3600 kg/ha, dependent upon the crop management for abiotic and biotic constraints and environmental conditions (Gan *et al.*, 2006).

Nowaday, *Cicer arietinum* is cultivatedin more than 50 countries across southern Europe, the North Africa,Americas, the Middle East, Australia andIndian subcontinent.

During 2006 to 2009, the chickpea production was about 9·6 million metric tonnes with an average yield of about 849 kg/ha and production area was 11.3 million ha (FAOSTAT, 2011).

The largest chickpea producing country is the India, producing 6.38 million metric tonnes during the years 2006–09, which is the 66% of total world chickpea production (FAOSTAT, 2011). The other main chickpeaproducing countries include Pakistan, Turkey, Australia, Mexico, Myanmar, Iran, Ethiopia, USA and Canada (Jukanti,*et al.,* 2012).

1.4. Chickpea Production in Pakistan

Chickpea is the most important pulse crops, mainly grown in the irrigated and rainfed areas of Punjab by resource-poor farmers in drought prone areas, specifically. Considerable progress has been achieved in developing improved varieties of chickpea that fit specific niches in thecropping pattern. Fallow areas were brought under chickpea cultivation as the crop could now escape terminal drought. However, large-scale adoption could not be sustained due to several socio-economic and technological constraints (Nisar *et al* ., 2007).

In terms of production and area of chickpea, Pakistan ranks 2nd after India. Chickpea is the crop that is grown in Barani parts of the country as Rabi crop. It participate about ¾ of the pulses crop cultivated in Pakistan (Ali *et al*., 1991). Chickpea is the largest Rabi pulse crop, accounting for 76 percent of total production of pulses in the country and occupies about 5 percent of cropped area. During 2017-18, gram production witnessed an increase of 3 percent on account of increase in area sown and favourable weather condition prevalent at the time of sowing. In Pakistan,chickpea is cultivated in an area of about 978 thousand hectares with yield of 340 thousand tonnes (Pakistan Economic Survey, 2017-18).

In Pakistan, it is grown under 3 cropping systems, first one is the rain fed system which covers about 88% of total chickpea cultivation area. In these areas,it is sown singly or mixed with other crops. The second one is the rice founded system, which constituteabout 11% of the total chickpea sowing area. In this system, the chickpea is cultivated after rice. The last one is the irrigated system, which covers only 1% of the total area (Hassan *et al*., 2003).

In the last few years, chickpea production has been significantly increased in Pakistan. It is due to the better adaptation to local agro-climatic situations, improved satisfactoriness through enhanced nutritive grade i-e, fatty acids and anti-nutritional feature

outlines and the development of new chickpea cultivars with higher yields, through the increase of export markets and by stronger gratitude of the assistances of alternative cropping systems (Muhammad *et al.*, 2007).

In Indo-Pakistan subcontinent, mainly Desi chickpea is cultivated. Thal is the major chickpea producing area. In Pakistan during year 2005-06, total area of chickpea production was 1028.90 thousand hectares (4.3% of total cultivated area), which is about 6% of the entire area under pulses crop in the country. The annual yield is about 479.5 thousand tones with an average yield of 466 kg/ha. During 1996-2006, yield of the chickpea was reduced from 617 to 466 kg/ha. On an average, Punjab produced about 80% of the total chickpea and NWFP, Sindh and Baluchistan provinces produced the lasting 20% (Govt. of Pak., 2006).

Punjab produces 84% of gram or chickpeas, 77% of wheat and 95% of potato - three key Rabi crops of the country's total production (FAO, 2018). In Pakistan (Punjab), district khusab contributed 28% of chickpea yeild while the remaining came from all other areas of the Punjab. In Noorpur (Thal desert of Khushab), where other crops are not grow so effectively, it plays an important role for the survival of the farmers. In dry areas of the Thal desert, chickpea maintains soil fertility, by playing a key role in the Nitrogen fixation (ICRISAT, 2005).

Chickpea is a cash cropfor the people of Noorpur Thal. All the public events of rural societies like illness of human being, marriages, livestock rearing and education are directly or indirectly linked with the chickpea. There was a wide gap between actual and potential yield, which may be attributed to many limitations, e.g, labour management,infrastructural and crop management restrictions (Sharif, 2004; Pankaj *et al.*, 2001).

1.5. Nutritional Value of Chickpea

Chickpea is rich in its nourishing standards; it comprises carbohydrates (64%), protein (23%), dietary fibre (19%), vitamins and minerals (Abdul *et al.*, 2014). There is an increasing demand for this crop, due to its notable nourishing value (Gul *et al.*, 2007, Jukanti *et al.*, 2012). In the semi-arid areas, it is important part of foods for those peoples who are vegetarian or those who cannot afford animal protein. Chickpea is a rich source of protein and carbohydrates, together founding 80% of the entire dry seed mass (Geervani, 1991; Chibbar *et al.*, 2010) in contrast to other crop. Chickpea is lipid free and is a best source of

minerals, vitamins, dietary fibre and trace elements (Williams and Singh, 1988; Wood and Grusak,2007; Agriculture and Agri-Food Canada, 2006).

Chickpea seeds are eaten as fried, green vegetables, snack food, boiled, parched,roasted, sweetmeats and condiments. About a dozen food preparations e.g. besan, dry gram (putanas), dhal and hummus are made from chickpeas (Hunsigi and Krishna, 1998).

Other than carbohydrates and protein, oil (including essential fatty acids) are also important organic compounds of the chickpea (Gul, 2007). The chickpea cannot be known as an oil containing seed; as the oil concentration of chickpeas are low. Oil of the chickpea consists of sterols, tocotrienols and tocopherols (Akihisa, *et al* 1992; Gopala, *et al* 1997; Akihisa, *et al.,*2000). All these ingredients have their particular medicinal and nutritional importance. Tocotrienols have been described to be higher antioxidant properties than the tocopherols and also have the biologically important assets e.g. inhibition of biosynthesis of lipid (Seher and Ivanov, 1973; Qureshi, *et al.,*1986; Yamaoka., *et al* 1991).

Phytosterols lowers cholesterol levels in humans (Jones, *et al.*, 1997) and they also show antibacterial, antiulcerative, anti-inflammatory, antitumor and antifungal activities (Akihisa, *et al.,* 2000, Arisawa, *et al.,*1985, Ling and Jones, 1995). In the human diet, micronutrients (e.g. copper, ferric, manganese, zinc) and macronutrients (e.g. phosphorous, potassium, magnesium and copper) are required. A 100 gram of cooked chickpea can provide 24 % phosphorous, 39% copper and 43% manganese of the recommended dietary allowance (RDA) (Wood and Grusak2007). That's why, chickpea has become a chief source of vitamins and minerals to the cereal based everyday food of millions of people living in under developed countries (Jukanti *et al.*,2012).

1.6. Health Benefits Due to Chickpea

Health benefits of chickpea include high *in vitro* protein digestibility and low allergenic properties (Ulloa *et al.*, 1988: Morrow 1991:Cordle 1994: Clemente, *et al.*, 1999:Tharanathan and Mahademavamma 2003: Amjad *et al.*, 2006: Shad *et al.,* 2009).

The oil ingredient in chickpea is the maximum among other pulses and contains 3-10% of the total dry seed weight (Williams and Singh, 1987; Almeida *et al.*, 2006; Wood and Grusak, 2007). Generally chickpea oil is composed of unsaturated fatty acids (Gül, *et al.*, 2008). Omega-6 linoleic fatty acid is the main part of chickpea oil (46% to 62% of total acids) followed by omega-9 oleic acid (Wood and Grusak, 2007). Omega-6 fatty acid is an

important unsaturated fatty acids that is necessary for human metabolism (Simopoulos 1999). Omega-9 oleic fatty acid is present in seeds; during storage it lowers oxidation properties (Turkulov *et al.*,1996). Chickpea lowers total serum cholesterol levels (Pittaway, *et al.*, 2008).

1.7. Disease of Chickpea

Chickpea is vulnerable to a enormous number of abiotic and biotic stresses, which can be disturbing to crop production by about 1/3rd each year (Haware, 1993). More than 54 insect pests and 50 pathogens have been described on chickpea, from different areas of the world (Dawar *et al.*, 2007; Singh *et al.*, 1994; Van Rheenen, 1991). In the biotic stress, many fungal species e.g. *Aspergillus flavus*, *A. amstelodami*, , *A. fumigates, A. sydowi*, *A. niger*, *A.wentii*, *A. nidulans, Alternaria alternata, A. porri, Cladosporium macrocarpum,Curvularialunata, Botrytis cinerea, Fusarium oxysporum, F. equiseti, F. semitectum, F. moniliforme, Myrothecium roridum, Penicillium notatum, Rhizopus arrhizus, Macrophomina phaseolina and Rhizoctonia* sp., have been described from chickpea (Dawar et al., 2007; Ahmad *et al.*, 1993). Of different diseases, Alternaria blight is caused by *Alternaria alternata* and Collectotrichum blight is caused by *Collectotrichum dematium* (Vishwakarima and Chaudhary, 1974). Ascochyta blight is produced by *Ascochyta rabiei* (Nene, 1980b), and grey mouldis produced by *Botrytis cinerea* (Dawar *et al.*, 2007). Mycotoxins are also produced by some Mould fungi (Rodricks, 1976). In stored grains, many researchers have identified toxin production ability of some mold fungi which destroy stored goods (Afzal *et al.*, 1979).

In Pakistan, Fusarium wilt, Ascochyta blight and stunt virus disease are the major diseases of chickpea (Ansari, 1982). Ascochyta blight is the most important and damaging disease which can decreases up to50 to 70% yield of chickpea (Malik and Bashir 1984).

1.7.1. *Ascochyta rabiei*

Ascochyta blight, a disease produced by *Ascochyta rabiei*, the main biotic factor restraint limiting chickpea yield worldwide (Nighat *et al.*, 2012; Siddique *et al.*, 2000; Singh *et al.*, 1998) This disease can occur in all the chickpea cultivated areas of the world (Chongo

et al., 2003; Kaiser *et al.*, 2000; Akem, 1999; Khan *et al.*, 1999; ICARDA, 1996; Kaiser and Muehlbauer, 1989; Nene and Reddy, 1987).

Ascochyta blight significantly reduces chickpea quality and yield, and in some situations, the chickpea yield losses for vulnerable verities are almost about 100% (Reddy and Singh, 1990). Financial losses due to disease damage have been extensive in several areas including West Asia (Akem *et al.,* 2000) Australia (Knights and Siddique, 2002; Ackland *et al.,* 1998), Latin America (Kaiser *et al.*, 2000), southern Europe (Trapero- Casas and Jime´nez-Dı´az, 1986), United States of America (Kaiser and Muehlbauer, 1989) and Canada (Chongo and Gossen, 2001). All the aerial parts of the plants are affected by ascochyta blight. In the seed borne infection, chocolate colored lesions appear at the base of seedling. Then, the lesions increase and girdle the shoot causing its rupture or death of the whole plant. On the green pods, the lesions are round with dark borders and have pycnidia in concentric circles. On the leaflets, the scratches are usually elongated or circular, having asymmetrical brown spots and are encircled by brown red boundary. In generally, the infected seed convey lesions. On the petiole and stem, the lesions are elongated, chocolate colored, having black spots and girdle the affected part. As the disease spread, the lesions of the effected plants become prominent and gradually feast, including the whole field. The initial infection of the ascochyta blight is recognized from infected seeds or from debris. In the rainy seasons, at a temperature 20°C and at 85 to 90% humidity, the fungus spread rapidly on the crop (Chauhan and Sinha, 1973). The disease plants produce spores on necrotic scratches that distribute plentiful inoculums by air or rain to other plants into field (Alam *et al.,* 1987).

The disease spread in irrigated and Barani areas of the country that may results in the complete failure and destroy chickpea crop. If the ecological circumstances favor disease, the losses may spread up to 100% (Wise *et al.*, 2008). Infection during pod development stage frequently results in shrunken infected seeds (Nene, 1980).

1.8. Genetic and Biochemical Basis of Resistance in chickpea

1.8.1. Genetic basis of resistance

The use of the resistant cultivars is the efficient method to control Ascochyta blight and it is a main objective of chickpea crop stability over the world (Reddy and Singh, 1990). The breeding for resistance varities through the last 60 years did not produce too many

resistant cultivars. But, the wild types of the genus "*Cicer*" i.e, *Cicer echinospermum* have few resistant varities. *Cicer echinospermum* and *Cicer reticulatum* both are cross-compatible with *Cicer arietinum* therefore they could deliver foundation of resistance (Singh *et al.*, 1998). An effort, in joining with breeding for effective resistance bases was made at the global center for agronomic research in the dry areas but abandoned after helpful results could not be attained. Recent research was made on gene pyramiding by merging many resistant genes into single variety (Singh *et al.*, 1994).

Multiple quantitative trait loci recognized on linkage groups 1, 2, 3, 4, 6 and 8 are participate in resistance mechanism of chickpea against *Ascochyta rabiei* (Tar'an *et al.*, 2007; Iruela *et al.*, 2006; Flandez-Galvez *et al.*, 2003; Huettel *et al.*, 2002).Many defense related mechanisms have also been described previously (Tar'an *et al.*, 2007). The expression profiles of many host genes, which are involved in defense mechanisms, have already been characterized in pathosystem of chickpea (ICC3996) (Coram and Pang 2005a, 2006).

Pathogenesis related (PR) protein, e.g. chitinase and glucanase are produced by pathogen derived elicitors (Edereva 2005; Kombrink and Schmelzer 2001). β1,3-glucanase (PR-2B) secrete glycosidic remains that enhance host defense mechanisms, decomposes and weakens fungal cell wall containing glucans (Edereva 2005; Kombrink and Schmelzer 2001). The rapidity and harmonization of the pathogen observation of the host also play important role to achieve effective defense. The resistance hosts respond effective and produce higher amount of defense related compounds than susceptible. For example, PR protein, chitinase and β-1,3-glucanase are quickly produced in resistant verities (Vaghefi *et al.*, 2013; Coram and Pang 2006; Hanselle and Barz 2001; Vogelsang and Barz 1993). In the same case, glutathione S-transferases that is a multi-gene family detoxify numerous complexes (Dixon *et al.*, 2002; Edwards *et al.*, 2000; Marrs 1996), protect uninfected cells from the poisonousness of oxygen and defeat apoptosis (Coelho *et al.*, 2010). The down-regulation of GST shows arise in cellular H_2O_2 from a probable oxidative rupture (Neill *et al.*, 2002).

The role of many genes were identified by Ichinose *et al.*, (2000) by comparing, isolating and sequencing cDNAs of chickpea after inoculation with *Ascochyta rabiei* with other plants genotypes. It is considered that the functions of cDNAs involved:

1) Genes for expression e.g. translation and transcription
2) Defense related pathway genes e.g. PR-proteins, reactive oxygen species scavenging enzymes, reinforcement of cell wall and phytoalexin biosynthetic enzymes.
3) Genes involved in signal transduction.
4) Genes encoding proteins for the metabolism

5) Genes for the catabolic pathway (Ichinose *et al.*, 2000).

1.8.2. Biochemical basis of resistance

Disease resistance in plant is the capacity of plants to retard, limit or stop disease progress and it happens at moderate, low or high level (Bell, 1981). Plants prevent themselves from the attack of biotic and abiotic stresses by the action of many chemical and physical barriers (Agrios, 2005). Many biochemicals e.g. amino acids, phenolic compounds, minerals and phytoalexins are necessary in disease resistance. Phytoalexins are the low molecular mass elements which are produced in the plants after the motivation by many plant pathogens and by mechanical or chemicals damage (Strange, 2003). Hundreds of phytoalexins have been identified from about 900 plant genotypes (Harborne, 1999). Many categorized groups are the isoflavans, pterocarpans and isoflavanones of pulses (Dixon *et al.*, 2002).

The resistance of chickpea to *A.rabiei* dependson the increase of phenolic contents (Vir and Grewal, 1975). The increased level of pterocarpon phytoalexins were observed in the stem and leaves of susceptible and resistance chickpea varities (Dolar and Gurcan 1993; Jamil *et al.*, 1990). After inoculation with *A.rabiei* the total phenolic contents accumulation in 2-3 months old plants was less in the susceptible as compared to resistant plants.

Many enzymes also participate in the resistance of chickpea plants to Ascochyta blight; e.g. diamine oxidase, polyamines, Phenlalanine ammonia lyase (PAL) and peroxidase. All these have been identified to show a great role in regulating the accumulation of phytoalexins and phenolic in answer to any attack on plant (Peltonen *et al.*, 1998; Okay *et al.*, 1997). Highest PAL content was detected 12-24 hours post inoculation which matches with the most rapid gathering of phytoalexins (Sarwar *et al.*, 2001). The resistant varities have the higher concentration of Cadaverine and Diamine oxidase (DAO) in the fourth node as associated to susceptible cultivars. Putrecine and DAO and POD activities markedly increased after post inoculation in the both verities as compared to control cultivars. But the higher levels of both enzymes activities were observed in the resistant lines (Angelini *et al.*, 1993). The specific enzyme polyphenol oxidase (PPO) activity was observed in the resistant cultivars after the inoculation with both the pathogens isolates from six to ten days. But, the PPO activity abruptly decreased after ten days of inoculation (Khirbat and Jalali, 1998).

The role of different minerals has also been revealed in relation to resistance against *A.rabiei*. Randhawa (1994) observed that the value of S and K was more in the susceptible

varities as compared to resistant. However, the amount of Zn, N, Fe and P did not change in the susceptible and resistant varities of chickpea. Excluding the regaining of Fe and Cu, all other elements were increased after inoculation on over all basis, in all the cultivars. The noticeable increase of K was observed after inoculation in the resistant varities but it was reverse in case of Mg, S and P concentration. Regarding amino acids contents, the value of arginine, threonine, lysine, aspartic acid, tyrosine, glutamine, leucine and iso-leucine were higher in resistant varities, while the higher concentration of proline, valine and alanine was observed in the susceptible verities and the level of glysine, serine, polyalanine and methionine were almost same in the both verities. As compared to susceptible, the resistant cultivars had higher level of amino acids. After the inoculation, there was overall decrease in the amount of amino acids. The phenolic contents were almost same in all the cultivars before the inoculation. After inoculation, the concentration of phenol was increased. As compared to the susceptible, the resistant cultivars produced more phenols after inoculation.

