Dedication

Affectionately dedicated
to Missile Man...

...Sachin

IDENTIFICATION AND CHARACTERIZATION OF PATHOGENESIS RELATED PROTEINS INVOLVED IN *Fusarium* wilt RESISTANCE IN CHICKPEA

by

SHRI. RASKAR SACHIN TUKARAM

(Reg. No. 20128)

A Thesis Submitted in Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE (AGRICULTURE)

In

BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY,

POST GRADUATE INSTITUTE,

MAHATMA PHULE KRISHI VIDYAPEETH,
RAHURI - 413722, DIST. AHMEDNAGAR
(MAHARASHTRA), INDIA

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CANDIDATE'S DECLARATION

I hereby declare that this thesis or part there of has not been submitted by me or any other person to any other University or Institute for a Degree or Diploma.

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This is to certify that the thesis entitled, **"Identification and characterization of pathogenesis related proteins involved in *Fusarium* wilt resistance in chickpea"**, submitted to the Faculty of Agriculture, Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist Ahmednagar, Maharashtra, India, in partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **BIOCHEMISTRY**, embodies the results of a piece of a *bonafide* research work carried out by **Shri. Sachin Tukaram Raskar**, under my guidance and supervision and that no part of the thesis has been submitted for any other degree, diploma or publication in any other form

The assistance and help received during the course of this investigation and sources of literature referred have been acknowledged

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Place : M P K V, Rahuri
Date : 30 /06/ 2003

(S.T.Raskar)

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ABSTRACT

Identification and characterization of pathogenesis-related proteins involved in *Fusarium* wilt resistance in chickpea

by

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A candidate for the degree
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MASTER OF SCIENCE (AGRICULTURE)
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A pot culture experiment was conducted to identify the pathogenesis-related proteins (PRs) and other biochemical markers involved in resistance to *Fusarium* wilt in chickpea. Three resistant, 'Vijay', 'Vishal', and 'Phule G-93009' and three susceptible 'Phule G-5', 'Chafa' and 'JG-62' cultivars of chickpea were separately grown in sterilized (control) and wilt-sick soils during the *rabi* season of 2002 The healthy and infected leaves, stems and roots of these six cultivars were analyzed for various PRs and other biochemical constituents after 25 days of sowing when the visual symptoms of the disease appeared on the susceptible cultivars grown in wilt-sick soil.

The acidic PRs were separated by native and SDS-PAGE while the basic PRs were separated by native-PAGE. The results obtained in the present investigation indicated that in the root portions of the resistant cultivars, a maximum number of the PRs bands were synthesized followed by stem portions as compared to the susceptible cultivars Marked differences in PR bands were not observed in the leaves of susceptible and resistant cultivars. Acidic PR bands of ~25 7 kD, 23 kD, 33.5 kD and 66kD and a basic PR band of ~22 kD appeared

to be synthesized in the root portions of the resistant cultivar, 'Vijay' In another resistant cultivar, 'Vishal' acidic PR bands of ~66 kD in root portions and of ~32 kD in stem portions were found to be synthesized The leaves, stems and roots of resistant cultivars *viz*, 'Vijay' followed by 'Vishal' had comparatively higher levels of total phenolics and chlorogenic acid than all the susceptible cultivars However the leaves, stems and roots of susceptible cultivars had higher levels of soluble proteins After invasion of pathogen, the contents of phenols and chlorogenic acid increased in resistant cultivars An increase in soluble proteins, however was of higher magnitude in the susceptible cultivars.

Thus the synthesis of higher number of novel PR bands and higher levels of phenols and chlorogenic acid in 'Vijay' is indicative of the fact that this variety is more tolerant to the *Fusarium oxysporum* fungi followed by 'Vishal' However, another resistant cultivar, 'Phule G-93009' did not show marked synthesis of PR bands and therefore may be classified as susceptible rather than resistant as categorized earlier based on the results obtained in the present investigation

INTRODUCTION

1. INTRODUCTION

Plant proteins are inexpensive as compared to animal proteins. Pulses are rich sources of plant proteins and often called as 'poor mans meat' (Kewate, 1986). Among the various pulses, chickpea (*Cicer arietinum* Linn) is the third most important pulse crop after dry bean (*Phaseolus vulgaris* L.) and dry peas (*Pisum sativum* L.) (Pushpamma and Geer Vani, 1980.) on a global basis. In addition to proteins and calories, chickpea is also rich in vitamins, *viz*, ascorbic acid and minerals, like calcium and iron. Since the chickpea seed coat contributes about 70 per cent of the total seed calcium, the consumption of whole seed would be nutritionally desirable where calcium deficiency exist. It is even a good source of iron as compared to other legumes (Williams, 1987). Malic acid and oxalic acid secreted by leaf, fruit wall and young stem of chickpea plants are prescribed for intestinal disorders. A germinated chickpea seed has been recommended against scurvy (Kewate, 1986)

Chickpea is of immense significance as a rotation crop in restoring and enriching soil fertility It is adapted well to the friable soil of agriculture, some what cloudy and stony rather than fine tilled. The crop is drought resistant, but genetic variation exists. It is mostly grown on conserved soil moisture and is rarely irrigated (Van Der Maesen, 1987) Owing to improvement in price and relatively low demand for inputs, chickpea has staged a comeback in rainfed or partially irrigated areas. It is traditionally grown in many parts of the world, such as India, Pakistan, Mexico, Turkey, Ethiopia and Myanmar In India Madhya Pradesh, Utter Pradesh, Rajasthan, Maharashtra and Andhra

Pradesh are the important chickpea producing states (Jodha and Subba Rao, 1987) It is a cool season crop of the semi-arid tropics and spring crop of the warm temperate zones (Van Der Maesen, 1987)

According to FAO (2000) around 12.03 million hectare area in the world is under chickpea, and the total chickpea production in the world is around 92 44 MT. In India, it is grown over an area of 6 86 million hectares, with a total estimated production 5 35 MT. India ranks first in chickpea cultivation (64 41%) and production (68 14%) among the various chickpea producing countries of the world In Maharashtra, it is grown over an area of about 0 932 million hectares with total estimated production of about 0.6 MT (FAO, 2000 and Anonymous, 2000).

The low per hectare grain yield of chickpea in Maharashtra has been attributed to many factors such as lack of irrigation, lack of timely and appropriate cultural practices, lack of adequate fertilizer application and lack of pest and disease management Amongst these factors, disease management is one of the most important one for successful cultivation of chickpea

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceri* is one of the most destructive diseases, causing severe losses in chickpea grain yield. According to a rough estimate, about 10 per cent loss in yield due to wilt was considered to be of regular occurrence in chickpea growing states of India (Singh and Dahiya, 1973), whereas Grewal *et al* (1974) believed that the loss may be between 2 and 5 per cent Haware and Nene (1980) reported that early wilting may cause 60-70 per cent loss in grain yield which may even go upto 100 per cent in wilt-sick plot (Pawar *et al.*, 1991)

The fungal disease affects quantity more than quality of the produce It decreases the potentiality of the water conducting vessels in the seedlings and during or after flowering stages The disease is seen in highly susceptible cultivars within 25 days after sowing Affected seedlings show yellowing of the leaves and stems and dropping of the leaves The plant may then collapse and lie flat on the ground Such seedlings when removed from the soil show shrinkage of the stem Roots when split vertically from collar region downward, show brown discolouration of the internal tissue (Nene, 1987) The fungus can survive in the soil for as long as five years and also the symptomless plants carry the fungus It is therefore not possible to control the disease through normal crop rotation or cultural practices The chemical applications to the soil are not feasible and economical to control the disease Use of resistant cultivars is the obvious answer

The biomolecules are known to be involved in the genetic variability of the crop plants, which influence the susceptibility or resistance of the host plants against pathogen. By changing their physiological conditions, higher plants protect themselves from various stresses, such as pathogen attacks, wounding, application of chemicals including phytohormones and heavy metals, air pollutants like ozone, ultra violet rays, and harsh growing conditions This reaction is known as the defense responses of the higher plants, and a series of proteins actively synthesized with this reaction are called "defense- related proteins" (Bowles, 1990) In particular, protective plant proteins specifically induced in pathological or related situations have been intensively studied from the agricultural interests and are called "pathogenesis related proteins,-PRs"

In plants resistance to a pathogen may be due to the presence of some barriers which prevent the entry of pathogen into host cells or some biochemical mechanism which can nullify the effect of pathogen The studies of biochemical research on disease resistance will furnish two prime informations first, the biochemical mechanisms by which plants inhibit or destroy potential pathogens in general and secondly, establishment of the specific factor/s responsible for the mechanism of compatibility or incompatibility in particular host-pathogen interaction. In the present investigation, the concern is with the second theme of identifying and characterizing the pathogenesis related proteins and correlating these with wilt resistance in chickpea Pathogenesis related proteins (PRs) were first described by Van Loon and Van Kammen (1970) in tobacco leaves infected with the tobacco mosaic virus (TMV) PRs are found in numerous plant and pathological situations (Bol *et al*, 1990a) PRs accumulate abundantly at the site of infection, but some also accumulate in the uninoculated parts of an infected plant, but to a lesser degree than in inoculated parts Thimmaiah and Ashoka (1999) reported that the inoculation of rice seedlings with blast fungus induced synthesis of new acidic and basic PRs The PRs resolved on SDS-PAGE showed three and one newly synthesized minor bands of acidic PRs in the range of 14-20 kD after 20 days of inoculation in the resistant and susceptible varieties, respectively over control. On the 13th day after inoculation, 5 and 2 new basic PR protein bands were expressed in resistant and susceptible cultivars, respectively over the control

The changes occur in the PRs in the roots of resistant and susceptible chickpea upon *Fusarium oxysporum* infection Six proteins were identified which were induced or up-regulated in roots of resistant cultivar compared to the susceptible cultivar upon infection (Giri *et al*,

2000). PRs which represent major quantitative changes in soluble protein during the defense response. They have typical physiochemical properties that enable them to resist to acidic pH and proteocleavage and thus survive in the harsh environment where they occur in vacuolar compartment of cell wall or intercellular spaces. (Stintzi *et al.*, 1993). The defense proteins are drawing much attention of plant breeders because defense related proteins usually provide a plant with resistance to stresses, varieties that are apt to intensively induce such proteins are agriculturally valuable (Bowles, 1990). Since wilt caused by *Fusarium oxysporum* f. sp. *ciceri* (FOC), has seven races worldwide of which race 1 and race 2 are prevalent in central India (Giri *et al.*, 2000). Although excellent resistant plant sources are available in chickpea germplasm, success in breeding for resistance is limited, mainly due to region-specific races of FOC and gradual breakdown of resistance in chickpea cultivars. Therefore the present investigation is an attempt to study the pathogenesis- related proteins synthesized in the resistant cultivars against the local strain of FOC, yet to be identified. It is thus proposed to undertake the research work entitled "Identification and characterization of pathogenesis related proteins involved in *Fusarium* wilt resistance in chickpea (*Cicer arietinum* L.)" with the following specific objectives:

1. To identify and characterize the acidic and basic PRs synthesized in different plant parts of resistant and susceptible chickpea cultivars at *Fusarium* infectional stages and
2. To analyze the phenols, chlorogenic acid and soluble proteins from the different plant parts of the resistant and susceptible cultivars of chickpea.