1.9. Aims and Objectives

The purpose of this study was to

- Identify biochemical markers to describe the susceptibility of chickpea against Ascochyta blight.
- Analyze different defense related genes to check their expression and regulation in different chickpea cultivars.
- Evaluate the response of desi and kabuli chickpea varities against the Ascochyta blight at molecular and biochemical level.

MATERIALS & METHODS

2. MATERIALS AND METHODS

2.1. Seed Collection and Nicking of Seed

In this study, a total of six desi varieties (Dasht, AUG-424, Punjab-2008, Paidar, Punjab-2000, Bittle-16) and four kabuli varieties (DG-92, Noor-91, K-14024, K-01248) were used (Table 2.1). Healthy seeds of chickpea cultivars were obtained from NIAB Faisalabad and NARC, Islamabad, Pakistan. For all verities of chickpea, 25 seeds of each variety were washed with running tap water. After this, nicking of seeds was done with the help of nail clipper.

2.2. Soil Preparation

Soil, sand and peat moss were mixed in 1:1:1 ratio and autoclaved after sieving. For the sowing, germination and plant growth purpose, this soil was used. Each pot was filled with almost 250 g of soil.

2.3. Seed sowing and germination

After nicking, the seeds were sown in the pots. In each pot, 5 seeds were sown and 5 pots were used for each treatment. Watering of plants was done after every five days and small amount of fertilizer was provided after 10 days of sowing.

2.4. Biochemical parameters

After 21 days of sowing, following biochemical parameters were studied:

2.4.1. Protein Contents

Protein contents of the leaves were determined by following the method of Lowry *et al.*, (1951), in which Bovine Serum Albumin were taken as standard phosphate buffer (stock solution). It was prepared by the following way;

I. Monobasic sodium phosphate (27.6 grams was dissolved into one liter distilled water).

II. Dibasic sodium phosphate (53.6 grams was dissolved into 1000 ml of water).

Sixteen milliliter of monobasic sodium phosphate solution and 84 milliliter of dibasic sodium phosphate were mixed and adjusted at specific pH (7.5).

Reagent A: Two grams sodium carbonate (Na_2CO_3)

One gram of Na-K tartrate and 0.4 gram of NaOH (0.1 N) were dissolved into100 ml of water.

Reagent B: 0.5 gram of $CuSO_4.5H_2O$ was dissolved into 100 ml of distilled water.

Reagent C: 50 ml of Solution A was dissolve in 1 ml of Solution B.

Reagent D: distilled water and Folin phenol reagent was mixed in 1:1 ratio.

Procedure

In one ml of phosphate buffer (pH 7.5), 0.1g of chickpea leaves were grind with the help of pestle and mortar. The sample was centrifuged at 3000 rpm for ten minutes. The supernatant (0.1 ml) were taken into the test tube and water was added up to 1 ml. In this mixture, 1 ml of reagent C was added. Shaken well for ten minutes and 0.1 ml of reagent D was added. It was kept for 30 minutes and absorbance was checked at 650nm. Different concentration of Bovine Serum Albumen e.g. 20, 40, 60, 80, 320 and 640 mg were made and absorbance of these was checked at 650nm. Protein concentration was determined by using the following equation

Protein content(mg/g) =K value ×Dilution factor × Absorbance/Weight of the sample

K value= 19.6, Wt. of sample= 0.1 g, Dilution factor = 2

2.4.2. Sugar Estimation

Sugar concentration of chickpea leaves was determined bythe modified method of Dube *et al.*, (1956). Plant leaves (0.5g) were grind into 5ml water and centrifuged for 5 minutes at 3000 rpm. In supernatant (0.1 ml), 80% phenol (1ml) was mixed. After Incubation at 25°C, carefully added 3ml conc. H_2SO_4. The samples were heated for 4 hours and absorbance of each sample waschecked at 420 nm by using spectrophotometer.

Sugar content mg/g =K value × Dilution factor × Absorbance/Weight of the sample

Dilution factor= 5

K value= 20

Weight of sample= 0.5 g

2.4.3. Proline Contents of Leaves (ug/g)

Proline content of leaves was calculated according to Bates *et al.* (1973). Fresh leaves (0.1gm) were grind in 3% sulphosalicylic acid (4ml) and kept at 5°Cfor one night. The mixture was centrifuged at 25^0c at 3,000rpm for 5minutes. 2ml of supernatant was taken in tubes and mixed with acidic Ninhydrin Reagent. These tubes were heated in water bath at 100°C for one hour. Tubes were cooled, 4ml of toluene was added and mixed well. The absorbance was measured at 520nm, toluene was taken as blank.

The proline content was calculated by using the formula.

Proline ug/g = dilution factor × k value× absorbance/sample wt.

K value= 17.52

2.4.4. Carotenoid and Chlorophyll Content

Carotenoids and chlorophylls (a, b and total) were determined according to Lichtenthaler (1988) and Hassanzadeh *et al.*, (2009), respectively. Fresh plant leaves (100mg) were taken and grind into 6 ml acetone (80%). The extracts were centrifugedfor 10 minutes at 6000 rpm at 4°C. The supernatants were taken into the tube and the pellets were resuspended and centrifuged with acetone until they turned colorless. The absorbance of samples was recorded at 645, 663 and 470 nm by using spectrophotometer. Pigment concentrations were expressed as mg g^{-1}DW.

2.4.5. Estimation of Phenols

Phenol concentration in the leaves of chickpea was determined by Ciocalteau Reagent method (Swain and Hillis, 1959). One gram of leaves sample was grind in 10 ml 0.3 N HCL and shaken for 60 minutes. After this, the extract was centrifuged for ten minutes at 4^0C at 8000 rpm. Supernatant got was evaporated to dryness on water bath. In the extract, hot water was added and final volume was adjusted to 10 ml. One ml of extract was taken to which 1 ml Folin-ciocalteau reagent was mixed followed by the addition of 1 ml Na_2CO_3 (35%) solution. The final volume was adjusted to5 ml by adding water. Absorbance was recorded at 620 nm.

2.4.6. Amino Nitrogen

The pH of alcoholic extract was adjusted at 7.0 (by adding HCL/NaOH 0.1 N). In 1 ml of alcoholic extract, ninhydrin reagent (1ml) was added. This solution was heated for 20 minutes and cooled. After this, 5 ml distilled water was added and the absorbance was taken at 475 nm by using spectrophotometer.

2.4.7. Screening of Flavonoid

The concentration of flavonoid in the chickpea leaves was determined by using spectrophotometer method (Quettier *et al.*, 2000). One milliliter of methanolic extract of leaves sample was made in concentration of 1 mg/ml. In the methanolic extract,one ml of 2% $AlCl_3$mixture was added. Then the reaction mixture were kept for 60 minutes at 25^0C. The absorbance of the samples wasmeasured at 415 nm by using spectrophotometer.

2.5. Enzymes Tests

2.5.1. POD activity

POD activity in the chickpea leaves was measured by the modified method of Reddy *et al.*, (1985). One gram of the chickpea leaves was groid in ten ml of phosphate buffer solution (pH 6.5, 0.1 M). The reaction mixture was centrifuged for 5 minutes at 5000 rpm and absorbance of the supernatant was recorded at 430nm. 1% H_2O_2 (500 ul) was prepared in the phosphate buffer and it was taken as blank. At 430nm, the change in the optical density per minutes was taken as one unit of peroxidase.

Change in A430 = Af-Ai

2.5.2. Catalase Activity

Catalase was determined by calculating the value of H_2O_2 disappearance (Teranishi *et al.*, 1974). Enzyme extract (0.1 mL), 20mM H_2O_2 and phosphate buffer (pH 7.5; 50 mM) was mixed to make the reaction mixture. Titanium reagent (two mL) was mixed to end the reaction after 5 min, which showed the formation of colored compound with H_2O_2 deposit.

This mixture was centrifuged at 10000 rpm for ten minutes at 4^0C. The supernatant was used to measure the absorbance at 410nm by using spectrophotometer.

2.5.3. Phenylalanine ammonia-lyase (PAL)

PAL in the chickpea leaves was identified by using the procedure of Whetten and Sederoff (1992). One gram of leaves was grind at 4°C in five ml of sodium borate buffer (pH 8.8, 0.1 M). The reaction mixture was centrifuged at 10000 rpm for 5 minutes at 4°C and the supernatant was used as enzyme extract. PAL activity was identified by mixing 0.5 ml of 0.02 M phenylalanine, 2 ml of boric acid buffer and 200 ul of enzyme extract and this material was incubated at 30°C for 30 minutes. HCL (200 ul) was added to stop the reaction. Absorbance was recorded at 290 nm by using the spectrophotometer.
One enzyme unit showed the quantity of enzyme that produces one nmol of cinnamic acid $min\text{-}^1gFW^{-1}$.

2.6. Physiological Parameters

2.6.1. Shoot Length

Length of the freshly harvested shoots of chickpea plants were measured with the help of measuring tape.

2.6.2. Root Length

Length of the freshly harvested roots of chickpea plants were measured with the help of measuring tape.

2.6.3. Fresh weight of plants shoot

Fresh weights of the chickpea plants shoots were noted by using electrical weighing balance.

2.6.4. Freshweight of plants roots

Fresh weights of the chickpea plant roots were noted by using electrical weighing balance.

2.7. Molecular Characterization

2.7.1. Total RNA Extraction

Total RNA from the chickpea was extracted by thermo scientific mini kit. The RNA extraction procedure included the following steps;

1. Pipette 500 ul of Plant RNA Lysis Solution in the 1.5 ml eppendorf tube.
2. Take small amount (about 50mg) of white sand into the mortar and pestle.
3. Cooled the mortar and pestle with the liquid nitrogen.
4. Weight the fresh chickpea leaves up to 100 mg and grind into the liquid nitrogen with the help of mortar and pestle (contain cooled white sand).
5. Immediately transfer the plant sample into 1.5 ml eppendorf tube containing the Plant RNA Lysis solution. Vortex it for 20 to 30 sec.
6. Incubate for 5 minutes at 56°C into the water bath. Centrifuge for 8 minutes at 14000 rpm.
7. Collect the supernatant and transferred into the new 1.5 ml Eppendorf tube. Add 250 ul of 96% ethanol into the supernatant, mix it by pipetting.
8. Transferred the mixture to a purification column, inserted in a collection tube.
9. Centrifuged for 1 minutes at 12000 rpm. Discard the flow through mixture.
10. In the purification column add 700 ul wash buffer 1 (WB 1). Placed the column in the collection tube, centrifuge for 1 minute at 12000 rpm. Discard the flow through solution.
11. Take 500 ul of wash buffer 2 (WB 2) in the purification column. Placed it into the new collection tube. Centrifuged for one minutes at 12000 rpm.
12. Repeat the step 11 and centrifuged at 13000 rpm for 1 minutes.
13. Discard the collection tube with flow through solution.
14. Placed the purification column to an RNase free 1.5 ml Eppendorf tube.
15. Add 30 ul of nuclease free water in the center of the purification column membrane, wait for 30 sec and centrifuged for 1 minutes at 12000 rpm.
16. Discard the purification column and immediately store the purified RNA at -80oc.

Nanodrop was used to check the integrity of RNA.

2.7.2. cDNA Synthesis

cDNA was made by using Thermo-scientific Revert-Aid First Strand cDNA synthesis kit.

2.7.2.1.Procedure:

- Take the components of the kit, mix, thawing and centrifuge for a short time. Store on ice.
- Into the nuclease free, sterile PCR tube, add the following reagents.
- Take the nuclease free water (volume vary) in the PCR tube, in this pipette the RNA template (vary) and add 1 ul of Oligo (dt)18 Primer. The total volume should be 12 ul.
- Reaction mixture was incubated for five minutes at 65°C. Quickly cool on ice and place the tube on ice.
- After this, following components were added in the specified order.

1.	5X Reaction Buffer	4 ul
2.	RiboLock RNase Inhibitor (20U/ul)	1 ul
3.	10 Mm dNTP Mix	2 ul
4.	RevertAid M-MuLV RT (200 U/uL)	1 ul

The total volume should be 20 ul.

- Mix gently and incubate for one hour at 42°C.
- Stop the reaction by heating for five minutes at 70°C.

The integrity of cDNA checked by Nanodrop.

2.7.3. Polymerase Chain Reaction

cDNA was used to amplify the fragment of different defense related genes in chickpea. For amplification of genes, specific primer primer were used (Table 2.2).

2.7.3.1. Primer Dilution

Primers were dissolved in 300 ul PCR water kept at 4°C, overnight. This mixture was further diluted 10 times andstored at -20°C, until further used.

2.7.3.2. PCR Strategy and Amplification

PCR was done in the reaction mixture of 20ul. The template of the cDNA were denatured at 94°C for 5 minutes, followed by 40 second at 95°C, 40 second at 56°C, 40 second at 72°C and 7 minutes at 72°C followed by 35 cycles. PCR reaction of 20ul contained the 0.5 ul of cDNA, 2 ul dNTPs, 1ul $MgCl_2$, 2ul of 10x buffer, 2 ul of mixed primers, 0.3 ul of *Taq* polymerase enzyme and 12.2 ul of PCR water, vortexed for 20 second and were subjected to thermal cycling.

2.7.3.3. Agarose Gel Electrophoresis

The amplified PCR fragments were run on 1% agarose gel and bands were examined under UV light.

2.7.3.3.1. Preparation of Agarose Gel

Two grams of the agarose was taken in the flask, 100 ml of 1X TBE buffer was added in the agarose and heated for 3 minutes in the microwave oven. After this, the agarose was cooled and ethidium bromide (3ul) was added in it. Poured this agarose into the tray and solidified. The general apparatus composed of plastic box filled with 1X TBE buffer and having the platinum electrode. Gel was poured in the suitable tray. The Gel tray was dip in the buffer filled tank. Loading dye (3 ul) was added in the 10 ul of each PCR product, separately. Then it was loaded in the wells and current was applied. The DNA samples were migrated across the gel, according to size.

2.7.3.3.2. Running of the Agarose Gel

Gel tray was placed into the electrophoresis chamber. PCR product mixed with bromophenol blue dye and loaded in the Gel. The electrophoresis chamber was connected to the electricity and made sure that the polarity is correct. The PCR product was migrated toward the positive (red) electrode. Gel was run about 1 hour at 85 volt.

2.7.3.4. Visualizing the PCR product in Gel

Ethidium bromide has higher ability for binding with the nucleic acid. One molecules of the DNA could bind with many molecules of ethidium bromide. Upon the exposure to ultraviolet light, the ethidium bromide made bright fluorescence. This technique also used to visualize the small fragments of DNA. Gel was taken in the dark and placed in the transilluminator to see the DNA bands.

2.8. Fungal Isolates

The purified strain of *A. rabiei* used in this experiment was obtained from the molecular plant pathology lab,Quaid-i-Azam University Islamabad.

2.8.1. Microscopic Identification of Fungus

Slide of the pure culture of *A. rabiei* was prepared for the microscopic identification. One drop of the lactophenol cotton blue stain was placed on the center of the slide and with the help of mounting needle, a small piece of mycelium from the edges of colony of fungus were put on the slide. With the help of needle, the mycelium was spread gently, on the slide. A cover slip was put over the slide and observed at 10 x, 40 × and 100 × magnifications.

2.9. Multiplication and Preparation of Inoculum

The purified strain of the *A. rabiei* was multiplied on Sabouraud Dextrose Agar (SDA) in petri dish at 25±1°C. The SDA media was prepared by adding 65.5 grams of Sabouraud Dextrose Agar in one liter of distilled water, mixed well and autoclaved at 121°c, 15psi for 20 minutes. The media was cooled and poured into the petri dish (90 mm in diameter) in laminar flow hood. The fungus stain was transferred into the petri dish containing SDA media with the help of sterilized saputula and placed into the incubator at 25±1°C for 10 days, to induce sporulation.

The conidia of the distinct stains were collected from 10 days old cultures on SDA media, by swamping pycnidial containing colonies with sterile distilled water and removing spore with the help of sterilized glass rod. Haemocytometer were used to account the number of spores

in the spore suspension and the conidial concentration was adjusted to 2×10^5 pycnidiospores mL^{-1}.

2.10. Inoculation of Seedlings

The minidome method already defined by Chen and Muehlbauer (2003) was used to record the pathogenic difference among the varities. One month old plants were inoculated with the spore suspension of *A. rabiei* using 3 pots of each variety. The plants was sprayed with this spore to run-off (estimated 3ml each plant) and instantly enclosed with plastic cover by forming a mini-dome to produce equal relative high moisture for 72 hours to enable infection. Then the plants were sited in growth chamber that was set at 22°C night (12 h) and 25°C night (12 h) at 100% relative humidity (Figure 2.1).

2.11. Measurement of Disease Severity

Disease severity was checked after 21 days of inoculation, by the method based on 1-9 assessment scale, which was improved by Reddy and Singh (1984), as follows:

1. Plant having no disease.
2. Lesions are present on plant, but they was inconspicuous and small.
3. Lesions were simply seen on plant, but plant was generally green.
4. Many lesions present on plant that's are clearly observe.
5. Lesions girdle stems, many leaves of plant show lesions.
6. Tips die back, plant crumpling.
7. Plant dying, but minimum three green leaves exist on plant.
8. No green leave remain on the plant but still having green shoot, almost dead.
9. And dead plant, practically no green parts were seen on the plant.

The trial was done twice in the growth chamber.

2.12. Statistical analysis

Data was statistically represented in the form of means and standard errors of triplicates. Statistix 8.1 was used to apply one-way ANOVA for the determination of significant differences at $P<0.05$.

Table 2.1: Experimental Treatments:

Treatment	variety
D1	Dasht
D2	AUG-424
D3	Punjab-2008
D4	Paidar
D5	Punjab-2000
D6	Bittle-16
K1	DG-92
K2	Noor-91
K3	k-14024
K4	K-01248

Table 2.2: Gene-specific primers to amplify the defense-related genes in chickpea

GenBank accession number	Defense related genes in chickpea	Forward (5'-3') primer	Reverse (3'-5') primer
X70660	chitinase	GCTGCAGGAATTGCAGTGTA	CAATGGCCAGCAAGGTTTAT
AJ012691	Superoxide dismutase	GATCCCTCTCACTGGACCAA	TATCCCGGAGTTGAGAGTGG
AJ005950	b-Glucosidase	ATGCGGATCTTTGTTTCCAC	CTGTGCCAGAATTTCCACCT
AJ012685	Actin	GGCATCTCTCAGCACTTTCC	CAGCTCTCAGTGCTCCATGA

Figure 2.1: Mini-dome techniques used for the inoculation of seedling.

RESULTS

3. RESULTS

After the 21 days of sowing, biochemical, physiological, enzymatic and molecular analysis of healthy plants prior to disease inoculation was carried out (Figure 3.1 & 3.2).

3.1. Biochemical parameter

3.1.1. Protein content

In the desi chickpea cultivars, all the susceptible plants (D2, D4 and D5) had low contents of protein as compared to moderate resistant and resistant varieties. The same trend was observed in kabuli chickpea. In kabuli, the K3 treatment plants have low concentration of protein, when compared with the other kabuli treatments. Overall result showed that the desi chickpea cultivars have the higher level of protein as compared to kabuli chickpea plants (Figure 3.3 A).

3.1.2. Sugar Content

In desi types the susceptible varieties (D2, D4, D5), higher concentration of total sugar was observed, as compared to resistant cultivars. Kabuli susceptible variety (K3) was also having higher content of sugar when compared to other (Figure 3.3 B).

3.1.3. Proline Concentration

The proline concentration varied in all susceptible and resistant varieties. Higher level of proline was recorded in D4 (in desi) and K2 (kabuli) plants. In desi cultivars D2 and kabuli cultivar K4, low concentration of proline was observed (Figure 3.3 C).

3.1.4. Chlorophyll a, b and Carotenoid

Before inoculation, overall results showed that in desi D5 and D6 treatments, plants were having low concentration of chlorophyll pigment "a" and high contents were observed in D3. In kabuli, K2 treatment plants exhibited low concentration of chlorophyll a and higher concentration was seen in K4 treatment plants. Higher level of chlorophyll b recorded into the D3 in desi and K4 in kabuli treatment plants. Chlorophyll b was low in D2 (desi) and K2

(kabuli). In D3 (desi) and K3 (kabuli), high content of carotenoid were observed while these are low were recorded in D5 and K2 (Figure 3.3 D).

3.1.5. Phenolic Content

Before inoculation, highest phenolic content were observed in the D1 (desi) and low phenolic content were recorded in D2 (desi) treatments. When we compared Kabuli varieties, K2 treatment plants showed the lowest concentration of phenol and highest were seen in K3 (Figure 3.4 A).

3.1.6. Amino Nitrogen Content

Before inoculation, amino nitrogen concentration vary in all plants. In desi chickpea cultivar D6, the lowest concentration of amino nitrogen was observed. It was the highest in D1 treatment. In Kabuli, the highest amino nitrogen was observed in K3 and lowest was observed in K1 plants (Figure 3.4 B).

3.1.7. Flavonoid Content

It was seen that the susceptible varieties were containing higher levels of flavonoid than resistant and moderate resistant cultivars. In desi chickpea cultivar, highest flavonoid contents were observed in D2, D4 and D5 treatment plants. In kabuli type, the highest flavonoid contents were observed in K3 treatment plants while K2 plants were containing the lowest concentration of flavonoid (Figure 3.4 C).

3.2. Enzymatic Assay

3.2.1. POD Activity

The susceptible varieties showed the lowest POD activity as compared to resistant and moderate resistant varieties. All susceptible desi lines (D2, D4, D5) showed lowest POD activity than the other cultivars. When we compared POD activity in Kabuli lines, the susceptible cultivar (K3) showed the lowest activity than others. Overall, the highest concentration of POD was seen in K3 and lowest were observed in D5 plants (Figure 3.4 D).

3.2.2. Catalase

The catalase activity varied in all susceptible and resistant varieties. In desi, the D2 treatment plants showed high catalase activity and lowest were seen in D5 treatment plants. In kabuli, the highest catalase activity was observed in K1 and lowest were seen in K4 plants (Figure 3.5 A).

3.2.3. PAL Activity

Desi lines D2, D4 and D5 and Kabuli line K3 exhibited lowest PAL activity. It was noted that the susceptible varieties had the lowest PAL activity than resistant and moderate resistant lines (Figure 3.5 B).

3.3. Physiological Parameter

3.3.1. Root and Shoot Length

Root and shoot length varied in all plants (Figure 3.6). Higher shoot height was observed in D6 plants and lowest were observed in K1 and D2 plants. Root length did not vary significantly in all Kabuli cultivars. In desi treatment D6, the highest root length and in D5, lowest root length was observed (Figure 3.5 C).

3.3.2. Fresh Weight of Roots and Shoots

In desi lines D2, D4, D5 and Kabuli line K3, low fresh weight of shoot was observed. It was observed that the susceptible varieties had low weight of shoot than the susceptible ones.The root weight varied in all varieties. In desi line D3 and in Kabuli line K3, higher weight of root was observed (Figure 3.5 D).

3.4. Molecular investigation

3.4.1. Expression Profiling of Chitinase gene by RT-PCR

Variation in the expression level of chitinase was observed in all the cultivars, when subjected to RT-PCR. Resistant and moderate resistant plants like D1, K1, K2 and K4 were having a higher expression of Chitinase. In RT-PCR Actin was used as internal control. All

the susceptible and resistance cultivars showed the same expression level of Actin (Figure 3.7).

3.4.2. Expression Profiling of SOD gene by RT-PCR

Higher expression pattern of SOD gene was seen in resistant cultivars. Expression of SOD was very low is highly infected line D4 (Figure 3.7).

3.4.3. Expression Profiling of β-glucosidasegene by RT-PCR

Expression of β-glucosidase gene could not be observed in any plant (Figure 3.7).

3.5. Microscopic Identification of *Ascochyta rabiei*

On PDA media, abundant growth of mycelium was observed. The mycelia on the edges were white in colour and in the center they appeared to be pinkish. Cottony colonies were observed (Figure 3.8). Microscopic studies revealed that presence of septate hyphae (Figure 3.9).

3.6. Measurement of Disease Severity

3.6.1 Disease Severity

Disease severity in chickpea plants was assayed after 21 days of inoculation (Figure 3.10 & 3.11). Desi lines D2, D4, D5 and Kabuli line K3 were observed to be highly susceptible. Desi lines D1, D3 and kabuli treatments K1 and K4 were found to be moderately susceptible. Desi line D6 and Kabuli cultivar K2 were found to be moderately resistant against *Ascochyta blight.* Overall, results showed that the desi types of chickpea plants were highly affected by Ascochyta, as compared to Kabuli type (Figure 3.12).

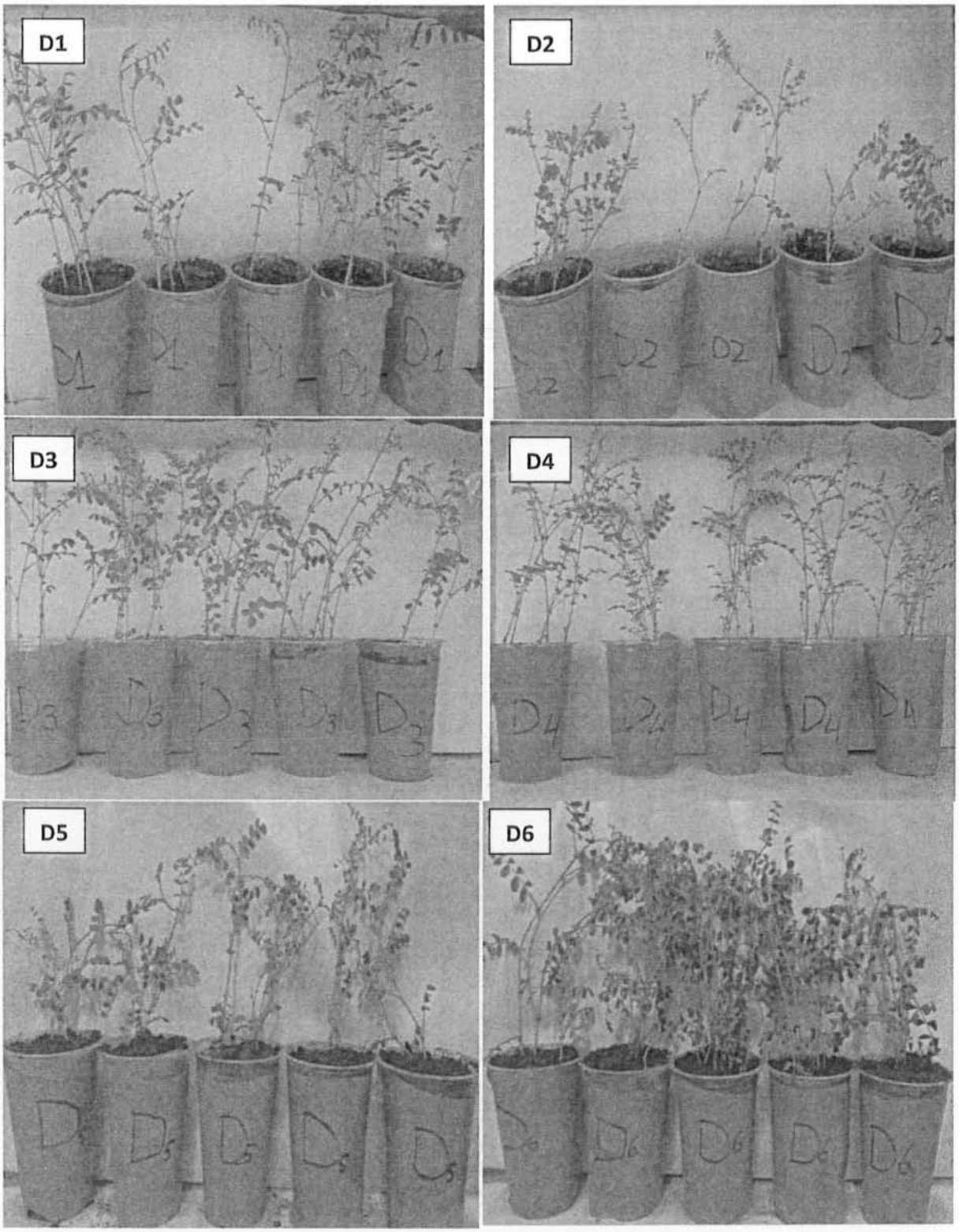

Figure 3.1. Plants seedling after 21 days of sowing. D1: Dasht, D2: AUG-424, D3: Punjab-2008, D4: Paidar, D5: Punjab-2000, D6: Bittle-16.

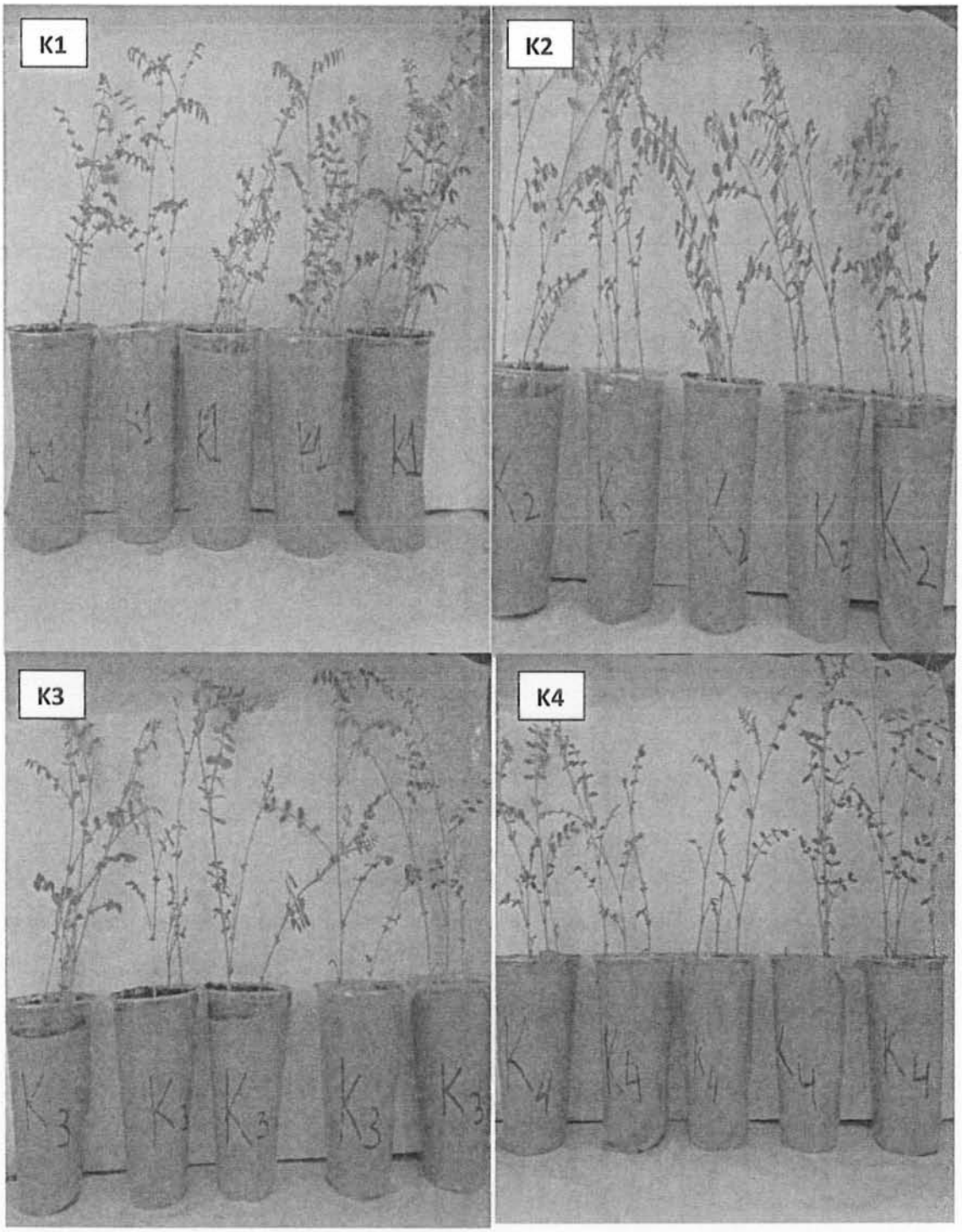

Figure 3.2. Plants seedling after 21 days of sowing. K1: DG-92, K2: Noor-91, K3: K-14024, K4: K-01248.

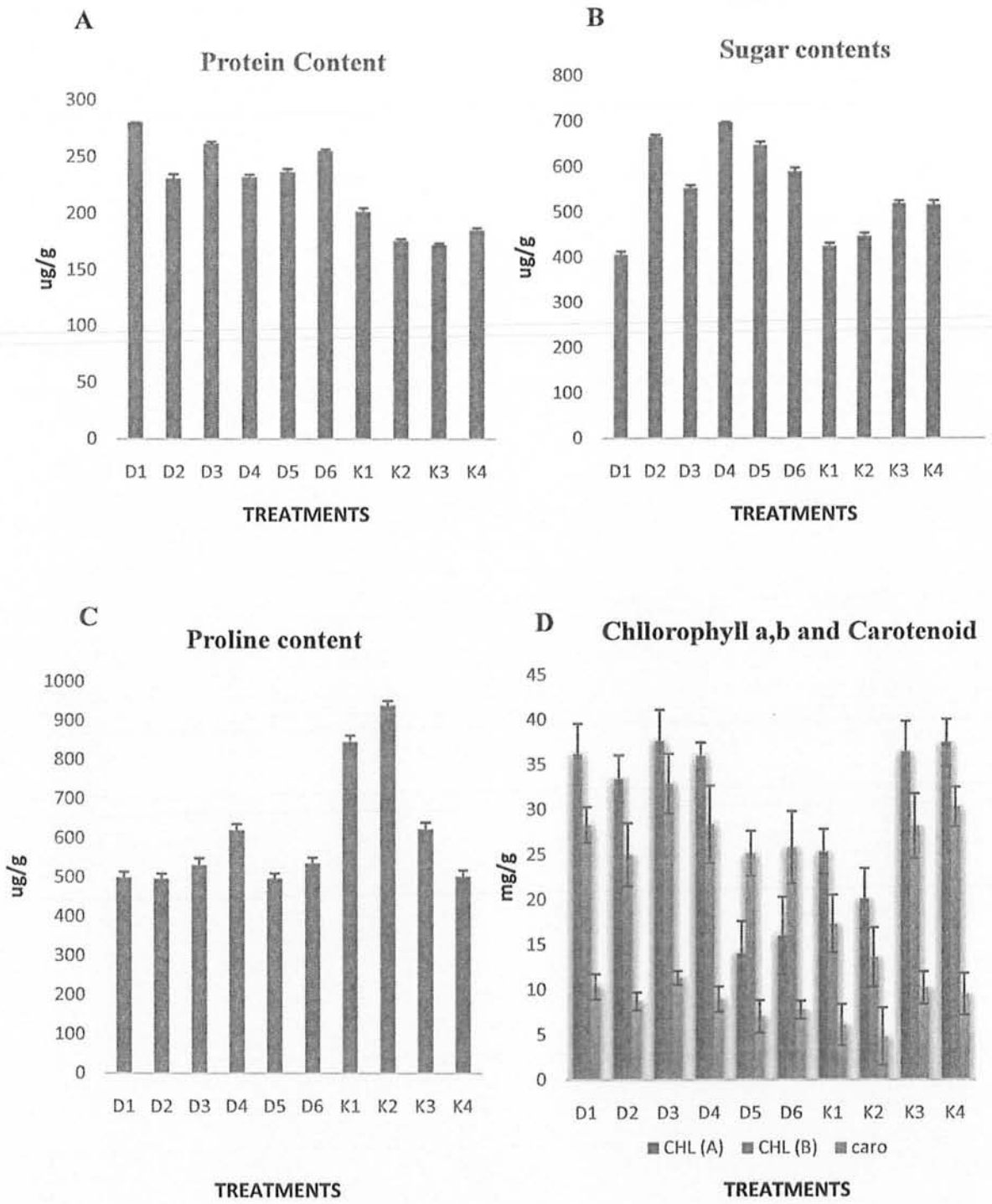

Figure 3.3: Estimation of Protein (A), Sugar (B), Proline (C), Chlorophyll a,b and Carotenoid (D) in Desi (D) and Kabuli (K) varieties of chickpea. D1: Dasht, D2: AUG-424, D3: Punjab-2008, D4: Paidar, D5: Punjab-2000, D6: Bittle-16, K1: DG-92, K2: Noor-91, K3: K-14024, K4: K-01248.