REVIEW
OF
LITERATURE

2. REVIEW OF LITERATURE

Higher plants by changing their physiological conditions, protects themselves from stresses, such as pathogen attacks, wounding, application of chemicals including phytohormones and heavy metals, and harsh growing conditions. This reaction is known as the defense responses of the higher plants. In incompatible host-pathogen interactions, damage caused by the pathogen remains restricted as a result of the plant's defensive response. Most effective is the hypersensitive reaction. This response is associated with coordinated and integrated set of metabolic alterations *viz,* altered ion fluxes across the plant cell membrane, generation of active oxygen species, changes in the phosphorylation state of regulatory proteins and transcriptional activation of plant defense systems, culminate in cell death at the site of infection, local accumulation of phytoalexins and cell wall rigidification as a result of callose, lignin and suberin deposition (Hammond-Kosack and Jones, 1996 and Young *et al.,* 1997). In addition, various novel proteins are induced which are collectively referred to as "defense related proteins" (Bowles, 1990).

The defensive proteins are drawing the much attention of plant breeders (Lee and Raikhel, 1995; Shah, 1997; Shewry and Lucas, 1997 and Datta and Muthukrishnan, 1999). Defense-related proteins usually provide a plant with resistance to stresses, varieties those are apt to intensively induce such protein are agriculturally valuable. Less toxic substance that cause crops to express defensive proteins are being investigated as a new type of agrochemical. Moreover, some defense-related proteins are going to be constantly produced in

genetically modified plants (Fritig *et al.*, 1998; Lee and Raikhel, 1995; Shah, 1997; Shewry and Lucas, 1997 and Datta and Muthakrishnan, 1999). However, several of them have been proved to be latex allergens and cross-reactive allergens in fruits, vegetables and pollen (Hanninen *et al.*, 1999, and Midoro-Horiuti *et al.*, 2001). Eventhough defense-related proteins can be useful agriculturally, their allergenicity should be evaluated carefully (Fu *et al.*, 2002; Lack *et al.*, 2002 and Taylor, 2002).

2.1 Classes of defense-related proteins

Defense related proteins are divided for ease of discussion into three classes based on their role in defense responses.

2.1.1 Structural proteins/enzymes

The first class involves products that are directly change the properties of the extracellular matrix and thereby affect the defense status of the plant by strengthening, repairing or altering the wall environment. Proteins within this class include the structural proteins: hydroxyproline-rich glycoproteins and glycine-rich glycoproteins, as well as wide range of enzymes involved in the construction and/or modification of other wall polymers including suberin, lignin, wall-bound phenolics and callose.

2.1.1.1 Extensions: Hydroxyproline-rich glycoproteins

These proteins are thought to play a central role in the primary wall organization, but are known also to accumulate in response to pathogen invasion and wounding. All extensions characterized to date are highly glycosylated, basic proteins. The salt-extractable cell wall glycoprotein purified from carrot consists of 3.5%

protein and 65% carbohydrate (Stuart and Varner, 1980). The extension polypeptides hydroxyproline-rich glycoproteins (HRGPs) are encoded by a multigene family and the expression of HRGP gene(s) is regulated both developmentally and by environmental stimuli that include wounding and pathogen invasion. Also, enzymes involved in the processing of the extension protein are induced by elicitor treatment (Bowles, 1990).

In studies on carrot, extension transcript levels increased 50-100fold on wounding, cold-stored roots, but only 2-3 fold if actively growing roots were injured. However, the effects of ethylen were different from those of wounding, since the former stimulus leads to the accumulation of two transcripts in carrot cells (1.8 and 4.0 kb) and the latter leads to three (1.8, 4.0 and 1.5 kb). This implies that ethylene and wounding are distinct signals in the carrot systems leading to differential activation of the same gene. (Eckert and Davis, 1987).

2.1.1.2 Glycine-rich proteins

This family of wall proteins is characterized by a high (60%) lysine content. Regulation of glycine-rich proteins (GRP) gene expression by developmental and environmental signals has been shown in two species, petunia and bean. In these species, transcripts levels were higher in young leaf and stem tissue compared with old, but rapidly increased in all tissues in response to wounding (Condit and Meagher, 1987). The activity of the bean GRP promoter was analysed in more detail using GRP-GUS gene fusions in transgenic tobacco (Kellre *et al.*, 1989). Interestingly in the transgenic tobacco, expression of the GRP gene peaked 30-40 min after wounding. Since this was much faster than wound regulation of the endogenous gene in bean plants, it was clear that the host plant species determines the kinetics of the wound response.

2.1.1.3 Peroxidases

The enzymes use H_2O_2 in a range of oxidations. Peroxidases have been studied extensively in higher plants for a number of years. The action of a highly anionic peroxidase is thought to be involved in the polymerization of phenolic monomers to generate the aromatic matrix of suberin (Bowles, 1990). Suberization is developmentally regulated process, but can also be induced site specifically within a defense context when a diffusion barrier must be constructed, such as in the locality of a wound or as an added defense within the apoplast (Espelie *et al.*, 1986). The final stages of lignin biosynthesis involved the dehydrogenative polymerization of cinnamyl alcohols to yield phenoxy radicals that couples non-enzymically to give oligomers of increasing size. The formation of phenoxy radicals is catalyzed by peroxidase (Bowles, 1990). Lignins are highly abundant polymers in the secondary wall of vascular plants, progressively replacing water in the wall matrix and increasing the stability, inertness, strength of the supportive structure. However, lignification is also induced in plant-pathogen responses, and has been correlated both with local resistance and induced systemic resistance (Bowles, 1990). The accumulation of a group of acidic peroxidases, inherited as a single locus has been correlated with induced resistance in cucumber and melon. Recently, the gene encoding the peroxidases putatively involved in lignin formation has been cloned from tobacco (Lagrimini, *et al.*, 1987).

The properties of the wall matrix can be greatly affected by covalent cross-linking of polymers. Peroxidases are known to play a crucial role in the formation of these intermolecular cross links. The process is fundamental to wall organization during growth and

development but an important role can also be envisaged during wound repair and wall strengthening in the immediate locality of an invading pathogen. There are two principle polymer bound phenolic groups that act as substrates for peroxidases. The first is the side chain of tyrosine, an amino acid is high abundance in HGRPs. The second are products derived from p-coumaric acid such as p-coumaryl groups, ferulate, and p-hydroxybenzoate. These phenolics are attached to wall polysaccharides and in particular, pectins have been shown to be feruloylated (Fry, 1983). The mechanism that transfers feruloyl groups to pectins is extremely site specific is known to occur within minutes of incorporation of sugar precursors into the polymers, that is in the golgi complex. The consequence of peroxidases action on these walls phenolics is two fold, first is the glycoproteins become cross-linked via isotyrosines, leading to an extremely stable, insoluble network and second is the gelling of polysachharides can be substantially increased by diferuloyl bridges (Fry, 1982; Cooper and Varner, 1984 and Epstein and Lamport, 1984). In principle, regulation of this cross-linking during defense response could arise from changes in levels of the relevant peroxidases, the temporal/spatial availability of the H_2O_2 in the wall microenvironment, and/or from the degree of feruloylation of the polysaccharides that secreted.

2.1.1.4 Cinnamyl alcohol dehydrogenase

Products of the reaction catalyzed by this cinnamyl alcohol dehydrogenase (CAD) enzyme are the cinnamyl alcohols, the direct precursors of lignin biosynthesis. In bean, the enzyme is encoded by a single gene that has recently been cloned (Walter *et al.*, 1988). The CAD gene is transiently activated (within 1.5h) in response to elicitor treatment leading to rapid increases in CAD enzyme activity. The

rapidity of the CAD response has lead to the suggestion that the enzyme may be responsible for the generation of signaling molecules, such as those related to dehydroceniferyl glucosides that exibit cell-division promoting activity (Walter *et al.*, 1988).

2.1.1.5 Callose synthetase

This enzyme catalyzes the formation of the β-1, 3-glucan called callose. Callose deposition is known to be a very rapid and much localized event in response to pathogen invasion or mechanical injury. The protein is a functional component of the plasma membrane (PM), and *in vitro* enzyme activity is strictly dependent on Ca^{2+}, in the presence of polyamino compounds and/or Mg^{2+} (Fink *et al.*, 1987). Invariably associated with the activation of callose synthetase was an efflux of K^+, and external alkalinization, and a net Ca^{2+} influx into the cells (Waldmann *et al.*, 1988). Activation can be caused by treatment of cells with chitosan, a known component of fungal walls and the exoskeleton of insects (Kohle *et al.*, 1985). The effectiveness of the elicitor increases up to 4000 degree of polymerization (Kauss *et al.*, 1989). This suggests that rather than acting through a specific receptor, the effect of chitosan on callose synthetase arises from damage to the membrane and/or a change in properties caused by interaction of the charged oligosaccharide with the negatively charged head groups of membrane phospholipids.

It has been suggested that callose synthetase and cellulose synthetase are the same enzyme. Thus, there would be on PM-located UDP glucose; glucosyl transferase that, dependant on the microenvironment at the cell surface, transfers glucose to the 4-OH (cellulose) or 3-OH (callose) of the terminal glucose in a nascent glucan

chain. If this model proves correct, switching from one polymer to another world is a highly elegant means of adaptation to circumstance. Turnover of callose has not been widely addressed as yet, but it is thought that β-1, 3-glucanases are involved, both those that are constitutively expressed in the wall and presumably, those are synthesized *de novo* and targeted to the wall in a defense response (Waldmann *et al.*, 1988).