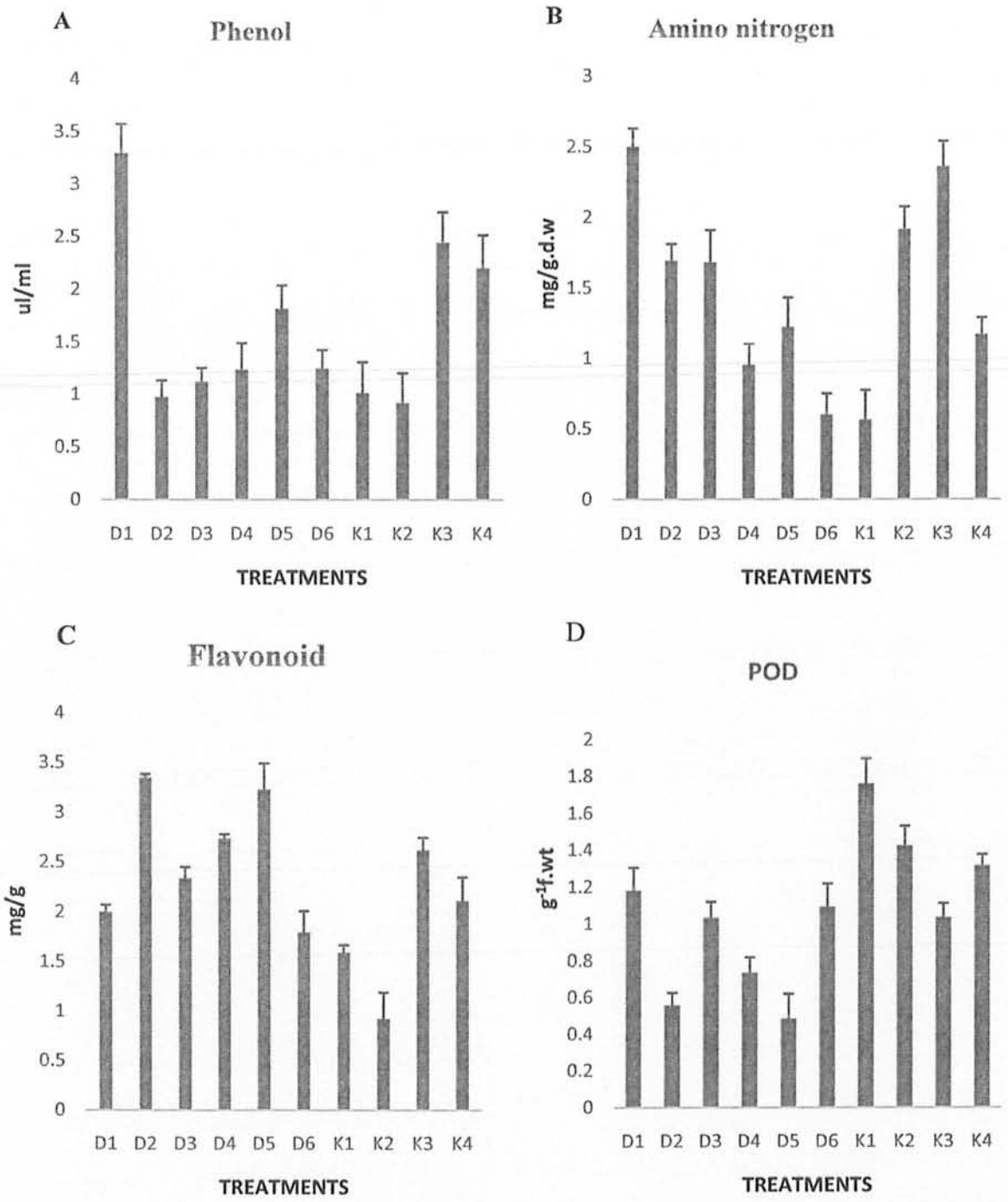

Figure 3.4: Estimation of Phenol (A), Amino Nitrogen (B), Flavonoid (C) and POD (D) in Desi (D) and Kabuli (K) varieties of chickpea. D1: Dasht, D2: AUG-424, D3: Punjab-2008, D4: Paidar, D5: Punjab-2000, D6: Bittle-16, K1: DG-92, K2: Noor-91, K3: K-14024, K4: K-01248.

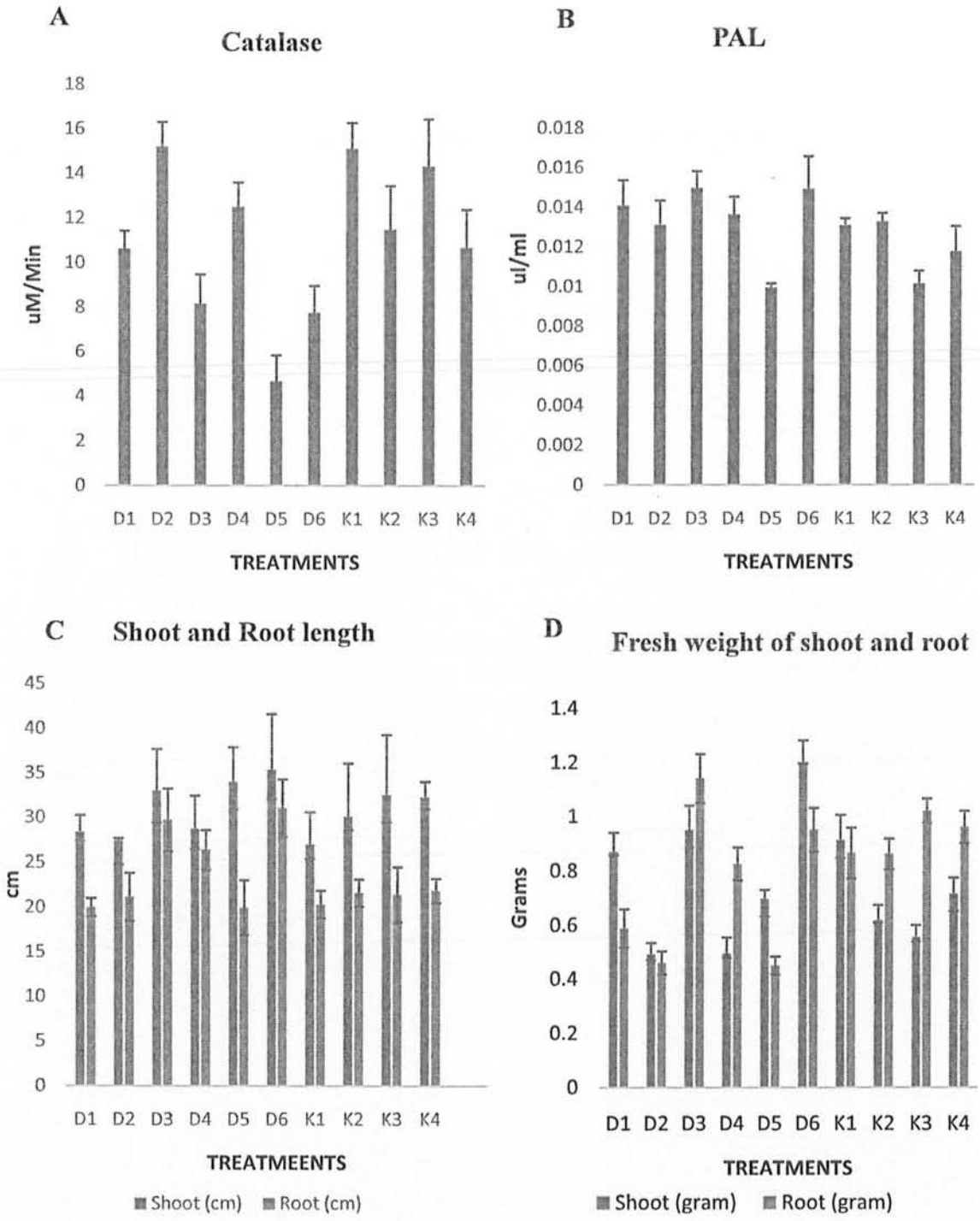

Figure 3.5: Estimation of Catalase (A), PAL (B), Shoot and Root Length (C), Fresh Weight of Shoot and Root (D) in Desi (D) and Kabuli (K) varieties of chickpea. D1: Dasht, D2: AUG-424, D3: Punjab-2008, D4: Paidar, D5: Punjab-2000, D6: Bittle-16, K1: DG-92, K2: Noor-91, K3: K-14024, K4: K-01248.

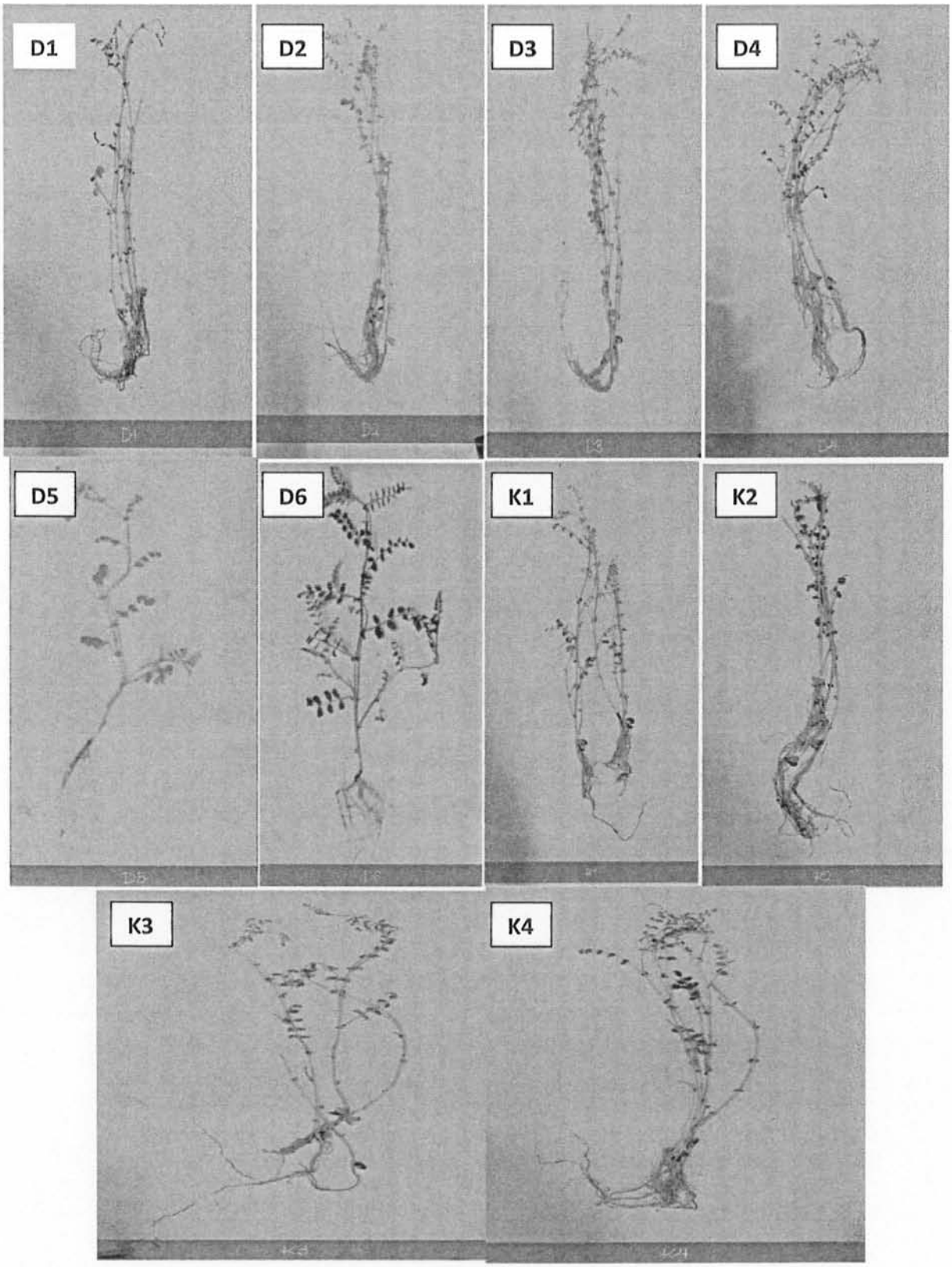

Figure 3.6: Root and shoot length of plants in Desi (D) and Kabuli (K) varieties of chickpea. D1: Dasht, D2: AUG-424, D3: Punjab-2008, D4: Paidar, D5: Punjab-2000, D6: Bittle-16, K1: DG-92, K2: Noor-91, K3: K-14024, K4: K-01248.

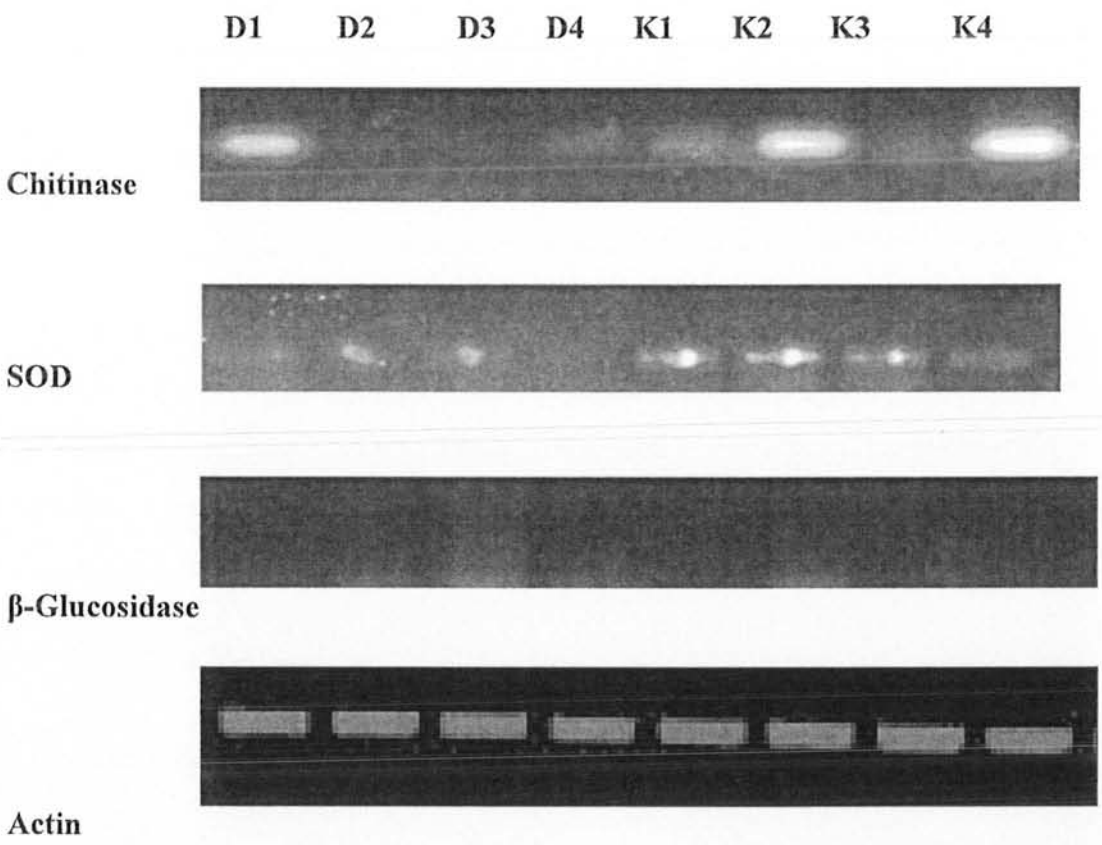

Figure 3.7: Expression profile of Chitinase, SOD and β-Glucosidase in Desi (D) and Kabuli (K) varieties of chickpea. D1: Dasht, D2: AUG-424, D3: Punjab-2008, D4: Paidar, K1: DG-92, K2: Noor-91, K3: K-14024, K4: K-01248.

Length of amplified sequences: Chitinase = 230 bp, SOD = 212 bp, β-Glucosidase = 192 bp, Actin = 210 bp.

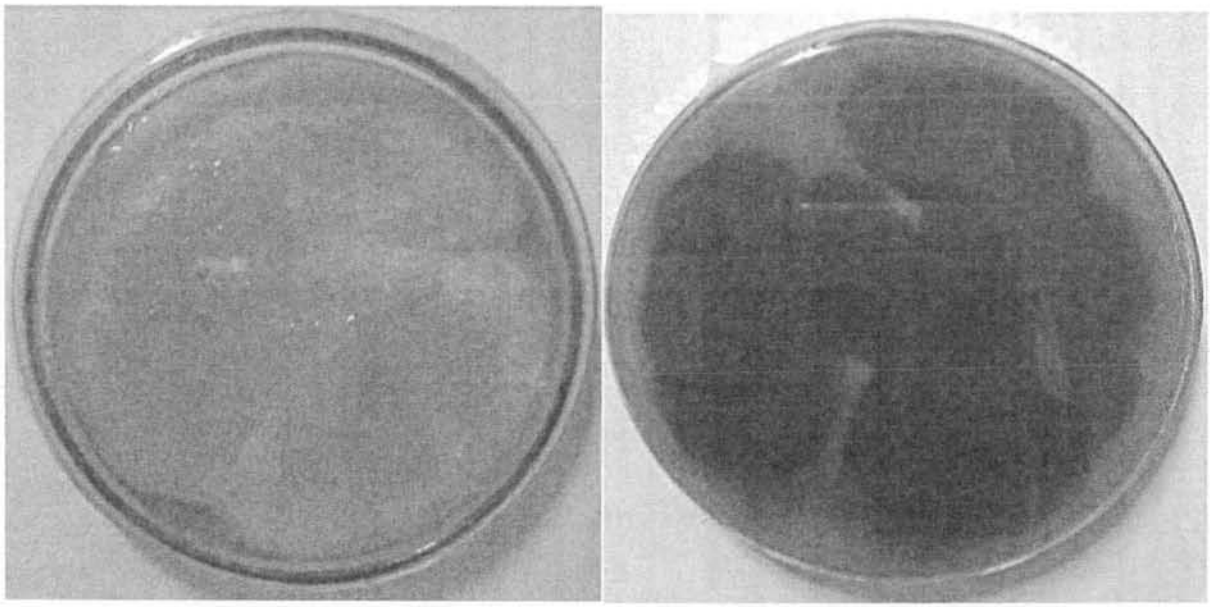

Figure 3.8: Growth of *Ascochyta rabiei* in Petri dishes at 25°C.
Front view (A), Back view (B).