2.1.2 Antimicrobial compounds/activity

The second class involves defense-related proteins that act directly as deterrence, exhibit antimicrobial activities, or catalyze the enzyme inhibitors, such as amylase(s) and proteinase inhibitors : toxic proteins, such as lectins and thionins, hydrolases such as chitinases, β-1,3-glucanases, and proteinases, and enzymes involved in the synthesis of oxidized phenolics, tannins, o-quinones, and the low-molecular weight antimicrobial compounds such as phytoalexins.

2.1.2.1 Lectins

The definition of lectin as a protein exhibiting multiple non-catalytic binding site for carbohydrates is 'operational' and provides as much insight into the function of the protein as the definition of an enzyme based on catalytic activity alone. The abundance of lectins in seeds ranges from >30% total protein e.g. concanavalin A (ConA) to less than 0.1% e.g. wheat germ agglutinin (WGA) a range that renders it unlikely the proteins can all be classified *per se* as storage reserves. Although simple sugars will act as competitive inhibitors, plant lectins can show extraordinary specificity toward the shape of oligosaccharides and this ability to transmit informational content makes them obvious candidates for mediators of molecular recognition (Bowles, 1990).

However, very few studies have addressed the possibility that lectin gene expression can be triggered in vegetative tissues by defense-related stimuli. There is one report that potato lectin accumulates on wounding. Certain lectins, e.g. ricin, phytohemagglutin (PHA), and ConA, are known to be toxic, and recently, an amylase inhibitors and the toxic protein arcelin from bean have been shown to be encoded by genes that are closely homologous to those encoding PHA (Walter *et al., 1988*).

2.1.2.2 Endohydrolases

Studies on defense responses have mainly focused on the endohydrolases that exhibit β-1, 3-glucanase and chitinase activities. Substrates for these enzymes are common components of the surface structures of pathogens and pests, β-glucans being major cellwall constituents of common fungal pathogens and chitin an abundant product of microbial walls and the exoskeleton of insects. There is good evidence that the action of the endohydrolases leads to detrimental effects, such as inhibition of the hyphal growth (Schnlumbaum *et al.,* 1986), as well as the probable release of signaling molecules (β-glucans and chitin/ chitosan oligomers) that activates defense genes (Keen and Yoshikawa, 1983). The proteins have been found in monocots (Fink *et al.,* 1988 and Swegle *et al.,* 1989) and dicots (Boller, 1987) and are known to accumulate under developmental regulation as well as in response to defense-related stimuli. Recently, groups 2 and 3 of the tobacco PR proteins have been identified as hydrolases (Kauffmann *et al.*, 1987 and Legrand *et al.,* 1987). Each tobacco hydrolase exists as acidic species: there are three acidic and one basic β-1,3-glucanases and two acidic and two basic chitinases. Equivalent hydrolases have also been well characterized in two other *Solanaceae* species, potato

and tomato. In both these species, proteins previously described as PR proteins have been recently identified as β-1, 3-glucanases and chitinases. In these species as in tobacco, the enzymes exist in acidic and basic isoforms and there is some evidence that the former are targeted to the apoplast and latter to the vacuole. Since the β-1, 3-glucanases and chitinases from all species so far investigated exist as isomer, it is probable the two enzymes are each encoded by multigene families. This already known for gene products of barley (Hoj *et al.*, 1989), bean (Broglie *et al.*, 1986), and the PR-proteins assigned hydrolase activity in tobacco (Bol *et al.*, 1988 and 1990a).

It has been suggested that hydrolases provide a dual mechanism of defense: the wall-located species being the first line of attack and the vacuolar species the second, when the plant cell membrane is breached. However, the systemic data imply a third role, not related immediate local effects of pathogen invasion but rather to longer-term and generalized protection, such as that underlying acquired resistance. (Swegle *et al.*, 1989).

2.1.2.3 Thionins

These are family of small basic proteins that have been identified in a number of monocots and dicots but are best characterized in barley. Originally extracted from the endosperm of the seeds and known to exhibit toxic properties to a range of organism they were assumed to play a protective role. More recently, other members of the multigene family have been shown leaf specific and are induced to accumulate in response to pathogen challenge and stress. The mode of action of thionin in a defense response may be related to their toxicity and/or their ability to exhibit thioredoxin activity (Bowles, 1990).

2.1.2.4 Thaumatinlike proteins

Thaumatin is the trivial name given to an intensely sweet protein found in the fruits of tropical shrub. The protein shares extensive homology to a bifunctional inhibitor from maize that shows potent *in vitro* activity against bovine trypsin and α-amylase (Richardson *et al.,* 1987).

2.1.2.5 Proteinase inhibitors

This discussion focuses mainly on the serine proteinase inhibitor (PI) 1 and 2 families, since they have been studied in detail as inducible defense-related proteins. PI 1 and 2 genes can be activated by wounding and pathogens. Wider protective role is envisaged, given the abundance of serine proteinase in digestive tracts and/or secretions of herbivores, pathogens, pests. To date, proteins of the inhibitor 1 family have been identified in several plant species, including the *Solanaceae* and monocots, but the PI that is wound induced in leaf tissue of tomato has been extensively studied. In studies on alfalfa, a PI of the Bowman-Birk family showed a typical pattern of accumulation in response to injury and PI activity 'ncreased rapidly in melon in response to fungal infection. Since constitutive expression of a Bowman-Birk class of PI from cow pea in transgenic tobacco led to enhanced resistance to insect larvae (Hider et al., 1987), it is probable that research into this family of defense-related genes and their products will increase for many years to come, particularly since the cow pea inhibitor conferred resistance to a tobacco pest.

2.1.2.6 Late enzymes of phytoalexin biosynthesis

Plant synthesizes low–molecular weight antimicrobial compounds during localized resistance plants to pathogen (Dixon, 1986

and Lamb *et al.*, 1989). Recent results involving enzymes used in the initial steps of isoflavonoid phytoalexin biosynthesis have been summarized in section 2.1.2. Less is known of the enzymes involved in later stages that are specific for isoflavonoid or furanocoumarin phytoalexins. Similarly, little is known of the second class of phytoalexins, the terpenoids. All terpenoids are formed from acetyl CoA, but the pathways leading from polyprenyl pyrophosphates to the different phytoalxins are complicated since at least three intracellular compartments have been shown to be capable of terpenoid biosynthesis. A terpenoid system that is readily amenable to molecular studies is the formation of casbene, the trivial name given to a diterpene phytoalxin in *Ricinus communis.* Casbene synthase, catalyzing formation of the cyclic diterpene from geranyl–geranyl pyrophosphate, has been purified and is located in proplastids. Enzyme activity is absent from healthy tissue, but rapidly increases in response to a pathogen polygalacturonase or a defined oligosaccharide (Bowles, 1990).

2.1.3 Additional defense-related proteins

The third class involves those proteins whose appearance can be correlated with a defense response, but which are of unknown function. Examples include members of the family of proteins defined initially as 'pathogenesis-related (PR) proteins' such as the products of the genes *win*1 and *win*2 that accumulate in a wound response, and examples of novel products that occur in response to elicitor treatment. Function has recently been assigned to certain PR-proteins that have been identified as β-1, 3-glucanases and chitinases.

2.2 Pathogenesis-related proteins

Typically the pathogenesis related proteins (PRs) described for a number of plant species are of low molecular weight, are acid soluble, proteolysis-resistant and have a high mobility in polyacrylamide gel electrophoresis (PAGE) (Pierpoint, 1986). Most PRs accumulate in the extracellular and basic PRs vacuolar, this is not true for all plant species. The vacuolar PRs are likely to exert an effect on the defense reaction after decompartimetalization of a cell, where as the extracellular PRs are directly in contact with the pathogen penetrating the tissue. PRs are found in numerous plants and pathological situations (Bol *et al.*, 1990b). Recently PRs defined as proteins coded for by the host plant but induced specifically in pathological or related situation (Antoniw and Pierpoint, 1978 and Van Loon *et al.*, 1994). Further, it does not accumulate locally in the infected leaf, but are also induced systemically, associated with the development of systemic acquired resistance (SAR) against further infection by fungi, bacteria and viruses. Induction of PRs has been found in many plant species belonging to various families (Van Loon, 1999), suggestive of a general role for these proteins in adaptation to biotic stress conditions. SAR, likewise, is a generally occurring phenomenon, that engenders and enhance the defensive capacity of plants in response to necrotizing infections (Sticher *et al.*, 1997). Since some of the tobacco PRs were identified as chitinases (Legrand *et al.*, 1987) and β-1, 3-glucanases (Kauffmann et al., 1987) with potential antifungal activity, it has often been suggested that the collective set of PRs may be effective in inhibiting pathogen growth, multiplication and spread, and responsible for the state of SAR (Kombrink and Somssich, 1997 and Ryals *et al.*, 1996).

Table 1. Recommended classification of pathogenesis-related proteins (PRs)

Family	Type number	Properties	Reference
PR-1	Tobacco PR-1a	Antifungal, 14-17kD	Antoniw *et al.* (1980)
PR-2	Tobacco PR-2	Class I, II, and III endo-β -1,3 glucanases, 25-35kD	Antoniw *et al.* (1980)
PR-3	Tobacco P,Q	Class I, II, IV, V, VI and VII endochitinases, about 30kD	Van Loon, (1982)
PR-4	Tobacco R	Antifungal, winlike proteins, endochitinase activity, similar to prohevein C-terminal domain, 13-19kD	Van Loon (1982)
PR-5	Tobacco S	Antifungal thaumatin-like proteins, osmotins, zeamatins, permeatins, similar to α-amylase /trypsin inhibitors	Van Loon (1982)
PR-6	Tomato inhibitor I	Protease inhibitors, 6-13kD	Green and Ryan (1972)
PR-7	Tomato P	Endoproteases	Vera and Conejero (1980)
PR-8	Cucumber chitinase	Class III chitinases, chitinase/lysozyme	Metraux *et al.* (1988)
PR-9	Lignin forming peroxidases	Peroxidases, peroxidases-like proteins	Lagrimini *et al.* (1987)
PR-10	Parsley PR-1	Ribonuclases, Bet v 1-related proteins	Somssich *et al.* (1986)

Table 1 contd....

Family	Type number	Properties	Reference
PR-11	Tobacco class V chitinase	Endochitinase activity	Melchers *et al.* (1994)
PR-12	Radish Rs-AFP3	Plant defensins	Terras *et al.* (1992)
PR-13	*Arabidopsis* TH12.1	Thionins	Epple *et al.* (1995)
PR-14	Barley LTP4	Non specific lipid transfer proteins (ns-LTPs)	Garcia–Olmeda *et al.* (1995)
PR-15	Barley OxOa (germin)	Oxalate oxidase	Van Loon and Van Strien (1999)
PR-16	Barley OxOLP	Oxalate-oxidase-like proteins	Van Loon and Van Strien (1999)
PR-17	Tobacco PRp27	Unknown	Van Loon and Van Strien (1999)

Originally, five main classes of PRs (PR-1-5) were characterized by both biochemical and molecular biological techniques in tobacco (Bol *et al.*, 1990b and Van Loon *et al.*, 1987). There upon in 1994 a unifying nomenclature for PRs was proposed based on their grouping into families sharing amino acid sequences, serological relationship, and enzymatic or biological activity. By then eleven families (PR-1-11) were recognized and classified for tobacco and tomato (Van Loon *et al.*, 1994). Recently 17 families were recognized (Table-1) (Van Loon and Van Strien, 1999).

Two criterias were used for the inclusion of new families of PRs, first, protein(s) must be induced by a pathogen in tissues that do not normally express the protein(s), and second, induced expression must have been shown to occur in at least two different plant-pathogen combinations, or expression in a single plant-pathogen combinations must have been confirmed independently in different laboratories (Van Loon and Van Strien, 1999). The tobacco PRs have been classified in major five groups on serological properties and later on sequence data. Group PR-1 contains the first discovered PRs of 15-17 kD molecular weight whose biological activity is still unknown, but some members have been shown recently antifungal activity (Antoniw *et al.*, 1980). Group PR-2 contains three structurally distinct classes of β-1, 3-glucanases with acidic and basic counterparts, with dramatically different specific activity towards beta glucans and with different substrate specificity (Antoniw *et al.*, 1980). Group PR-3 consists of various chitin lysozymes that belong to three distinct classes, are vacuolar or extra cellular, and exhibit differential chitinase and lysozyme activities. Some of them, either alone or in combination β-1, 3 glucanases, have been shown to be antifungal *in vitro* and *in vivo* (transgenic plant) probably by hydrolyzing their substrates as structural components in the fungal cell wall (Van Loon, 1982). PR-4 is the less studied, and in tobacco contains four members of 13-14.5kD of unknown activity and function (Van Loon, 1982). Group PR-5 contains acidic-neutral and very basic members with extracellular and vacuolar localization, respectively and all members show sequence similar the sweet-tasting protein thaumatin (Van Loon, 1982). Several members of the PR-5 group from tobacco and plant species were shown to display significant *in vitro* activity of inhibiting hyphal growth spore germination of various fungi probably by a membrane permeabilizing mechanism

(Stintzi *et al.*, 1993). Recently 22kD potato PR-5 was shown to the acting together with a 32kD basic chitinases, and it was suggested that the actin–binding complex might be involved in cytoplasmic aggregation, there by participating in the defense against *Phytophthora infestans* (Takemoto *et al.*, 1997).

PR-6 is proteinase inhibitors implicated in defense against insects and other herbivores, micro-organisms, and nematodes (Koiwa *et al.*, 1997 and Ryan, 1990). PR-7 has so far been characterized only in tomato, where it is a major PR and acts as an endoproteinase (Vera and Conejero, 1988). Because lysis of fungal cell walls often requires degradation of cell wall proteins in addition to hydrolysis of chitin and glucan (Goldman and Goldman, 1998 and Harman *et al.*, 1996), it seems reasonable to assume that PR-7 serves as an accessory to antifungal action. PR-8 and-11 are all classified as endochitinases, even though their specific activities on colloidal chitin vary over 100-fold (Burnner *et al.*, 1998). The PR-9 family of peroxidases is likely to function in strengthening plant walls by catalyzing lignin deposition in reaction to microbial attack (Van Loon and Van Strien, 1999). The PR-10 family is structurally related to ribonucleases (Moiseyev *et al.*, 1997) and although it is tempting to suppose that these intracellular PRs may be active against viruses, a capability to cleave viral RNA remains to be demonstrated. The PR-12 type defensins PR-13 type thionins and PR-14 type lipid transfer proteins (LTPs) all exhibit antifungal and antibacterial activity, exerting their effect at the level of the plasma membrane of the target micro-organism (Bohlmann, 1994; Broekaert *et al.*, 1997 and Garcia Olmedo *et al.*, 1995).

2.3 Pathogenesis-related proteins associated with defense response in crop plants

A ubiquitous feature of plant-pathogen interactions is termed the hypersensitive response (HR). In addition, local HR is often associated with the onset of systemic acquired resistance (SAR) in distal plant tissues. This typical resistance is generally effective against a broad range of pathogens and it is associated with the transcriptional activation of whole set of marker genes, many of which encode pathogenesis-related proteins. In this section PRs associated with different diseases in crop plant is reviewed briefly.

2.3.1. PRs associated with *Fusarium oxysporum* f. sp. *ciceri* (FOC)

Giri *et al.*, (2000) analyzed root proteins in established resistant (WR-315) and susceptible (JG-62) cultivars in response to FOC-race 1. Root tissues were harvested at 0, 5, 2, 6 and 12 days after inoculating 5-days-old chickpea seedlings with the pathogen. Two-dimensional gel electrophoresis was employed for monitoring changes in protein composition of roots. Six proteins were identified which are either induced or up-regulated in roots of resistant cultivar compared to the susceptible cultivar upon infection. Their approximate molecular masses are 70, 42, 34 (one major and one minor), 7 and 6kD, respectively. N-terminal sequencing of 6 and 7kD proteins yielded sequence homology with phenyalanine ammonia lyase and PR-10 proteins. The reports on PRs associated with FOC are scantly therefore the PRs associated with other pathogenic organism are described below.

2.3.2 PRs associated with different hosts

Thimmaiah and Ashoka (1999) identified PRs in rice seedlings inoculated with the blast fungus, *Pyricularia oryzae*. In their pot culture experiment, five days old seedlings of two rice varieties, namely IET-7191 (resistant) and HR-12 (susceptible) were inoculated with a 15 days old culture of the blast fungus for induction of acidic and basic PRs. The samples of the whole seedlings were analyzed at 6, 13, and 20 days after inoculation (DAI). The acidic PR proteins were identified by native-PAGE and SDS-PAGE and basic proteins by native-PAGE. The electrophoretogram of native–PAGE showed a variation in number and intensity of acidic PR proteins due to inoculation over control. A maximum of 15 bands were observed in both the varieties at 20 DAI. The SDS-PAGE showed a 3 and 1 newly synthesized minor bands in the range of 14-20kD at 20DAI in IET-7191 and HR-12, respectively over control. The native-PAGE showed a maximum of 12 and 13 basic PR-protein bands at 13 DAI in IET-7191 and HR-12 respectively. At 13 DAI, 5 and 2 new basic PR-protein bands were expressed in IET-7191 and HR-12, respectively over control. The results indicated that the inoculation of seedlings with the blast fungus induced synthesize and expression of new acidic and basic PR-proteins.

Deborah *et al.* (2001) inoculated rice leaf sheaths with *Rhizoctonia solani* (pathogen) and *Pestalotia palmarum* (non-pathogen) and analyzed for the accumulation of PRs. They observed that inoculation of plants with *R. solani* and *P. palmarum* resulted in a marked increase in activities of chitinase and β-1, 3-glucanase. The levels of both enzymes were higher in incompatible interactions than in compatible interactions. Western blot analysis indicated that two proteins with molecular weights of 33 and 35 kD cross-reacting with

barley chitinase antibody were induced in rice in response inoculation with *R. solani.* The appearance of these chitinases was correlated with increase in enzyme activity.

Increased phenolic content and accumulation of PRs; *viz,* chitinase, β-1, 3-glucanase and thaumatin-like protein (PR-5), were observed in sugarcane plants treated with a novel synthetic signal molecule, acibenzolar–5–methyl (CGA–245704; benzo (1, 2, 3) thiadiazole–7–carbothioic acid 5–methyl ester), inducing resistance in sugarcane against rot disease caused by the fungus *Colletotrichum falcatum* Went (Sunder *et al.*, 2001).

Jeun (2001) reported that accumulation and localization of the pathogenesis–related proteins AP24 in leaves of tomato plants exhibited systemic acquired resistance against *Phytophthora infestans* after pre-treatment with 3-aminobutyric acid or tobacco necrosis virus. In cotton stem, levels of mRNA for genes for cadinene synthase, acidic chitinase, basic chitinase, β-1, 3-glucanase, and at least one member of the family of the cotton *Ypr*10 gene homologues coding for potential PR-10 proteins increased after inoculation with *Verticillium dahliae.* However, levels of mRNA for a gene for phenylalanine ammonia lyase were similar in all samples (McFadden *et al.*, 2001). Feugey *et al.*, (2000) observed temporal changes in PAL activity and expression of genes encoding intercellular PRs were followed after inoculating birch roots with *Paxillus involutus* (isolates PO and Mi) and *Hebeloma cylindrosporum* (strains D1 and D105) fungi.

2.3.3 PRs associated with elicitor molecules

Elicitor molecules associated with PRs elicit the co-ordinated set of defense response reactions in the plants. Among the

different sets of defense response reactions, the synthesis of pathogensis related proteins in incompatible reactions is one of them.