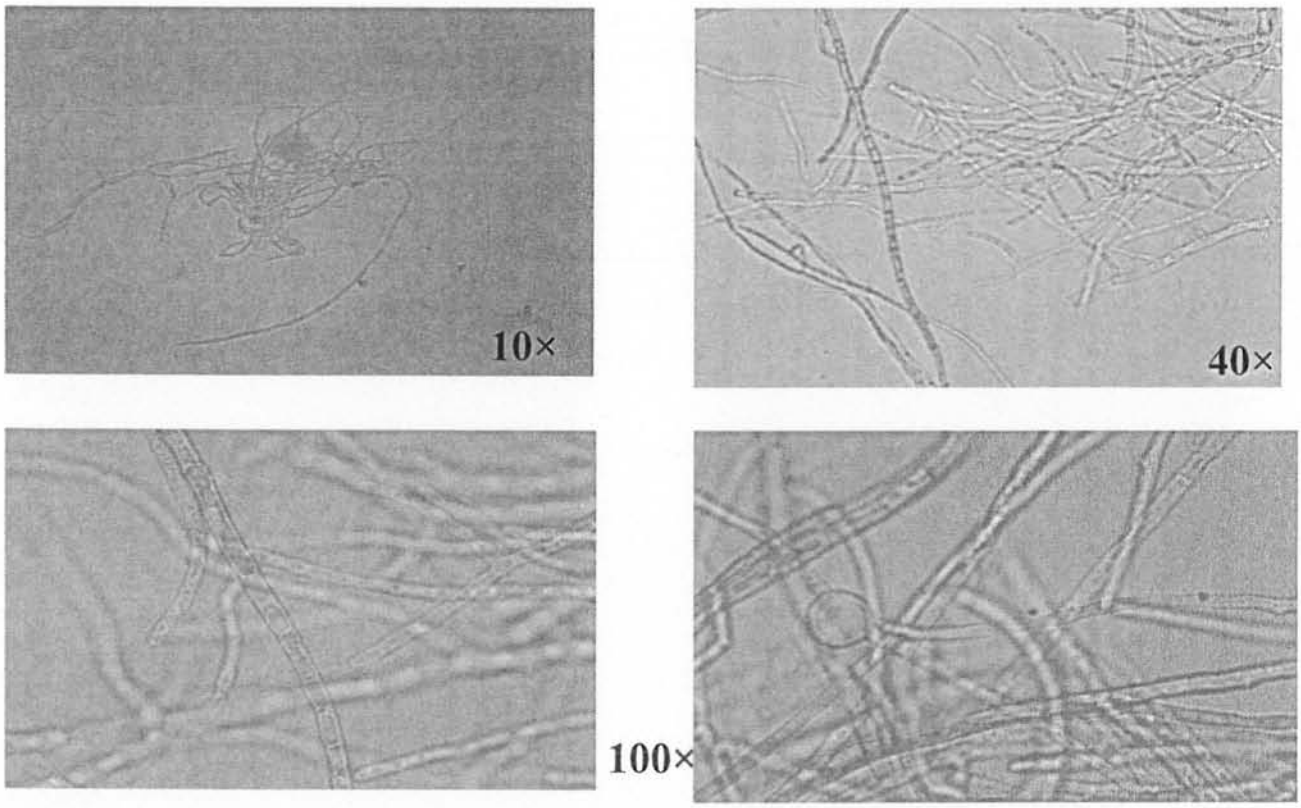

Figure. 3.9: *Ascochyta rabiei* under light microscope at 10X (A), 40X (B) and 100X (C) magnification.

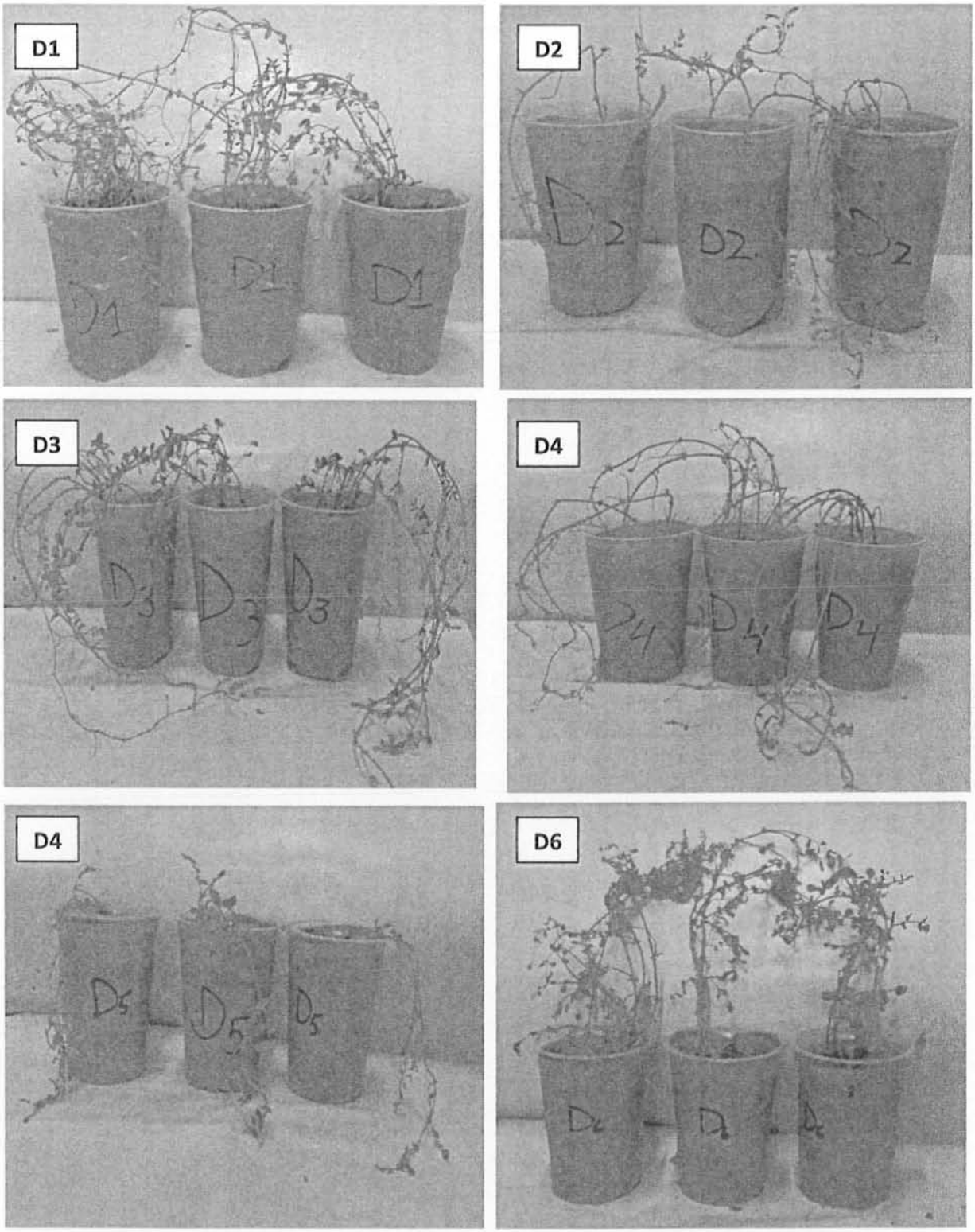

Figure 3.10: Disease severity after 3 weeks of foliar inoculation in Desi (D) varieties of chickpea. D1: Dasht, D2: AUG-424, D3: Punjab-2008, D4: Paidar, D5: Punjab-2000, D6: Bittle-16.

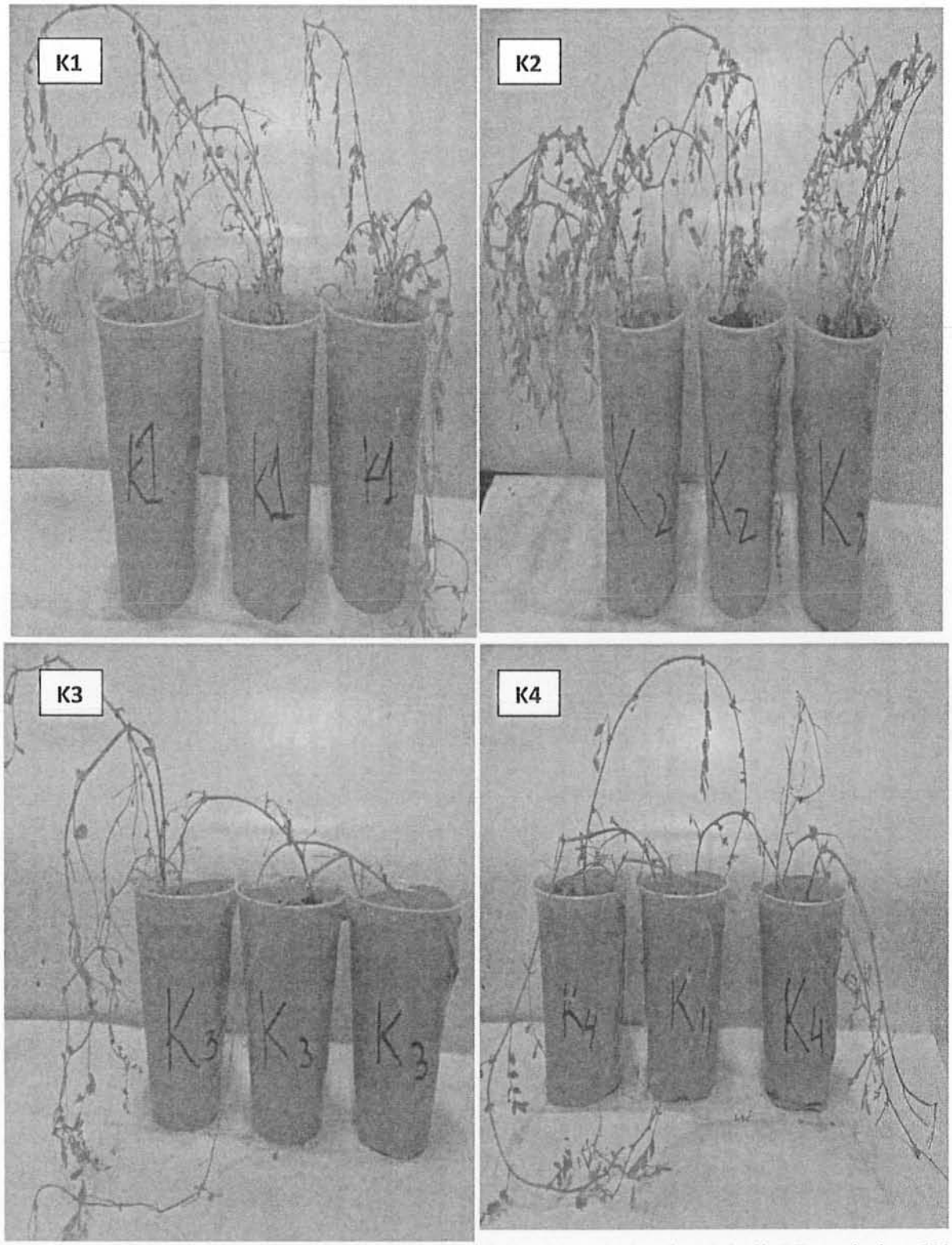

Figure 3.11: Disease severity after 3 weeks of foliar inoculation in Kabuli (K) varieties. K1: DG-92, K2: Noor-91, K3: K-14024, K4: K-01248.

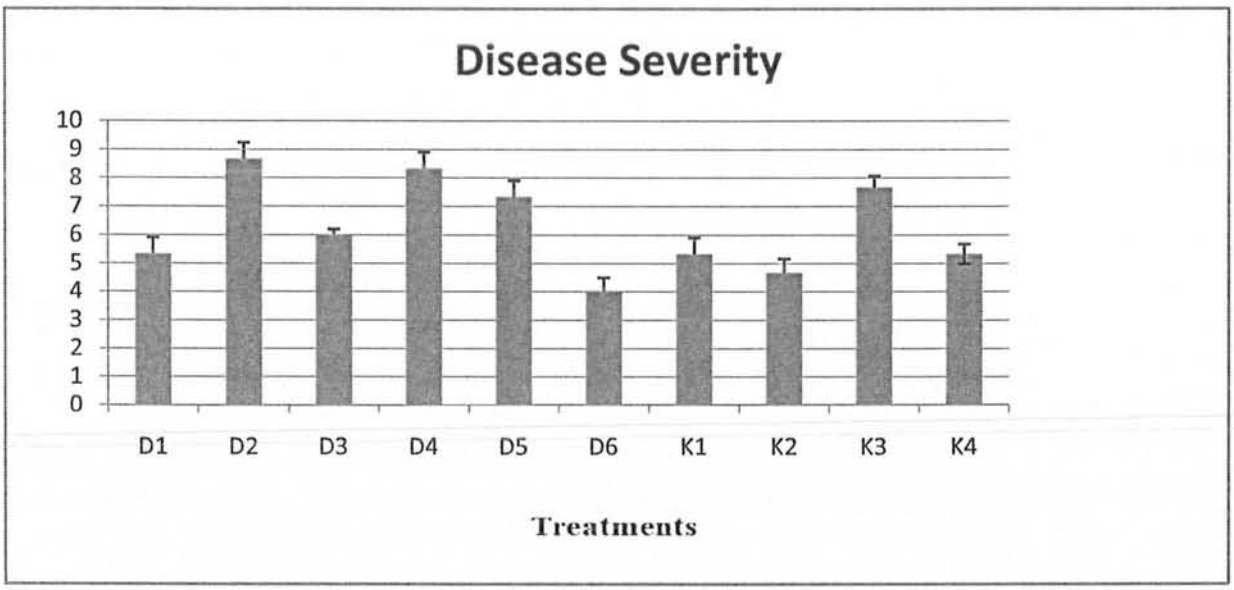

Figure 3.12: Measurement of disease severity in Desi (D) and Kabuli (K) varieties of chickpea. D1: Dasht, D2: AUG-424, D3: Punjab-2008, D4: Paidar, D5: Punjab-2000, D6: Bittle-16, K1: DG-92, K2: Noor-91, K3: K-14024, K4: K-01248.

DISCUSSION

4. DISCUSSION

The experiment was conducted to examine the role of different biochemicals, physiological and genetic indicators to assess the susceptibility of chickpea varieties against Ascochyta blight. Chickpea varieties revealed diverse levels of resistance to Ascochyta blight. Resistance of chickpea against Ascochyta blight was determined by varied levels of physiological, biochemicals and genetic characters. In the recent study all the biochemical, physiological and genetic indicators were observed before the inoculation to describe the resistance and susceptible behavior of cultivars, in a better way.

In present investigation the higher protein contents were observed in all the resistant and moderately resistant cultivars and all the susceptible cultivars having the lower concentration of total protein. In the desi type, D2, D4 and D5 varieties were having lower protein content. The kabuli line (K3) showed low concentration of protein. Our results are in conformity with those obtained by Pradeepkumar (2005) where resistant barley cultivar accumulated more soluble protein than susceptible ones. Kalappanavar and Hiremath (2000) also investigated numerous foliar diseases resistant sorghum genotypes having the higher concentration of protein, related to those of susceptible cultivars. Malhotra *et al.,* (2009) noted that there was significant higher protein content in resistant tomato cultivars, although it reduced expressively in susceptible genotypes when treated to Fusarium wilt.

The infection by pathogens may bring changes in the photosynthesis and respiratory pathway which are the procedure that takes place within the plant (Jaypal and Mahadevan. 1968). The disease response was linked with the sugar level in different plant crops. Usually, the high content of non-reducing sugars, total sugars, reducing sugars in the host plants are specified to be accountable for disease resistance. In the present study it was investigated that the susceptible cultivars have the high level of total sugar than the resistant and moderately resistant lines, the result are in the agreement with Phukan (1993) who investigated that there was a reduction in the starch and total sugar in the potato after treatment by *Phytophthora infestans* (Mount.). Higher level of sugar was found in the leaves of susceptible lines as related to resistant cultivars.

Bhat Tanmai (1997) also studied that there was higher levels of total sugars with increased severity of late leaf spot of groundnut. She described that the amount of increased

sugar was higher in susceptible line as compared to resistant. Likewise, Reeti Singh *et al.*, (1998) also investigated that there was increased level of reducing and total soluble sugar in rust susceptible plant of safflower as related to resistant. Sindhan *et al.*, (1999) stated that there was higher level of reducing, non-reducing and total sugar in the uninoculated leaves of the mungbean, vulnerable to Cercospora leaf spot than the susceptible varieties. Chakrabarthy *et al.*, (2002) also described that the healthy leaves of the higher vulnerable cotton contained significantly higher concentration of total sugar as compared to resistant.

Proline contents in the organelles and cytosol is important to maintain the osmotic and turgor pressure. Recent study showed that the Proline contents vary in all the treatments. Our results are in the agreement that the investigation of proline did not help to differentiate the susceptible and resistant cultivars. May be the proline accumulation may also be changed with the concentration of K^+ and other solutes (Ellis *et al.,* 2007). On the opposite,few findings show that Proline contents in plants play a expressively stress response against the disease attacks (Arie *et al.,* 2007; Grote *et al.,* 2006; Claussen, 2005; Fabro *et al.*, 2004) noted that proline is stored in the leaf of tomato inoculated with *P. syringae* pv. but it does not changed in leaves treated with isogenic virulent bacteria. For the accumulation of proline in plant, a specific stress level is required (Claussen, 2005).