Salicylic acid (SA) has been shown to act as a signal molecule that is produced by many plants subsequent to the recognition of potentially pathogenic microbes. Increase in levels of SA often trigger the activation of plant defenses and can result in increased resistance to subsequent challenge by pathogens. Yalpani *et al.* (2001) observed that the polyketide 6-methylsalicylic acid (6-MeSA), a compound apparently is not endogenous to tobacco, can mimic SA. Tobacco leaves treated with 6-MeSA showed enhanced accumulation of the PRs PR-1, β-1, 3 glucanase, chitinases and also develop increased resistance against tobacco mosaic virus. Murphy *et al.* (2000) treated tobacco with SA, and could delay the onset of disease caused by the necrotrophic fungal pathogen, *Botrytis cincera.* When components of the intercellular fluid with a molecular weight grater than 300 Da were removed, the intercellular fluid no longer inhibited mycelial growth suggesting that the inhibitor is one or more of the extracellular PRs. Schaller *et al.* (2000) treated tomato plants with fusicoccin (FC), an activator the plasma-membrane H^+ ATPase which maintains an electrochemical gradient across the plasma membrane, resulted in a dose–dependent accumulation of transcripts for intra-and extracellular PRs. Transcripts of PR proteins and SA started to accumulate 3h after FC treatment. The 2-Aminodan–2-phospheric acid an inhibitor of SA synthesis was found to suppress the accumulation of SA but not the induction of PR gene expression in response to FC treatment. Furthermore in the transgenic tobacco plants over expressing a bacterial salicylate hydroxilate gene (*nah* G-tobacco) and PRs transcripts accumulated after FC treatment to levels similar to those observe in control tobacco plants. The result indicated the role for the

proton gradient across the plasma membrane in the SA-independent induction of PR-gene expression.

2.4 Total phenolics and chlorogenic acid

The phenolic compounds particularly orthodihydroxy phenolic compounds and phytoalexins are chemicals responsible for confirming disease resistance. The possibility of plant extract containing phytoalexins/phenolic compounds needs to be explored in order to combat various diseases of agricultural crops (Bajaj, 1988).

The plant parts i.e. leaves or stems or roots contain higher levels of phenols in resistant than susceptible cultivars of chickpea against *Fusarium* wilt (Gupta and Kotasthane, 1984; Gupta and Khare, 1992 and Mandavia, *et al.*, 1997), in pigeon pea (Kotasthane and Gupta, 1984). In response to the infection by wilt fungus caused to the chickpea, the content of the total phenols increased and accumulated in the root and stem tissues of the both resistant and susceptible cultivars except in the leaf tissue of resistant genotype where it showed a declined (Gupta and Kotasthane, 1984; Gupta and Khare, 1992 and Mandavia *et al.*, 1997). Similar trend in the contents of phenols during pathogenesis was observed in chickpea in case of *Ascochytarabie* infection (Vir and Grewal, 1974), in rice against brown spot disease (Sathiynathan and Vidhyashekaran, 1981), in alfalfa during early stage of anthracnose disease (Lenseen *et al.*, 1991 in limabean against stem rot (Mandavia and Parameshwaran, 1993) In case of infection of *Helminthosporium* eaf spot to sorghum, concentration of total phenols and o-dihydroxyphenols increased in resistant line, however in the diseased leaves of susceptible cultivars, the

concentration of total phenols decreased, while O-dihydroxy phenols increased (Singh and Chand, 1982).

Certain phenols like catcheol and chlorogenic acid were found to be higher in stem rot resistant plants of limabean at all the stages of disease plant development (Mandavia and Parameswaram, 1995). Stevenson *et al.*, (1997) reported that *Fusarium oxysporum* f. sp *ciceri*, whether present in the soil or artificially applied to chickpea roots, induced an increase in the concentration of phenolic compounds such as medicarpin and maackiain in the roots particularly in the roots of resistant cultivars and showed antifungal activity against *F oxysporum* f. sp. *ciceri*.

It was observed that chlorogenic acid, p-coumaric acid and ferulic acid were present in 2-4 fold higher amounts in the root tissue of wilt resistance plants of chickpea at the early stage of wilt infection (Mandavia et al., 1997). At pre-infectional stage chlorogenic acid, p-coumaric acid, ferulic acid, salicylic acid, vanillic acid and umbelliaferone, etc., were present in lower amounts in the stem tissues of wilt resistant plants compared to that in susceptible plants. However hydroquinone and umbelliferone were present in higher amount in stem tissues of plant at diseased initiation stage (Mandavia *et al.*, 1997). At disease initiation stage both, chlorogenic acid and p-coumaric acid were present in higher amount in the leaves of resistant variety. At disease initiation stage, both chlorogenic acid and p-coumaric acid were present in higher amount in the leaves of resistant plants and chlorogenic acid p-coumaric acid and ferulic acid increased and were higher in root tissues of resistant variety. Hydroquinone and ambelliferone were higher in

stem tissues of resistant variety at disease initiation stage (Mandavia *et al.*, 1997). Similar results were obtained in cumin in relation to *Fusarium* wilt (Mandavia *et al.*, 1999).

At severe disease stage, destruction of cell structures occurs and some has been correlated to a rise in metabolic diseased plant tissues (Lakshminarayanan, 1955). The rise in levels of some of the phenolics in infected plants due to their release from the cell wall structures during their destructions.

2.5 Proteins

Protein contents in resistant and susceptible varieties of crop plants in relation to disease resistance were studied by several research workers. In relation to *Fusarium* wilt of tomato, protein content was found higher in healthy wilt susceptible plants than in resistant plants, but this trend was reversed by infection (Malhotra, 1993). Stutz and Bess (1998) observed a 2-3 fold increase in the level of hydroxyproline-rich glycoprotein in cell walls of resistant cultivar of tomato infected with *Fusarium oxysporum*. In case of *Fusarium* wilt of chickpea, soluble proteins increased in the infected leaves of susceptible variety as compared to the leaves of resistant variety, while in root tissues, there was no significant difference in the levels of protein between these two varieties (Maravia et al., 1993). The crude protein percentage was increased in wilted roots of as susceptible variety of chickpea, as compared to healthy susceptible and resistant varieties (Gupta and Kotasthane, 1982). Increase in protein content after infection may be due to synthesis of proteins in the form of certain carbohydrate degrading enzymes secreted by the infecting fungi (Prasad *et al.*, 1988). Increase in proteins in the form of enzymes such as

glucose-6-phosphate and 6-phosphogluconate dehydrogenase in maize leaves infected with *Heliminthosporium carbonum* was reported by Malca *et al.* (1964). However Jennings *et al.* (1969) reported the possible release of bound proteins during degradation of infected tissue of maize due to *H. carbonum* Pundir *et al.* (1990) suggested that degradation of host proteins into free amino acids by the enzyme protease from *Fusarium* wilt pathogen, increased the concentration of free amino acids in lentil. The concentration of ammonia was increased in infected leaves indicating the degradation of existing host proteins It was also concluded that rotting of fruit tissue in muskmelon results in degradation in the level of soluble proteins. The reduction may be due to utilization of host protein by fungal protease (Prasad *et al*, 1988).

More amount of proteins is correlated with more amount of nitrate nitrogen in the susceptible plants, which can not prevent the pathogen from infecting the host plant (Satyaprasad and Ramarao, 1983 and Saxena and Khare, 1988). High amount of nitrate nitrogen makes the host tissue succulent, which can be easily attacked by the pathogen (Saxena and Khare, 1988 and Gupta and Khare, 1992).

MATERIAL AND METHODS

3. MATERIALS AND METHODS

3.1 Material

3.1.1 Plant Material

The seeds of *Fusarium* wilt susceptible 'JG-62', 'Phule G-5' and 'Chafa' and resistant cultivars, 'Vijav'. 'Vishal' and 'Phule G-93009' were obtained from the Principal Scientist, All India Co-ordinated Pulses Improvement Project, Mahatma Phule Krishi Vidyapeeth, Rahuri.

3.1.2 Fungal Material

The samples of *Fusarium* infected chickpea plants were collected from the experimental site of Plant Pathology, Mahatma Phule Krishi Vidyapeeth, Rahuri and fungus was isolated, purified and maintained on agar slants for further studies.

3.1.3 Soils

Soil obtained from the Pulses Improvement Project, M.P.K.V, Rahuri was sterilized in autoclave at 15 lbs pressure for one hour to serve as control. In another set, the soil was infected with fungus by mixing purified culture grown on cotton seed at the rate of 100 g infected cotton seed per kg soil.

3.1.4 Chemicals

The various chemicals used in this investigation were of analytical grade and procured from Glaxo Laboratories, Mumbai, E-Merck (India) Ltd., Mumbai, s.d.fine chemicals Ltd., Mumbai, Sisco Research Laboratories Ltd., Mumbai.

3.2 Methods

3.2.1 Isolation and purification of *Fusarium oxysporum* f.sp.*ciceri*

Reagents

Preparation of potato dextrose agar medium (PDA medium)

Potato	200g
Dextrose	20g
Agar	20g
Water	1000ml

Method

The samples of *Fusarium* infected chickpea plants were collected from experimental site of Plant Pathology, Mahatma Phule Krishi Vidyapeeth, Rahuri. The affected portion of stems and roots of the samples were cut into suitable pieces, washed thoroughly in tap water so as to remove soil particles. The pieces were then disinfected by 0.1 per cent mercuric chloride solution for 2 minutes followed by consecutive several washings with sterilized water to remove the traces of mercuric chloride solution. Three to four such pieces were then plated aseptically on sterilized potato dextrose agar in each petriplate. The petriplates were incubated at room temperature.

Mycelia of the fungi, free from contamination was transferred to agar slants by hyphal tip method. By single spore isolation technique the fungus *Fusarium oxysporum* f.sp.*ciceri* was purified and agar slants showing pure fungal growth were maintained for further studies.

3.2.2 Multiplication and inoculation in to soil

Reagents

Richards broth

Potassium nitrate (KNO_3)	10g
Potassium dihydrogen phosphate (KH_2PO_4)	5g
Magnesium sulphate ($MgSO_4.7H_2O$)	2.5g
Ferric chloride ($FeCl_3$)	0.02g
Sucrose	50g
Distilled water	1000ml
pH	6.6 to 7.2

Method

The above pure culture of the fungus was multiplied on Richard's medium for 15 days at room temperature. Further it was transferred on sterilized crushed cotton seeds for multiplication about 15 days. The sterilized soil was inoculated with fungus by mixing crushed cotton seeds at the rate of 100 gm crushed seed infected with fungus per kg of soil. The inoculated soil was incubated for 30 days with frequently stirred and watered and allowed the maximum visible growth on soil surface. The earthen pots were filled in with this inoculated soil. Then seeds were sown in the earthen pot. The seeds sown in the sterilized uninoculated soil served as control. The pots were kept in glass house at the temperature between 26 to 30 ^{o}C and plant samples were collected after 25 days when visual symptoms of wilting observed.

3.2.3 Experiment

The pot culture experiment was carried out in two separate sets, using completely randomized block design, during Nov. 2002. In the first set, the seeds of resistant and susceptible cultivar were sown in

earthen pots containing 10 kg sterilized (control) soil and in the second set, the seeds of the resistant and susceptible cultivar were sown in the earthern pots containing 10kg wilt-sick (*Fusarium oxysporum* f.sp.*ciceri*) soil. The healthy and infected samples of leaves, stems and roots were collected from plants grown in autoclaved sterilized soil (Plate-I) and infected soil (Plate-II) respectively after 25 days of sowing when visual symptoms of disease occurred on susceptible plants. The PRs, soluble proteins, phenolics and chlorogenic acid content were estimated from the fresh samples. These plant parts were washed with water, blotted and acetone powder was prepared for analysis.

3.2.4 Methods of analysis

3.2.4.1 Acidic pathogenesis-related proteins

The acidic PRs were extracted as per the method described by Cordero *et al.* (1992) and were separated by native-PAGE (Davis, 1964) and SDS-PAGE (Laemmli, 1970). The vertical slab gel electrophoresis apparatus of Atto AE-6210 model of Japan make was employed for separation of the acidic and basic PRs from the leaf, stem and root portions.

Reagents

1. Extraction solution

i. Acetone (-20°C cold)

ii. Diethyl ether

iii. Extraction buffer : 0.1 M borox-sodium hydroxide buffer (pH 10.0) containing 0.1 per cent (w/v) polyvinyl pyrrolidone (PVP), 5 mM ascorbic acid, 10 mM sodium thiosulphate and 1 mM phenyl methyl sulphonyl fluoride (PMSF).

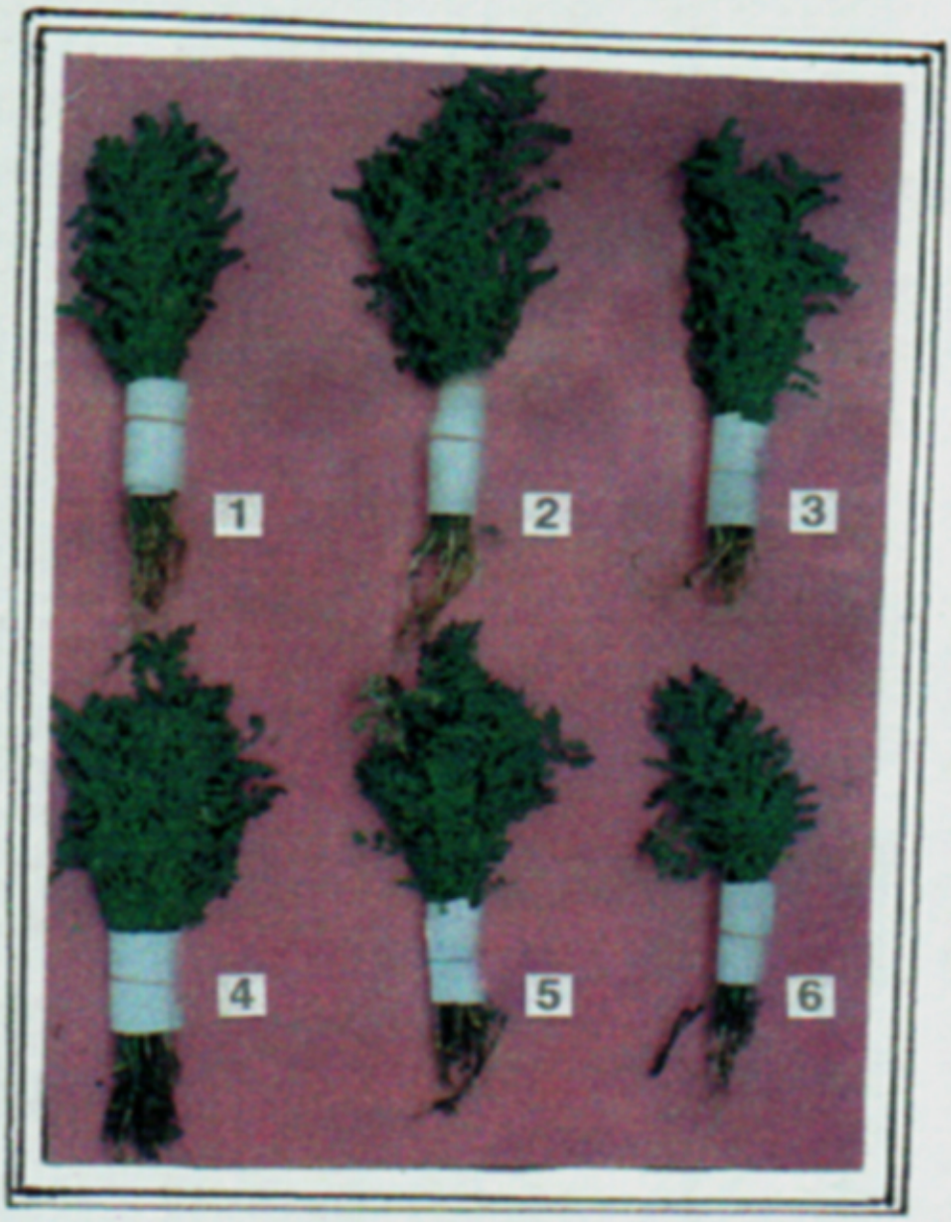


Plate 1. Chickpea resistant 1) Vijay, 2) Vishal and 3) Phule G093009 cultivars and susceptible 4) Phule G-5, 5) Chafa and 6) JG-62 cultivars grown in sterilized soil

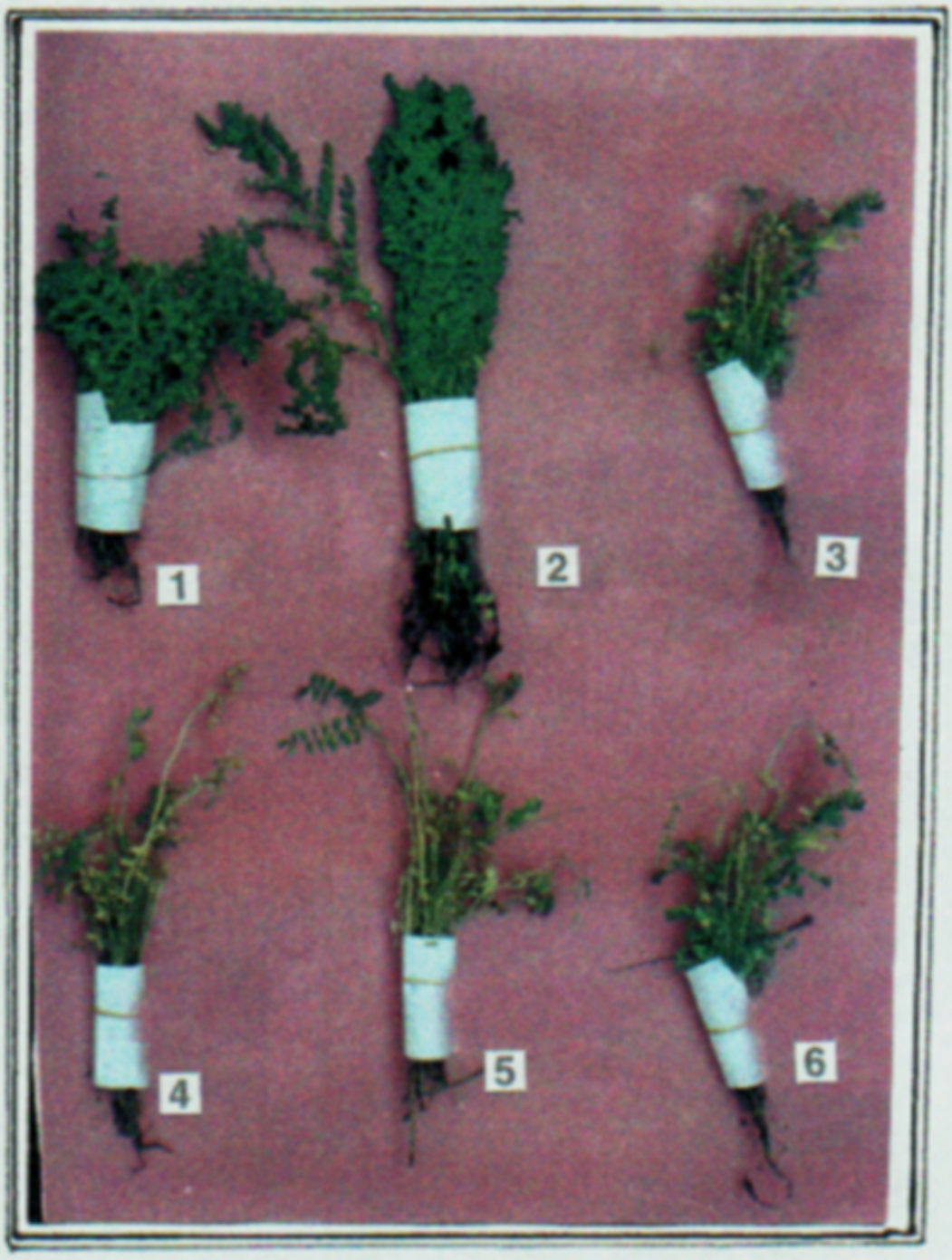


Plate 2. Chickpea resistant 1) Vijay, 2) Vishal and 3) Phule G093009 cultivars and susceptible 4) Phule G-5, 5) Chafa and 6) JG-62 cultivars grown in wilt-sick soil

2. Stock solutions for stacking gel for native-PAGE

A. Tris-HCl buffer (pH 6.8)

i. Tris : 5.98 g

ii. 1 M HCl : 49 ml

iii. TEMED : 0.46 ml
(N, N, N', N' tetramethylethylenediamine)

iv. Small quality of distilled water was added in it and pH was adjusted to 6.8. The final volume was made to 100 ml. The solution was filtered and stored in brown bottle at 4°C

B. Acrylamide solution

i. Acrylamide : 20 g

ii. Bisacrylamide : 1 g
(N, N' methylenebisacrylamide)

iii. Dissolved in distilled water and final volume was made to 100 ml. The solution was filtered and stored in brown bottle at 4°C.