Recent study showed that chlorophyll pigments varied in all the susceptible and resistant lines. There were no clues found that prior to inoculation, chlorophyll pigments play a significant role to describe the susceptible and resistant nature of plant against the biotic stress. Might be after inoculation, the chlorophyll pigments play a significant role in plant defense. As the previous studied showed that the resistant cultivars show higher level of chlorophyll pigments than the susceptible ones as the biochemical parameters play a significant role in defense mechanism of plants against the biotic stresses (Charitha and Reddy 2001; Rajasekaran and Nagarajan 2005). In the salt stress there were decreases in photosynthesis pigment in pepper (Chookhampaeng 2011). Other studied showed that the increased level of chlorophyll content are might be due to the increase in photosynthesis,rate of transpiration, stomatal conductance and increased plant growth (Rajasekaran *et al.*, 2006; Sampathkumar and Ganeshkumar 2003).

Before inoculation the total phenolic contents varied in all the treatments. Prior to the inoculation, no important role of phenol has been observed in all resistant and susceptible cultivars. In desi type D2, which was the susceptible according to our study, exhibited lowest

value of total phenolic contents and the higher contents of these compounds were observed in D1 plant, which is the moderately susceptible. When we observed Kabuli types, the lowest phenolic contents were seen in the K2 treatments plant, which was resistant according to our study and the highest value were observed into the K3 treatments plants and they were susceptible according to recent study. The results of the present study are in agreement with those obtained by Davis *et al.,* (1955), Seevers *et al.,* (1971), Daly *et al.* (1971) and Rammah and El-Hayatmy (1983) who recommended that there were no connection between the increase in the phenolic compounds and the host resistance. On the contrary, the findings of Gupta (1981), Sindhan and Parashar (1981), Rathi (1984), Kalia and Sharma (1988), Chander (1989) in powdery mildew of pea and Bhargava and Khare (1988), Singh *et al.* (2003) on chickpea against Ascochyta blight showed that the resistant cultivars having higher level of phenol as compared to susceptible.

The concentration of amino nitrogen may vary in all the resistant and susceptible cultivars of chickpea. Amino nitrogen might not play significant role for describing the susceptible and resistant nature of cultivars toward the biotic stress. The leaves of plant are the major site for the utilization of nitrates. The nitrates that are absorbed by plants are reduced to nitrites and then ammonia. Finally, they are converted to amino nitrogen and proteins. Rajeswari (2014) observed that the control plant of chickpea had higher content of amino nitrogen than the treated with *fusarium oxysporum*.

In the recent investigation, it was observed that the susceptible cultivars of desi (D2, D4, D5) and kabuli (K3) had higher level of flavonoid, as compared to resistant ones. In contrast, in the previous studied, it was observed that the resistant varieties have higher level of flavonoid than the susceptible cultivars. Proanthocyanidin oligomers precisely prevent fungal enzymes such as stillbene oxidase and protein kinase (Polya and Foo 1994; Goetz *et al.*, 1999). Flavan-3-ols, such as epicatechin, oligomeric proanthocyanidins and catechin have antifungal and inhibitory properties against *B. cinerea* (Hébert *et al.*, 2002; Goetz *et al.*, 1999) and other fungi (Veluri *et al.*, 2004; Colmenares *et al.*, 1998). Flavanol concentration was high in resistant lines as compare to susceptible. Murthy and Bagyaraj (1980) and Mahadevan and Sridhar (1986) but Khirbat (1992), could not detect any alteration between resistant and susceptible cultivars for flavanol concentration.

Prior to inoculation, the POD enzyme activity were low in all the susceptible cultivars of desi and kabuli lines (D2, D4, D5, K3). It might be due to the reason that the POD

enzyme may be involved in host-pathogen resistance mechanisms. Previous studied also supported that in the resistant cultivar, the POD activity highly increased after 72-216 hpi and also increased in susceptible after the 9 days of post inoculation. The POD is considered to be involved to yield lignin (Vance *et al* 1980: Oliveira e*t al* 1997). Therefore this compounds act as barriers against plant pathogens and participate in host resistance mechanism (Okay *et al.*, 1997). The POD activity was considered as an indicator for resistance in plant cultivars against pathogens (Malolepsza and Vrbanek, 1994: Shimoni *et al.*, 1996: Barcelo *et al.*, 1996: Krstic *et al.*, 1997: Martinez *et al.*, 1998: Rothe *et al.*, 1988).

Present study showed that there was no relation of catalase activity and the host resistance. Catalase content was varied in all susceptible and resistant chickpea cultivars. Our result did not meet with the study of Vir and Grewal (1975). They observed that catalase activity expressively increased in the resistant chickpea varieties and other plant-pathogens combination (Barna, 1995: Niebel *et al.*, 1995: Chamnongpol *et al.*, 1996: Piqueras *et al.*, 1996). It was also considered that the catalase may involve in the defense mechanism of chickpea against the *A. rabiei* by detoxifying the toxic oxygen (Foyer *et al.*, 1994) and it was highly increased after inoculation in resistant cultivars and 144-216 hpi in susceptible cultivars.

In the present investigation, prior to inoculation in the desi type, the lowest PAL activity was observed in D2 and D5 treatments plant, which are the susceptible cultivars against the blight and the highest PAL was observed in the moderately resistant and resistant lines. When we examined Kabuli types, susceptible line K3 also showed low PAL activity. Previous studies also described that the PAL activity was higher in resistant cultivars of many plant (Chakraborty *et al.*, 1993; Awan *et al.*, 1997; Koike and Nanbu 1997; Okay *et al.*, 1997; Gläßgen *et al.*, 1998; Peltonen *et al.*, 1998). Higher level of PAL activity was considered as a marker for resistance in plant cultivars to pathogens (Fritzemeier *et al.*, 1987: Lawton and Lamb, 1987: Jahnen and Halbrock, 1988: Maher *et al.*, 1994: Zhang *et al.*, 1997).

Present study investigated that the physiological parameters could also be used as indicator to describe the susceptibility of plant against the biotic stress. It was noted that the root and shoot length are vary in all the susceptible and resistant line. No correlation was found in the physiological parameters of the susceptible and resistant cultivars. However, fresh weight of plant shoot plays a significant role to describe the susceptibility of plant. Our result showed that all the susceptible varieties were having the low fresh weight of shoot as

compared to resistance ones. According to Abdalall. (2010) the pathogen fungi significantly reduces dry root, root length, and shoot length, shoot weight and yield in wheat and Barley.

The preliminary studied were conducted on 10 varieties (six desi and four kabuli) of chickpea. But the expressions of different genes were checked of eight varieties (four desi and four kabuli). The expression level of D5 and D6 were not checked. The reason behind this is that the biochemical investigation and disease severity indicated that D5 is mostly behave as like D2 and D6 resembles with K2 and D1.

The expression pattern of different genes may be used as an indicator to describe susceptibility and resistance of a plant against disease. Molecular investigation by RT-PCR results showed that resistant plants like D1, K1, K2 and K4 were having a higher expression of both Chitinase and SOD genes. Expression of Chitinase gene was more descriptive as all the resistant lines were having higher expression of Chitinase. Our investigation are also in agreement of Nehra *et al.*, 1994; Cho and Muehlbauer, (2004) who described that numerous pathogenesis-related proteins are involved in chickpea resistance against Ascochyta blight, chitinase and *β*-1,3-glucanase (Hanselle and Barz, 2001). Chitinase gene can be used to improved fungal resistance in plant such as tea, tobacco, rice and clover (Kirubakaran and Sakthivel. 2007). Generally, gene expression and enzymatic activity of chitinase may be stimulated by pathogens attacks (Ferreira *et al.*, 2007). Overexpression of chitinase in many species of transgenic plants showed resistance to fungus pathogens and delayed disease signs in plants (Jach *et al.,* 1995; Lorito, 1998; Hong and Hwang, 2006).

Expression of SOD was very low is highly infected line D4. Expression of β-glucosidase gene could not be observed in any plant which is might be due to reason that β-glucosidase is typically involved in HR and SAR and it is expressed after infection. Our results are in agreement with Cho and Muehlbauer, 2004 reported that anti-oxidant stress related genes such as glutathione reductase (GR), ascorbate peroxidase and SOD did not show the higher expression. The production of SOD was known after *Ascochyta rabiei* inoculation in chickpea but no difference in expression was observed in two cultivars. While, blight resistance was apparently provided by hypersensitive responses (Otte *et al.,* 2001). No direct sign was observed to support the suggestion of HR-initiated blight resistance in chickpea.

Expression of β-glucosidase gene could not be observed in any plant which is might be due to reason that β-glucosidase is typically involved in HR and SAR and it is expressed

after infection. Cho and Muehlbauer 2004 also reported that expression of β-glucosidase gene could not be noted in control plants. The expression of this gene started after 24 hours of post inoculation and its expression was maximum after 72 hpi in chickpea when treated with *A. rabiei* and *F. oxysporum*. Putative glucosytransferase and β-glucosidase are apparently involved in the metabolism of conjugated complexes of glucoside which further produce aglycons (anti-microbial agent), were down and up regulated, respectively, after inoculation. (Park *et al.*, 1995 and Mackenbrock and Barz, 1991).

4.1. CONCLUSION

- Different chickpea varieties showed different levels of resistance against *A. rabiei*. The present studies were done to understand the biochemical and genetic basis of resistance in chickpea in contradiction of Ascochyta blight.
- It was concluded that prior to inoculation, protein, sugar, flavonoid, POD, PAL and fresh weight of shoot are the factors on which the susceptibility and resistant behaviors of varieties could be observed.
- Whereas chlorophyll pigments, phenol, amino nitrogen, catalase, root/ shoot length don't give any clue on which we could describe the resistance and susceptibility of plants.
- The molecular investigation showed that the expression of both Chitinase and SOD genes was higher in resistant plants but the expression of Chitinase was extremely high and more conclusive in resistant lines like D1, K2, and K4 which depicts the role of this gene in defense mechanism of chickpea.
- Higher expression of Chitinase gene is probably the reason of disease resistance. β-glucosidase gene probably plays no role before inoculation.
- This study concludess that defense related genes (chitinase and SOD) are important in defense mechanism of chickpea and these could be cloned and transformed into chickpea to create resistance against Ascochyta blight.

REFERENCES

REFERENCES

Abdalall, A.H.A. 2010. Assessment of yield loss caused by root rots in wheat and barley. *Journal of Food, Agriculture and Environment.* 8:638-641.

Abdul, R., Younas, M. U., Ehetisham-ul-Haq, M., Farooq, M., Waris, I.H., Ahmad, E.P.F., Ltif, M. and Ahmad, M. 2014. Screening of chickpea (*Cicer arietinum* L.) varieties against Ascochyta blight and its management through biopesticides. *Archives of Phytopathology and Plant Protection.* 47(8):938-943.

Ackland, S., Moore, K., Schwinghamer, M. and Sykes, J. 1998. Chickpea Foliar Disease Update. *AGNOTE-NSW Agriculture, Australia.* 1-4.

Adám, A,L., Bestwick, C.S., Barna, B.J. and Mansfield, W. 1995. Enzymes regulating the accumulation of active oxygen species during the hypersensitive reaction of bean to *Pseudomonas syringae* pv. Phaseolicola. *Planta.* 197(2):240-249.

Afzal, M.R., Cheema, A. and Chaudhary, R.A. 1979. Incidence of aflatoxin producing fungi in animal feed stuff. *Mycopathologia,* 69(3):149-51.

Agriculture and Agri-Food Canada. 2006. Chickpea: Situation and outlook. Bi-weekly Bulletin. 19.

Agrios, G.N. 2005. Plant Pathology, 5th edition. Academic Press, New York.

Ahmad, I., Iftikhar, S. and Bhutta, A.R. 1993. Seed borne microorganism in Pakistan. A checklist 1991. Pakistan Agricultural Research Council, Islamabad, Pakistan. 32.

Akem, C.N. 1999. Ascochyta blight of chickpea: Present status and future priorities. *International Journal of Pest Management.* 45:131-137.

Akem, C.N., Malhotra R.S., Bayaa B., Mouselli, M.N. and Kabbabeh, S. 2000. Taking blight off chickpea. ICARDA-CARAVN. 2:18.

Akihisa T., Yasukawa K., Yamaura M., Ukiya M., Kimura Y.,Shimizu N. and Arai K. 2000. Triterpene alcohol and sterol formulates from rice bran and their anti-inflammatory effects. *Journal of Agricultural and Food Chemistry.* 48:2313-2319.

Akihisa, T., Nishismura, Y., Nakamura, N., Roy, K., Gosh, P., Thakur, S. and Tamura, T. 1992. Sterols of *Cajanus cajan* and three other leguminosae seeds. *Phytochemistry.* 31:1765-1768.

Alam, S.S., Strange, R.N and Qureshi, S.H. 1987. Isolation of *Ascochyta rabiei* and a convenient method for copious inoculum production. *The Mycologist.* 21:20.

Ali, A., Aftab, M., and Tufail, M. 1991. Criterion for the selection of the high yielding genotypes and pure lines of chickpea. *Pakistan Journal of Agricultural Research.* 12(4):90-94.

Ali, A., Mahmood, K. and Afzal, M. 2003. Thal mein chaney ke kasht [In Urdu]. *Zaratnama* 42:16-21

Almeida, D., Costa, G.E., Queiroz-Monici, D.S., Machado, R. K.P., and Oliveira, C.D. 2006. Chemical composition, dietary fibre and resistant starch contents of raw and cooked pea, common bean, chickpea and lentil legumes. *Food Chemistry.* 94:327-330.

Amjad, I., Iqtidar, A.K., Nadia, A. and Khan, M.S. 2006. Nutritional quality of important food legumes. *Food Chemistry.* 97:331-33.

Angelini, R., Bragaloni, M., Fedevico,R., Infantino, A. and Puglia., A.P. 1993. Involvement of polyamine, diamine oxidase and peroxidase in resistance of chickpea to *Ascochyta rabiei. Journal of Plant Physiology.* 142:704-709.

Anonymous, 2004. Pakistan Statistical Pocket Book, Federal Bureau of Statistics, Statistics Division, Government of Pakistan, Islamabad.

Ansari, M.A. 1982. Pulses, the poor man meat. *Pakistan Agriculture.* 4:30-31.

Arie, T., Takahashi, H., Kodama, M. and Teraoka, T. 2007. Tomato as a model plant for plant pathogen interactions. *Plant Biotechnology Journal.* 24:135-147.

Arisawa, M., Kinghorn, D.A., Cordell, G.A., Phoebe, C.H. and Fansworth, R.N. 1985. Plant anti-cancer agents xxxvI, schottenol glucoside from Accharis cordi folia and Ipomopsis aggregate. *Plant Medicine.* 6:544-555.

Arumugam, R., Rajasekaran, S. and Nagarajan, S.M. 2010. Response of Arbuscular mycorrhizal fungi and Rhizobium inoculation on growth and chlorophyll content of Vigna unguiculate (L) Walp Var. Pusa 151. *Journal of Applied Sciences and Environmental Management.* 14:113-115.

Awan, M.F.M., Maciejewska, U., Kleczkowski, K. and Wielgat, B. 1997. Differential Responses of Potato Cell Suspensions to a Culture Filtrate of *Phytophthora infestans. Zeitschrift fur Naturforschung Section C. Journal of Biosciences.* 52(5-6):333-338.

Baird, N.A., Etter, P.D., Atwood, T.S., Currey, M.C., Shiver, A.L., Lewis, Z.A., Selker, E.U., Cresko, W.A. and Johnson, E.A. 2008. Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS ONE.* 3(10):3376.

Barcelo, A.R., Zapata, J.M. and Calderon, A.A. 1996. A basic peroxidase isozyme, marker of resistance against *Plasmo paraviticola* in grepevines is induced by an elicitor from Trichoderma viride in susceptible grepevines. *Journal of Phytopathology.* 144(6): 309-313.

Barna, B. 1995. Production of disease tolerant and stress tolerant plants by increasing their juvenility and antioxidant capacity. *Novenytermeles.* 44(5:6):561-567.

Bates, L., Waldren, R. and Teare, I. 1973. Rapid determination of free proline for water-stress studies. *Plant Soil.* 39:205-207.

Bell, B. A. 1981. Biochemical mechanisms of disease resistance. *Annual Review of Plant Physiology.* 32:21-81.

Bhargava, P.K. and Khare, M.N. 1988. Factors influencing mechanism of resistance in Alternaria blight of chickpea. *Indian Phytopathology.* 41:363-366.

Bhat Tanmai. 1997. Source – sink relationship as influenced by late leaf spot in groundnut genotypes. M.Sc.(Agri.) Thesis, University of Agricultural Sciences, Dharwad. 69-72.

Boschin, G. and Arnoldi, A. 2011. Legumes are valuable sources of tocopherols. *Food Chemistry.* 127:1199-1203.

Chakrabarthy, P. K., Mikewar, D.M., Sheoraj and Sravankumar, V. 2002. Biochemical factors governing resistance in diploid cotton against grey mildew. *Indian Phytopathology.* 55:140-146.

Chakraborty,U., Chakraborty, B. N. and Kapoor, M. 1993. Changes in the levels of peroxidase and phenylalanine ammonia-lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans. Folia Microbiologica.* 38:491.

Chamnongpol, S., Willekens, H., Langebartels, C., Vanmontagu M., Inze, D. and Vancamp, W. 1996. Transgenic tobacco with a reduced catalase activity develops necrotic lesions and induces pathogenesis related expression under high light. *The Plant Journal.* 10(3):491-503.

Chander, M.S. 1989. Biochemical properties associated with rust and powdery mildew resistance in pea. *Plant Disease Research.* 4:19-23.

Charitha, D.M. and Reddy, M.N. 2001. Growth response of groundnut to VAM fungus and Rhizobium inoculation. *Plant Pathology.*10:71-78.