C. Ammonium persulphate solution (0.15%,w/v) : Ammonium persulphate, 0.15 g was dissolved in distilled water to make 100 ml volume. The solution was prepared fresh at every time.

3. Stock solutions for separating / resolving gel for native-PAGE

D. Tris HCl buffer (pH 8.9)

i. Tris : 36.6 g

ii. 1 M HCl : 4.8 ml

iii. TEMED : 0.25 ml

iv. Small quantity of distilled water was added to it and pH was adjusted to 8.9 The final volume was made to 100 ml. The solution was filtered and stored in brown bottle at 4°C.

E. Acrylamide solution

i. Acrylamide : 30 g

ii. Bisacrylamide : 0.8 g

iii. Dissolved in distilled water to make the final volume 100 ml. The solution was filtered and stored in brown bottle at 4^0C.

F. Riboflavin solution : Riboflavin, 0.04 mg was dissolved in distilled water to make 100 ml volume and prepared fresh at the time of experiment.

4. Stock solutions for stacking gel for SDS-PAGE

G. Sodium dodecyl sulphate solution (10%, w/v) : Sodium dodecyl sulphate (SDS), 10 g was dissolved in distilled water with constant stirring and gentle heating. Final volume was made upto 100 ml with distilled water.

H. Ammonium per sulphate (5%, w/v) : Ammonium per sulphate, 0.5 g was dissolved in distilled water to make final volume 10 ml. It was prepared fresh at every time.

5. Working solutions for native - PAGE

a. Separation gel (8%, pH 8.9)

D	solution	:	3.75 ml
E	solution	:	7.5 ml
C	solution	:	15.0 ml
Distilled water		:	3.75 ml

b. Stacking gel (5%, pH 6.8)

B	solution	:	2.50 ml
A	solution	:	1.25 ml
F	solution	:	1.25 ml
Distilled water		:	5.0 ml

6. Working solutions for SDS-PAGE

a. Separation gel (12 %, pH 8.9)

D	solution	:	6.0 ml
E	solution	:	12.0 ml
H	solution	:	0.20 ml
G	solution	:	0.15 ml
Distilled water		:	11.25 ml

b. Stacking gel (5%, pH 6.8)

E	solution	:	2.0 ml
A	solution	:	1.50 ml
H	solution	:	0.40
G	solution	:	0.10 ml
Distilled water		:	6.0 ml

7. Tray / Electrode buffer

Stock buffer

i. Tris : 3.0 g

ii. Glycine : 14.4 g

iii. Dissolved in distilled water, the pH was adjusted to 8.3 and final volume was made to 1 litre. This buffer was used for 2-3 subsequent runs.

8. Staining solution

i.	Coomasie brilliant blue	:	0.1 g
ii.	Methanol	:	40 ml
iii.	Acetic acid	:	10 ml
iv.	Distilled water	:	50 ml

The dye was first dissolved in methanol. Then acetic acid and distilled water was added in it. The solution was filtered 2 to 3 times before use. Fresh solution was prepared and used every time.

9. Destaining solution

As above solution without dye was prepared.

10. Fixing solution

Trichloroacetic acid 150 g dissolved in distilled water to make final volume 1 litre.

11. Standard protein molecular weight marker

The mixture of protein molecular weight markers were obtained from the Genei Pvt. Ltd. Bangalore of the following proteins with their molecular weight :

Proteins used	**Molecular weight (kD)**
Phophorylase b	97
Bovine serum albumin	66
Ovalbumin	43
Carbonic anhydrase	29
Lactoglobulin	18
Aprotinin	6.5

Methods

Extraction of PRs

Preparation of acetone powder

About 5 g of fresh tissues of leaves, stems and roots were cut into small pieces of about 1-2 cm and transferred into pre-chilled pestle and mortor which was kept in an insulated ice-bucket. The tissues were homogenized with chilled acetone (- 20 ^{0}C). The homogenized material was filtered through Buchner Funnel with Whatman No. 1 filter paper under the suction. The residue was washed with diethyl ether and was spread over the Whatman No 1 filter paper

and air dried for about 1 hr. The powder was wrapped in aluminum foil and stored at 4 ^{o}C.

Extraction of acidic PRs

About 0.5 g of acetone powder was extracted in 5 ml of borax-sodium hydroxide buffer solution by mixing it thoroughly The mixed sample was centrifuged at 10000 Xg for 20 min at 4 ^{o}C and supernatant was collected and used for resolution of acidic PRs by electrophoresis.

Electrophoresis

The two glass plates with spacer in between it was fixed on the gelplatform of the electrophoresis unit with due care to remained it as leakproof. The separation gel solutions were mixed in appropriate proportion and degassed. The solution was immediately poured in between the glass plates mounted on a gel platform by Pasteur pipette so that about 2/3 of the space was filled with gel with due care that no air bubble was remained. Then small quantity of distilled water was carefully overlayered on the top of the gel to get horizontal gel surface and avoid contact of air. The plates with gel were kept for gel polymerization under fluorescent light for 60 min. After polymerization of gel, the water was removed with the filter paper strips. The stock solutions of stacking gel were mixed in appropriate proportions to get a working solution and the mixture was immediately poured over the stacking gel. The 13 well comb was immersed in it and the gel was kept under fluorescent light for 30 min for polymerization. The comb was removed after 30 min and the wells were washed with the electrode buffer. The traces of electrode buffer from the well was removed with filter paper strips. The plates were removed from platform and fixed on

the gel cassette. The cassette was placed in the buffer tank and electrode buffer was poured in such a way that the wells were completely dipped into buffer.

The twelve samples of each 25 µl (150 µg) were mixed with 2 µl of bromophenol blue to function as tracking dye and loaded by syringe in the twelve wells carefully. The unit was connected to power supply unit. Initially the current 1.5mA/ well was applied till the samples migrates into the spacer gel and then it was adjusted 2 mA/well. The electrophoresis apparatus except power supplier was kept in a refrigerator at constant temperature of 4°C. The electrophoresis was carried out until the bromophenol blue (tracking dye) migrated almost to the end of the gel and current was switched off.

The cassette was removed from the unit and the gel was taken out gently. It was placed in a staining tray and incubated overnight in fixing solution of 15 per cent trichloroacetic acid (TCA). It was thoroughly washed with fixing solution for removal of excess SDS which might be precipitated on the surface. Sufficient staining solution was poured to cover the gel uniformly. It was incubated for 18 hours and the stain was decanted. The destaining was carried by keeping the gel in the destaining solutions for one day with interchanging the destaining solution frequently. The band intensity was assessed visually by placing gel over transilluminator and recorded as faint, faint dark, dark and intense bands and as absence or presence of specific bands. The relative mobility (Rm) of resolved PR bands were calculated as follows :

$$Rm = \frac{\text{Distance migrated by PR band (cm)}}{\text{Distance migrated by tracking dye from the top of the separation gel (cm)}}$$

The respective separation gel and stacking gel solutions were used for resolution of acidic PRs by native-PAGE (reagent Sr. No. 5) and SDS-PAGE (reagent Sr. No. 6).

Calibration of standard curve

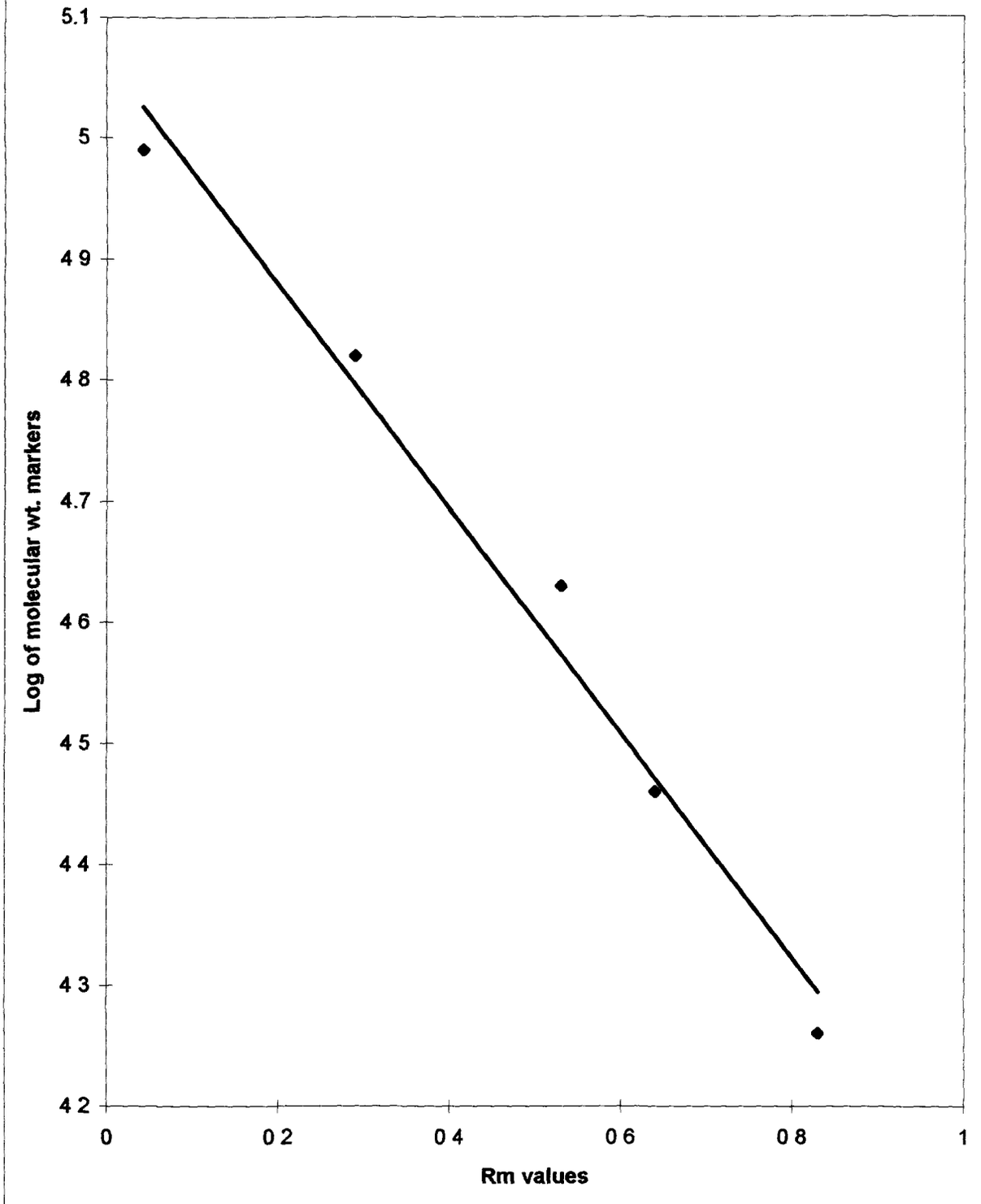
The 10 µl mixture of standard protein molecular weight markers, were diluted by adding 50 µl of extraction buffer and total 60 µl was loaded in the well of the gel in triplicate. The electrophoresis was carried as described above. The Rm values of each protein marker was calculated and the graph was plotted on graph paper, plotting Log mol. Wt. of protein versus Rm value. The molecular weight of PR bands were calculated from this standard graph.