Chauhan, R.K.S. and Sinha, S. 1973. Effect of varying temperature, Humidity and light during incubation in relation to disease development in blight of gram (*Cicer arietinum* L.) caused by *Ascochyta rabiei.* Proceed. National Science Academy, India. 37:473-482.

Chavan, J.K., Kadam, S.S., and Salunkhe, D.K. 1986. Biochemistry and technology of chickpea (*Cicer arietinum* L.) seeds. *Critical Reviews in Food Science and Nutrition.* 25:107-157.

Chen, W., Muehlbauer, F.J. 2003. An improved technique for virulence assay of *Ascochyta rabiei* on chickpea. International Chickpea and Pigeon pea Newsletter. 10:31-33.

Chibbar, R.N., Ambigaipalan, P. and Hoover, R. 2010. Molecular diversity in pulse seed starch and complex carbohydrates and its role in human nutrition and health. *Cereal Chemistry.* 87:342-352.

Cho, S. and Muehlbauer, F. 2004. Genetic effect of differentially regulated fungal response genes on resistance to necrotrophic fungal pathogens in chickpea (*Cicer arietinum* L.). *Physiological and Molecular Plant Pathology.* 64:57-66.

Chongo, G. and Gossen, B.D. 2001. Effect of plant age on resistance to *Ascochyta rabiei* in chickpea. *Canadian Journal of Plant Pathology.* 23:358-363

Chongo, G., Buchwaldt, L., Gossen, B.D., Lafond, G.P., May, W.E., Johnson, E.N. and Hogg, T. 2003. Foliar fungicides to manage ascochyta blight (*Ascochyta rabiei*) of chickpea in Canada. *Canadian Journal of Plant Pathology.* 25:135-142.

Chookhampaeng, S. 2011. The effect of salt stress on growth, chlorophyll content proline content and antioxidative enzymes of pepper (*Capsicum annuum* L.) seedling. *European Journal of Scientific Research.* 49:103-109.

Claussen, W. 2005. Proline as a measure of stress in tomato plants. *Plant Science.* 168:241-248.

Clemente P., Vioque, A., Sanchez-Vioque, J., Predroche, R., Bautista J. and Millan, F. 1999. Protein quality of chickpea (*Cicer arietinum* L.) protein hydrolysates. *Food Chem*istry. 67:269-274.

Coelho, L.P., Peng, T. and Robert, F.M. 2010. Quantifying the distribution of probes between subcellular locations using unsupervised pattern unmixing. *Bioinformatics.* 26:7-12.

Colmenares, Ramirez-Martinez, N.G., Aldana, J.R., Ramos-Nino, J.O., Clifford, M. N.E. and Pekerar, S. 1998. Isolation, characterisation and determination of biological activity of coffee proanthocyanidins. *Journal of the Science of Food and Agriculture.* 77:368-372.

Coram, T.E. and Pang, E.C. 2005b. Isolation and analysis of candidate ascochyta blight defense genes in chickpea. Part II. Microarray expression analysis of putative defense-related ESTs. *Physiological and Molecular Plant Pathology.* 66:201-210.

Coram, T.E. and Pang, E.C. 2006. Expression profiling of chickpea genes differentially regulated during a resistance response to *Ascochyta rabiei. Plant Biotechnology Journal.* 4:647-666

Coram, T.E., Pang, E.C. 2005a. Isolation and analysis of candidate ascochyta blight defence genes in chickpea. Part I. Generation and analysis of an expressed sequence tag (EST) library. *Physiological and Molecular Plant Pathology.* 66:192-200.

Cordle, C.T. 1994. Control of food allergies using pro- tein hydrolysates. *Journal of Food Technology.* 48: 72-76.

Cubero, J.I. 1987. Morphology Chickpea. In: The Chickpea (Eds.: Saxena, M.C. and Singh, K.B). CAB International, London, *UK.* 35-67.

Daly, J.M., Ludden, P. and Seevers, P. 1971. Biochemical comparison of stem rust resistance controlled by the Sr 6 or Sr 11 allele. *Phyiology and Plant Pathology.* 1:397-407.

Davis, D., Waggoner, P.E. and Dimond, A.E. 1955. Conjugated phenols in the Fusarium wilt syndrome. *Nature.* 172:959.

Dawar, S., Syed, F., and Ghaffar, A. 2007. Seed borne fungi associated with chickpea in Pakistan. *Pakistan Journal of Botany.* 39(2):637.

Dixon, D.P., Lapthorn, A. and Edwards, R. 2002. Plant glutathione transferases. *Genome Biology.* 3:3004

Dixon, R., Achnine, L., Kota, P., Lui, C., Reddy, M. and Wang, L. 2002. The phenylpropanoid pathway and plant defense - a genomics perspective. *Molecular Plant Pathology.* 3:371-390.

Dolar, S.F. and Gurcan, G.A. 1993. The role of the phytoalexins on the resistance to chickpea blight *Ascochyta rabiei* (Pass.) Labr. in chickpea. *Journal of Turkish Phytopathology.* 22:17-26.

Dube, M., Giles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. 1956. Calorimeteric method of sugar and related substance. *Analytical Chemistry.* 28:350-356.

Duke, J.A. 1981. Handbook of legumes of world economic importance. Plenum Press, New York. 52-57.

Edereva, A. 2005. Pathogenesis-related proteins: research progress in the last 15 years. *General and Applied Plant Physiology.* 31:105-124.

Edwards, R., Dixon, D.P. and Walbot, V. 2000. Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. *Trends in Plant Science.* 5:193–198

Ellis, J.G., Dodds, P.N. and Lawrence, G.P. 2007. The role of secreted proteins in diseases of plants caused by rust, powdery mildew and smut fungi. *Current Opinion in Microbiology.* 10:326-331.

Fabro., I., Kovacs, V., Pavet, L. and Szabados, M.E. 2004. Alvarez, Proline accumulation and AtP5CS2 gene activation are induced by plant-pathogen incompatible interactions in Arabidopsis. *Molecular Plant Microbe Interaction.* 17:343-350.

FAO, 2004. Production Year book 2003. Food and Agricultural Organization of the United Nations, Rome. 58.

FAO. 2018. Punjab Food Outlook Report. http://www.fao.org/pakistan/news/detail-events/en/c/1179181/.

FAOSTAT. 2011. http://faostat.fao.org/site/567/Desktop Default.aspx (accessed 12 December 2011).

Ferreira, R.B., Monteiro, S., Freitas, R., Santos, C.N., Chen, Z., Batista, L.M., Duarte, J., Borges, A., and Teixeira, A.R. 2007. The role of plant defense proteins in fungal pathogenesis. *Molecular Plant Pathology.* 8:677-700.

Flandez-Galvez, H., Ades, P.K., Ford, R., Pang, E.C.K. and Taylor, P.W.J. 2003. QTL analysis for ascochyta blight resistance in an intra-specific population of chickpea (*Cicer arietinum* L.). *Theoretical and Applied Genetics.* 107:1257-1265.

Foyer, C.H., Descourvieres, P. and Kunert, K. J. 1994. Protection against oxygen redicals An important defense mechanism studied in transgenic plants. *Plant cell and Environment.* 17(5):507-523.

Frimpong, A., Sinha, A. and Tar'an, B. 2009. Genotype and growing environment influence chickpea (*Cicer arientinum* L.) seed composition. *Journal of the Science of Food and Agriculture.* 89:2052-2063.

Fritzemeier, K.H., Claude, C., Erich, K., Frauke, R., Janet T., Dierk,S. and Klaus, H. 1987. Transient Induction of Phenylalanine Ammonia-Lyase and 4-Coumarate: CoA Ligase mRNAs in Potato Leaves Infected with Virulent or Avirulent Races of *Phytophthora infestans*. *Plant Physiology.* 85:34-41.

Gan, Y.T., Siddique, K.H.M., MacLeod, W.J. and Jayakumar, J. 2006. Management options for minimizing the damage by ascochyta blight (*Ascochyta rabiei*) in chickpea (*Cicer arietinum* L.). *Field Crops Research.* 97:121-134.

Gecit, H.H. 1991. Chickpea utilization in Turkey. In Proceedings of a Consultants Meeting, 27–30 March 1989. Patancheru, AP: ICRISAT. 69-74.

Geervani, P. 1991. Utilization of chickpea in India and scope for novel and alternative uses. In Proceedings of a Consultants Meeting. ICRISAT. 47-54.

Gläßgen, W., Rose, A., Madlung, Wolfgang, K.J. and Gleitz, H.U.S. 1998. Regulation of enzymes involved in anthocyanin biosynthesis in carrot cell cultures in response to treatment with ultraviolet light and fungal elicitors. *Planta.* 204:490.

Goetz, G., Fkyerat, A., Metais, N., Kunz, M., Tabacchi, R. and Pezet, R. 1999. Resistance factors to grey mould in grape berries: identification of some phenolics inhibitors of *Botrytis cinerea* stilbene oxidase. *Phytochemistry.* 52:759-767.

Gopala, K., Prabhakar, J.V. and Aitzetmuller, K. 1997. Tocopherol and fatty acid composition of some Indian pulses. *Journal of the American Oil Chemists' Society.* 74:1603-1606.

Government of Pakistan, 2006. "Agricultural Statistics of Pakistan (district-wise)". Economic Wing, Ministry of Food, Agriculture and Livestock, Islamabad.

Government of the Punjab 2005. "Punjab Development Statistics, Government of the Punjab, Lahore.

Graham, P.H. and Vance, C.P. 2003. Legumes: Importance and Constraints to Greater Use. *Plant Physiology.* 131(3):872-877.

Grote, D., Schmit, R. and Claussen, W. 2006. Water uptake and proline index as indicators of predisposition in tomato plants to *Phytophthora nicotianae* infection as influenced by abiotic stresses. *Physiological and Molecular Plant Pathology.* 69:121-130.

Gül, M.K., Egesel, C.M. and Turham, H. 2008. The effects of planting time on fatty acids and tocopherols in chickpea. *European Food Research and Technology*. 226:517-522.

Gul, M.K., Egesel, C.O. and Turhan, H. 2007. The effect of planting time on fatty acids and tocopherols in chickpea. *European Food Research and Technology*. 226(3):517-522.

Gupta, S.K., 1981. Investigation into some biochemical aspects of pea powdery mildew (Erysiphe polygoni DC). M.Sc. Thesis, HPKVV, Oachaghat, Solan.

Hanselle, T. and Barz, W. 2001. Purification and characterisation of the extracellular PR-2b β-1, 3-glucanase accumulating in different *Ascochyta rabiei* infected chickpea (*Cicer arietinum* L.) cultivars. *Plant Science.* 161:773-781.

Harborne, J.B. 1999. The comperative biochemistry of phytoalexins induction in plants. *Biochemical Systematics and Ecology*. 27:335-367.

Hassan, G., Naqibullah, K. and Haroon, K. 2003. Effect of zero tillageand herbicides on the weed density and yield of chickpea underrice-based conditions of D.I. Khan. *Pakistan Journal of Weed Science*. 9:193-200

Hassanzadeh, M., Ebadi, A., Panahyan-e-Kivi, M., Eshghi, A. G. Jamaati-e-somarin, M., Saeidi and Zabihie-Mahmoodabad, R. 2009. Evaluation of drought stress on Relative water content and chlorophyll content of Sesame (*Sesamum indicum* L.) genotypes at early flowering stage. *Research Journal of Environmental Sciences.* 3(3): 345-360.

Haware, M. P. 1993. International Chickpea Newsletter. 29:1.

Hébert, C., Charles, M. T., Gauthier, L., Willemot, C., Khanizadeh, S. and Cousineau, J. 2002. Strawberry proanthocyanidins: Biochemical markers for *Botrytis cinerea* resistance and shelf-life predictability. *Acta Horticulturae*. 567:659-662.

Hong, J.K. and Hwang, B.K. 2006. Promotor activation of pepper class II basic chitinase gene, CAChi2, and enhanced bacterial disease resistance and osmotic stress tolerance in the CAChi2-overexpressing Arabidopsis. *Planta*. 223:433-448.

Huettel, B., Santra, D., Muehlbauer, F.J. and Kahl, G. 2002. Resistance gene analogues of chickpea (*Cicer arietinum* L.): isolation, genetic mapping and association with a Fusarium resistance gene cluster. *Theoretical and Applied Genetics.* 105:479-490.

Hulse, J.H. 1991. Nature, composition and utilization of pulses. In Uses of Tropical Grain Legumes, Proceedings of a Consultants Meeting, 27–30 March 1989. Patancheru, AP: ICRISAT. 11-27.

Hunsigi, G. and Krishna, K.R. 1998. Science of field crop production. Oxford and IBH Publishing Company, India. 176.

Ibrikci, H., Knewtson, S.J.B. and Grusak, M.A. 2003. Chickpea leaves as a vegetable green for humans: evaluation of mineral composition. *Journal of the Science of Food and Agriculture.* 83:945-950.

ICARDA, 1996. Field Survey of Chickpea Diseases. Legume Annual Report 1996. International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria.

Ichinose, Y., Tiemann, k., Schwenger-Erger, C., Toyoda, K., Hein, F., Hanselle, T., Cornelsand, H., Barz, W. 2000. Genes expressed in *Ascochyta rabiei*-inoculate chickpea plantsand elicited cell cultures as detected by differential cDNA-hybridization. *Journal of Biosciences.* 55:44-54.

ICRISAT. 2005. "Chickpea" International Crops Research Institute for the Semi-Arid Tropics webmaster-icrisat@cgiar.org. 2005.

Iqbal, S.M., Rauf, C.A., Ayub, N. and Ghafoor, A. 2002. Morphological Characters of Chickpea Cultivars Related to Resistance Against Blight. *International Journal of Agriculture and Biology.* 4(4):496-499.

Iruela, M., Rubio, J., Barro, F., Cubero, J.I., Millán, T. and Gil, J. 2006. Detection of two quantitative trait loci for resistance to ascochyta blight in an intra-specific cross of chickpea (*Cicer arietinum* L.): development of SCAR markers associated with resistance. *Theoretical and Applied Genetics.* 112:278-287.

Jach, G., Gornhardt, B., Mundy, J., Logemann, j., Pinsdorf, E., Leach, R., Schell, J. and Maas, C. 1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different Barley antifungal proteins in transgenic tobacco. *The Plant Journal.* 8:97-109.

Jahnen, W. and Hahlbrock, K. 1988. Cellular non localization of non-host resistance reaction of parsley (*Petroselinum crispum)* to to fungal infection. *Planta.* 173:197-204.

Jamil, F.F., Bashir, N. and Haq, I. 1990. Biochemical basis of resistance in chickpea against ascochyta blight. In: Proc. Nati.Chem. Conf II., 114-117.

Jaypal, R. and Mahadevan, A., 1968. Biochemical changes in Banana leaves in response to leaf spot pathogens. *Indian Phytopathology.* 21:43-48.

Jones, P.H., MacDougall, D.E., Ntanios, F. and Vanstone, C.A. 1997. Dietary phytosterols as cholesterol-lowering agents in humans. *Canadian Journal of Physiology and Pharmacology.* 5:217-227

Joshi, M.M. and Singh, R.S. 1969. A *Botrytis* grey mould of gram. *Indian Phytopathalogy.* 22:125-128.

Jukanti, A.K., Gaur, P.M., Gowda, L.L.C. and Chibbar, R.N. 2012. Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): A review. *British Journal of Nutrition.* 108:11-26.

Kaiser, W.J., Muehlbauer, F.J. 1989. An outbreak of ascochyta blight of chickpea in the Pacific Northwest, USA. International Chickpea Newsletter. 18:16-17.

Kaiser, W.J., Ramsey, M.D., Makkouk, K.M., Bretag, T.W., Acikgoz, N., Kumar, J. and Nutter, F.W. 2000. Foliar diseases of cool season food legumes and their control. In: Knight, R. (Ed.), Linking Research and Marketing Opportunities for Pulses in the 21st Century. Kluwer Academic Publishers. The Netherlands. 437-455.

Kalappanavar, I.K. and Hiremath, R.V. 2000. Biochemical factors for multiple resistance to foliar diseases of sorghum. *Madras Agricultural Journal.* 87:66-70.

Kalia, P. and Sharma, S.K. 1988. Biochemical genetics of powdery mildew resistance in pea. *Theoretical and Applied Genetics.* 76:795-799.

Khan, M.S.A., Ramsey, M.D., Corbiere, R., Infantino, A., Porta-Puglia, A., Bouznad, Z. and Scott, E.S. 1999. Ascochyta blight of chickpea in Australia: identification, pathogenicity and mating type. *Plant Pathology.* 48:230-234.

Khirbat, S.K and B. L. Jalali. 1998. Polyphenoloxidase and bound phenol content in the leaves of chickpea (*Cicer arietinum* L.) after inoculation with *Ascochyta rabiei. Legume Research.* 21(3-4):198-200.

Khirbat, S.K. 1992. Studies on the chickpea blight caused by *Ascochyta rabiei* (Pass.) Lab. Ph.D. Thesis. CCS Haryana Agricultural University, Hisar.

Khokar, S.N., Muzaffar, A.K. and Chaudhri, M.F. 2001. Some charactersof chickpea-nodulating rhizobia native to Thal soil. *Pakistan Journal of Biological Sciences.* 4:1016-1019.

Kirubakaran, S.I. and Sakthivel. N. 2007. Cloning and overexpression of antifungal barley chitinase gene in *Escherichia coli. Protein Expression and Purification.* 52:159-166.

Knights, E.J. and Siddique, K.H.M. 2002. Chickpea status and production constraints in Australia. In Integrated Management of Botrytis Grey Mould of Chickpea in Bangladesh and Australia. BARI: Joydebpur, Bangladesh and CLIMA Crawley, Summary Proceedings of a Project Inception Workshop. 33-41.

Koike, M. And Nanbu, K. 1997. Phenylalanine ammonia-lyase activity in alfalfa suspension cultures treated with conidia and elicitors of *Verticillium albo-atrum. Biologia Plantarum.* 39:349.

Kombrink, E. and Schmelzer, E. 2001. The hypersensitive response and its role in local and systemic disease resistance. *European Journal of Plant Pathology.* 107:69-78.

Krstic, B., Vico, I., Tosic, M. and Stojanovic, G. 1997. Peroxidase isoenzymes in strawberry root infected with binucleate *Rhizoctonia* sp. And their implication in disease resistance. *Journal of Phytopathology.* 33:275-297.

Lawton, I.A. and Lamb Christopher, j. 1987. Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. *Molecular and Cellular Biology.* 7:335-341.

Lev-Yadun, S., Gopher, A. and Abbo, S. 2000. The cradle of agriculture. *Science.* 288:1062-1063.

Lichtenthaler, H.K., (Ed.). 1988. Application of Chlorophyll Fluorescence. Kluwer Academic Publishers, Paris. 325-332.

Lindbeck, K.D., Richardson. H. and Hobson, K. 2010. Staying ahead of chickpea Ascochyta blight. Southern Pulse Bulletin. Australia. 15.

Ling, W.H. and Jones, P.J. 1995. Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Science.* 57:195-206

Lorito, M. 1998. Chitinolytic enzymes and their genes. *Trichoderma and Gliocladium.* Vol. II, 1st edn (Kubicek, CP and Harman GE, eds). 2:73-99.

Lowry, O.H., Poesenbrough, N.J., Fal, A.L. and Randall, R.J. 1951. Protein measurement with folin phenol reagent. *Journal of Biological Chemistry.* 193:265-275.

Mackenbrock, U. and Barz, W. 1991. Elicitor-induced formation of pterocarpan phytoalexins in chickpea (*Cicer-arietinum* L.) cellsuspension cultures from constitutive isoflavone conjugates upon inhibition of phenylalanine ammonia-lyase. *Zeitschrift für Naturforschung C.* 46:43-50.

Mahadevan, A. and Sridhar, R. 1986. Methods in Physiological Plant Pathology (3rd ed.) Sivakami Publication. Madras. 328.

Maher, E.A.T., Nicholas, J., Bate, Weiting, N., Yonatan E., Richard A., Dixont, and Chris J.L. 1994. Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. Proceedings of the National Academy of Sciences of the United States of America. 91:7802-7806.

Mahmood, K., Munir, M. and Rafique, S. 1991. Rainfed Farming Systems and Socio-economic Aspects in Kalat Division (Highland Balochistan). *Pakistan Journal of Agricultural Sciences.* 5:15-20.

Malhotra, S. K. 2009. Biochemical components of tomato genotypes in relation to Fusarium wilt. *Indian Society Of Mycology And Plant Pathology.* 18:221-222.

Malik, B. and Bashir, M. 1984. Strategies for controlling gram blight. *Program Farming.* 4:21-23.

Malolepsza, U. and Urbanek, H. 1994. Change in peroxidase activity in bean suspension culture after *B. cinerea* and elicitor treatment. *Journal of Phytopathology.* 141(3):322.

Marrs, K.A. 1996. The functions and regulation of glutathione S-transferases in plants. *Annual Review of Plant Biology.* 47:127-158.

Martinez, C., Montillet, J.L., Bresson, E., Angel, J.P., Dai, G.H., Danial, J.P., Geiger and Nicole, M. 1998. Apoplast peroxidase generates superoxide anions in cell of cotton cotyledons under going the hypersensitive reaction to *Xanthomonas campestris* pv. *Malvacearum* race-18. *Molecular Plant-Microbe Interactions.* 11(11):103-104.

Moreno, M. and Cubero, J.I. 1978. Variation in *Cicer arietinum* L. *Euphytica.* 27:465-485.

Morrow, B. 1991. The rebirth of legumes: Legume pro- duction, consumption and export are increasing as more people become aware of legumes nutritional benefits. *Food Technology.* 9:715-723.

Muehlbauer, F.J. and Tullu, A. 1997. *Cicer arietinum* L. In New CROP Fact SHEET, Seattle, WA: *Washington State University, USDA-ARS.* 6.

Muhammad, Z.U.H., Mansoor, A., Shahid, I., Shakeel, A. and Hakoomat. A. 2007. Characterization and Compositional Studies of Oil from Seeds of Desi Chickpea (Cicer arietinum L.) Cultivars Grown in Pakistan. *Journal of the American Oil Chemists' Society.* 84:1143-1148.

Murthy, G.S. and Bagyaraj, D.J. 1980. Flavanol and alkaloid content of pigeonpea cultivars, resistant and susceptible to *Fusarium udum. Indian Phytopathology.* 33: 633-634.

Nasir, E. and Ali, S.I. 1972-1994. Flora of Pakistan National Herbarium, NARC., Islamabad. Deptt. of Botany, University of Karachi, Pakistan.

Nehra, K., Chugh, L., Dhillon, S. and Singh, R. 1994. Induction, purification and characterization of chitinases from chickpea (*Cicer arietinum* L.) leaves and pods infected with *Ascochyta rabiei. Journal of Plant Physiology.* 144:7-11.

Neill, S.J., Desikan, R., Clarke, A., Hurst, R.D. and Hancock, J.T. 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. *Journal of Experimental Botany.* 53:1237-1247.

Nene, Y.L. 1980. A Review of Ascochyta blight of chickpea. *Tropical Pest Management.* 28:61-70.

Nene, Y.L. 1980b. A world list of pigeon pea (*Cajanus cajans* L.) and chickpea (*Cicer arietinum* L.) pathogens. *ICRISAT Pulse Pathology Progress Report.* 8:1-14.

Nene, Y.L. and Reddy, M.V. 1987. Chickpea diseases and their control. In: Saxena, M.C., Singh, K.B. (Eds.), The Chickpea. CAB International, Oxon, England. 233-270.

Niebel, A.K., Heungens, N., Barthels, D., Inze, M., Vanmontagu and Gheysen, G. 1995. Characterization of a pathogen induced potato catalase and its systemic expression upon nematod and bacterial infection. *Molecular Plant-Microbe Interactions.* 8(3):371-378.

Nighat, S., Akhtar, K.P., Shah, T.M. and Atta, B.M. 2012. Evaluation of Chickpea Advance Genotypes against Blight and Wilt Diseases under Field Conditions. *International Journal of Agriculture and Biology.* 1814-9596.

Nisar, A.S., Khalid, M.A., Mazher, A. and Khalid, M. 2007. Economics of Chickpea Production in the Thal Desert of Pakistan. *Pakistan Journal of Life and Social Sciences.* 5(1-2):6-10.

Okay, E.N., Duncan, E.J., Sirju-Charran, G. and Sreenivasan, T.N. 1997. Phytophthora resistance in Cacao: Role of peroxdise, polyphenoloxidase and phenylalanine ammonia lyase. *Journal of Phytopathology.* 145:295-299.

Oliveira, R.F., Pascholatic, F.S. and Leite, B. 1997. Papilla formation and peroxidase activity in *Mimosa scabrella* hypocotyl inoculated with the non-pathogen *Colletotrichum graminicola. Fitopatologia Brasileira.* 22(2):195-197.

Otte, O., Pachten, A., Hein, F. and Barz, W. 2001. Early elicitor-induced events in chickpea cells: functional links between oxidative burst, sequential occurrence of extracellular alkalinisation and acidification, KC/HC exchange and defense-related gene activation. *Zeitschrift für Naturforschung C.* 56:65-76.

Pakistan Economic Survey. 2017-18. Pakistan Bureau of Statistics. http://www.finance.gov.pk/survey/chapters_18/02-Agriculture.pdf.

Pande, S., Siddique, K.H.M., and Kishore, G.K. 2005. Ascochyta blight of chickpea: biology, pathogenicity, and disease management. *Australian Journal of Agricultural Research.* 56:317-332.

Pankaj, K., Deshmukh, P.S., Kushwaha, S.R. and Sunita-Kumari. 2001. Indian Society of Agricultural Science. Annals of Agricultural Research. Division of Plant Physiology. *Indian Agricultural Research Institute, New Delhi.* 110-112.

Park, H.H., Hakamatsuka, T., Sankawa, U.N. and Ebizuka, Y. 1995. Rapid metabolism of isoflavonoids in elicitor-treated cell-suspension cultures of *Pueraria lobata. Phytochemistry.* 38:373-80.

Peltonen, S., Mannonen, L. and Karjalainen, R. 1998. Elicitor induces changes of phenylalanine ammonia lyase activity in barley cell suspension cultures. *Plant Cell, Tissue and Organ Culture.* 50:185-193.

Peltonen, S., Mannonen, L. andKarjalainen, R. 1997. Elicitor-induced changes of phenylalanine ammonia-lyase activity in barley cell suspension cultures. *Plant Cell, Tissue and Organ Culture.* 50(3):185-193.

Phukan, S.N. 1993. Changes in carbohydrate content of potato varieties due to infection by *Phytophthora infestans. Indian Journal of Mycology and Plant Pathology.* 23(3):223-226.

Pieters, R. and Tahiri, A. 1986. Methods for screening resistance in chickpea to *Ascochyta blight. Euphytica.* 35:1007-1010.

Piqueras, A.J.A., Hernandez, E., Olmos, E., Hellin and Sevilla, F. 1996. Change in antioxidant enzyme and organic solutes associated with adaptation of citrus cells to salt stress. *Plant Cell, Tissue and Organ Culture.* 45(1):53-60.

Pittaway, J.K., Robertson, I.K. and Ball, M.J. 2008. Chickpeas may influence fatty acid and fiber intake in an ad libitum diet, leading to small improvements in serum lipid profile and glycemic control. *Journal of the American Dietetic Association.* 108:1009-1013.

Polya, G. M., and Foo, L.Y. 1994. Inhibition of eukaryote signal-regulated protein kinases by plant-derived catechin-related compounds. *Phytochemistry.* 35:1399-1405.

Pradeepkumar, M., 2005. Physiology and biochemical basis of resistance to leaf blight of Barley (Hordeum vulgare L.). M. Sc. (Agri.) Thesis, University of Agriculture Science. Dharwad, Karnataka (India).

Quettier, D.C., Gressier, B., Vasseur, J., Dine, T., Brunet, C., Luyckx, M.C., Cayin, J.C., Bailleul, F. and Trotin, F. 2000. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *Journal of Ethnopharmacology.* 72:35-42.

Qureshi, A.A., Burger, W.C., Peterson, D.M. and Elson, C.E. 1986. The structure of an inhibitor of cholesterol biosynthesis isolated from barley. *The Journal of Biological Chemistry.* 261:10544-10550.

Rajasekaran, S. and Nagarajan, S.M. 2005. Effect of dual inoculation (AM fungi and Rhizobium) on Chlorophyll content of *Vigna unguiculata* (L.) Walp. Var. Pusa. 151. *Mycorrhiza News.* 17:10-11.

Rajasekaran, S., Nagarajan. S., Arumugam, S.M., Sravanamuthu, K. and Balamurugan, S. 2006. Effect of dual inoculation (Rhizobium and AM fungi) on chlorophyll content of *Arachishy pogaea* L. CV. TMV-2. *Plant Archives.* 6:671-672.

Rajeswari, P. 2014. Control of *Fusarium Oxysporum* Causing Fusarium Wilt by *Trichoderma* Spp and *Pseudomonas fluorescens* on *Arachishy pogaea* L. *International Journal of Advanced Biotechnology and Research.* 6(1):57-65.

Rammah, A.M. and El- Hayatmy Y. 1983. Relations of polyphenols and free amino-nitrogen in alfalfa rust disease infection. *Journal of Agricultural Reseach.* 61:1-14.

Randhawa, M. A. 1994. Role of some morphological and chemical characters of gram in resistance to ascochyta blight. Ph.D Thesis, Department of Plant Pathalogy, Uni. Agri., Faisalabad, Pakistan. 191.

Rao, Parthasarathy, Birthal, P.S., Bhagavatula, S. and Bantilan, M.C.S. 2010. Chickpea and Pigeon pea Economies in Asia: Facts, Trends and Outlook. Patancheru, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics. 76: 978-992-530-534.

Rathi, A.S., 1984. Biochemical changes in pea leaves due to powdery mildew infection. *Indian Society of Mycology and Plant Pathology.* 28:330-333.

Reddy, K. P., Subhani, S.M., Khan, P.A., and Kumar, K.B. 1985. Effect of light and benzyl adenine on dark-treated growing rice (*Oryza sativa*) leaves II. Changes in peroxidase activity. *Plant and Cell Physiology.* 26(6):987-994.

Reddy, K.P., Subhani, S.M., Khan, P.A. and Kumar, K.B. 1995. Effect of light and benzyladenine on dark treated growing rice (*Oryza stiva*) leaves II. Change in Peroxidase activity. *Plant and Cell Physiology.* 26(6):987-994.

Reddy, M.V. and Singh, K.B. 1990. Relationship between ascochyta blight severity and yield losses in chickpea. *Phytopathologia Mediterranea.* 31:59-66.

Reddy, M.V., Singh, K.B. 1984. Evaluation of a world collection of chickpea germplasm accessions for resistance to Ascochyta blight. *Plant Disease.* 68:900-1.

Reeti Singh, Pandya, R.K. and Khare, M.N. 1998. Biochemical changes in sun flower leaves infected by rust (*Puccinia calcitrape* var centuraeae). *Journal of Mycology and Plant Pathology.* 28:164-167.

Rodricks, J.V. 1976. Mycotoxins and other fungal related food problems. Advances in Chemistry series 149. *American Chemicals Society.* Washington.

Rothe, G.M., Welschbilling, N. and Reiss. E. 1988. Molecular size and net charge of pathogenesis related enzyme from barley infected with *Drechslera teres f. teres.* Shoem. *Electrophoresis.* 19(5):745-751.

Sampathkumar, G. and Ganeshkumar. 2003. A. Effect of AM fungi and Rhizobium on growth and nutrition of *Vigna mungo* L. and *Vingnaun guiculata* L. *Mycorrhiza News.* 14.15-18.

Sarwar, N., Jamil, F.F. and Parveen, R. 2001. Accumulation of phytoalexins and phenalalnine ammonia lyase in chickpea after inoculation with *Ascochyta rabiei* and their role in defense mechanism. *Pakistan Journal of Botany.* 33(4):373-382.

Seevers, P.M. Daly, J.M. and Catedral, F.F. 1971. The role of peroxidase isoenzymes in resistance to wheat stem rust disease. *Plant Physiology.* 48:353-360.

Seher, A. and Ivanov, S.A. 1973. Natural antioxidants, antioxidative action of tocotrienols. *Fette Seifen Anstricluim.* 75:606-608.

Setti, B., Bencheikh, M., Henni, J. and Neema, C. 2009. Comparative aggressiveness of *Mycosphaerella pinodes* on peas from different regions in western Algeria. *Phytopathologia Mediterranea.* 48(2):195-204.

Shad, M.A., Pervez, H., Zafar, Z.I., Zia-Ul-Haq, M. and Nawaz, H. 2009. Evaluation of biochemical composition and physiochemical parameters of oil from seeds of desi chickpea varieties cultivated in arid zone of Pakistan. *Pakistan Journal of Botany.* 41:655-662.

Sharif, M. 2004. Wheat yield Gap Analysis: Future Options for Pakistan: A Report submitted in partial fulfillment of the requirement for intership programme in Agricultural Economics B.Sc. (Hons.) Agriculture.

Shimoni, M., Reuveni, R. and Bar-zur, A. 1996. Biochemical changes in groundnut leaves due to infection by early and late leaf spot pathogen. *Indian Society of Mycology and Plant Pathology.* 26(2):210-212.

Siddique, K.H.M., Brindsmead, R.B., Knight, R., Knights, E.J., Paull, J.G. and Rose, I.A. 2000. Adaptation of chickpea (*Cicer arietinum* L.) and Faba bean (*Viciafaba* L.) to Australia. In: 'Linking Research and Marketing Opportunities for Pulses in the 21st Century'. Ed. R knight, Kluwer. Academic Publishers, Dordrecht, The Netherlands. 289-303.

Simopoulos, A. 1999. Essential fatty acids in health and chronic disease. *American Journal of Clinical Nutrition*. 70:560-569.

Sindhan, G.S. and Parashar, R.D. 1981. Correlation studies on bacterial blight incidence in guar germplasm with mineral and phenolic contents of leaves. In: Proc. Third International Symposium Plant Pathology. 14-18:192.

Sindhan, G.S., Indrahooda And Parashar, R.D. 1999. Sources of resistance to*Cercospora* leaf spot in Mung bean and biochemical parameters for resistance. *Journal of Mycology and Plant Pathology*. 29:130-132.

Singh, K.B., Malhotra, R.S., Halila, M.H., Knights, E.J. and Verma, M.M. 1994. Current status and future strategy in breeding chickpea for resistance to biotic and abiotic stresses. *Euphytica*. 73:137-149.

Singh, K.B., Ocampo, B. and Robertson, L.D. 1998. Diversity for abiotic and biotic stress resistance in the wild annual Cicer species. *Genetical Research and Crop Evoluation*. 45:917.

Singh, R., Sindhu, A. and Singal, H.R. 2003. Biochemical basis of resistance in chickpea (*Cicer arietinum* L.) against *Fusarium* wilt. *Acta Physiologica Hungarica*. 38:13-19.

Strange, N. R. 2003. Introduction to Plant Pathology. John Wiley Sons Ltd. New York. USA. 301.

Swain, T. and Hillis. E.W. 1959. Phenolic constituents of *Prunus domestica*. I. Quantitative analysis of phenolic constituents. *Journal of the Science of Food and Agriculture*. 10: 63-68.

Tar'an, B., Warkentin, T., Tullu, A. and Vandenberg, A. 2007. Genetic relationships among Chickpea (*Cicer arietinum* L.) genotypes based on the SSRs at the quantitative trait loci for resistance to Ascochyta blight. *European Journal of Plant Pathology*.119:39-51

Teranishi, Y., Tanaka, A., Osumi, M., and Fukui, S. 1974. Catalase activities of hydrocarbon-utilizing Candida yeasts. *Agricultural and Biological Chemistry*. 38(6):1213-1220.

Tharanathan, R.N. and Mahademavamma, S. 2003. Grain legumesn A boon to human nutrition. *Trends Food and Science Technology*. 14:507-518.

Trapero-Casas, A., Jime´nez-Dı´az, R.M. 1986. Influence of sowing date on fusarium wilt and ascochyta blight of chickpea in Southern Spain. In: O'Keeffe, L.E., Muehlbauer,

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