3.2.4.2 Basic pathogenesis-related proteins

The basic PRs were extracted as per the method described by Granell *et al.* (1987) and were separated by native-PAGE (Reisfeld *et al.*, 1962).

Reagents

1. **Extraction buffer :** 0.1 M citrate buffer (pH 4.0) containing 0.1 per cent (w/v) PVP, 5 mM ascorbic acid, 10 mM sodium thiosulphate and 1 mM PMSE.



Standard graph of protein molecular weight markers

2. Stock solutions for stacking gel

A. Potassium hydroxide buffer (pH 6.8)

i. 1 N potassium hydroxide : 48 ml

ii. Acetic acid : 2.87 ml

iii. TEMED : 0.46 ml

iv. Dissolved by adding small quantity of distilled water and pH was adjusted to 6.8. The final volume was made to 100 ml. The solution was filtered and stored in brown bottle at 4°C.

B. Stock acrylamide solution

i. Acrylmide : 10 g

ii. Bisacrylamide : 25 g

iii. Dissolved in distilled water to make final volume 100 ml and stored in brown bottle at 4°C.

C. Riboflavin solution : Same as above given in F.

3. Stock solutions for separating gel

D. Potassium hydroxide buffer pH (4.3)

i. 1 N potassium hydroxide : 48 ml

ii. Acetic acid : 17.2 ml

iii. TEMED : 4.0 Ml

iv. Small quantity of distilled water was added in it and dissolved and pH was adjusted to 4.3. The final volume was made 100 ml and filtered. The solution was stored in brown bottle at 4°C.

E. Stock acrylamide solution : As described under acidic PRs 'E' solutions.

F. Ammonium persulphate solution : Ammonium persulphate 0.28g was dissolved in distilled water and final volume was made to 100 ml. It was prepared fresh.

4. Working solutions

a.	Separation gel	:	(8%, pH 4.3)
	R solution	:	3.75 ml
	E solution	:	7.50 ml
	T solution	:	15.0 ml
	Distilled water	:	3.75 m
b.	Stocking gel pH 96.8)		
	O solution	:	1.25 ml
	P solution	:	2.5 ml
	F solution	:	1.25 ml
	Distilled water	:	5.0 ml

5. Tray / Electrode buffer

Stock buffer

i. β-alanine - 3.12 g

ii. Acetic acid - 0.8ml

iii. Dissolved in distilled water and pH was adjusted to 4.5. Final volume was made to 1 litre.

6. Staining solution, destaining solution, fixing solution standard protein molecular weight marker etc. were used as described under acidic PRs.

Method

Extraction of basic PRs

The basic PRs were extracted as that described under extraction of acidic PRs except the extraction buffer. For extraction of basic PRs citrate buffer was used.

Electrophoresis

The basic PRs were resolved by carrying out the separation gel, stacking gel and electrode buffer solution as described under reagents. Rest of the reagents and protocol was followed as described under electrophoresis of acidic PRs.

3.2.4.3 Total phenolics

Total phenolics contents was estimated by using Folin-Denis reagent as described by AOAC (1990)

Reagents

1. Folin-Denis reagent

   Extractly 100g of sodium tungstate, 20g of phosphomolybdic acid and 50ml of concentrated phosphoric acid were added to 750ml distilled water. The mixture was allowed to reflux for 2 hr on hot water bath and volume was made to 1000 ml with distilled water.

2. Alkaline reagent

   Sodium carbonate of 350g was dissolved in 1000 ml distilled water at 80 ^{o}C for 30 min and allowed to stand for over night at room temperature and filtered through glass wool .

3. Standard stock solution of tannic acid

   Accurately 100 mg of tannic acid was dissolved in distilled water and volume was made to 100 ml.

4. Working standard solution of tannic acid

   Ten ml of stock solution was pipetted in 100 ml volumetric flask and volume was made to 100ml with distilled water, which contained 100µg Of tannic acid per ml.

Extraction of phenols

One gram of fresh sample was cut in to small pieces, macerated and boiled in 25 ml distilled water for 30 min. The contents were then filtered and volume was made to 25 ml. From this, 1 ml filtrate was used for colour development.

Colour development

One ml extract was pipetted in test tube, 0.5 ml of Follin-Dennis reagent and 1ml of alkaline reagent was added. The content was diluted to 10ml with distilled water, mixed on cyclomixer and kept at room temperature for 30 min for colour development. After 30 min optical density was read at 740 nm on spectronic–20.

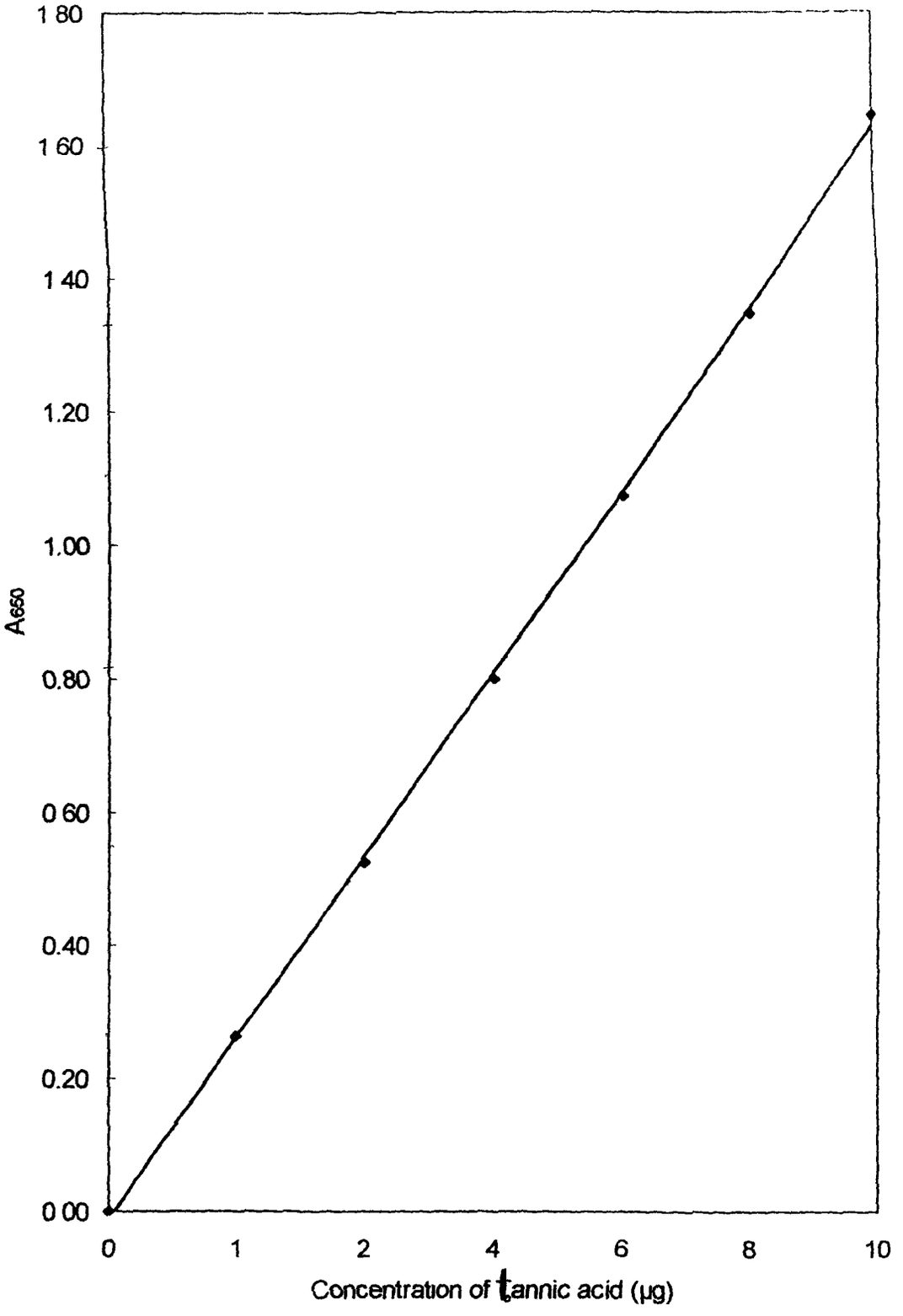
Calibration of standard curve

The working standard solution of tannic acid 0, 0.1, 0.2----- 1 ml was pipetted into a series of test-tube in triplicate and volume was made 1ml with distilled water. The colour was developed as described above and standard curve was plotted on graph paper, plotting absorbance versus concentration of tannic acid.

The total phenols were calculated from the standard graph and results were expressed as mg/ 100g fresh weight of sample.

3.2.4.4 Chlorogenic acid

The ground defatted 2g sample was boiled with 10ml water for 10 min. The boiled contain were cooled. filtered through Whatman No.1 filter paper and volume was made to 10ml. The chlorogenic acid in 5ml of this filtrate was determined by adsorbtion technique and described by Zucker and Aherns (1958) with slight modification.



Standard graph for estimation of total phenolics

The extract (5 ml) was mixed with 10g activated alumina gel for 10min on a mini column. The white gel turned yellow after adsorbtion of chlorogenic acid on gel. The gel was washed with 4ml of freshly mixed solution (1:1) of 0.5% sodium nitrate and 0.5% acetic acid .The yellow gel turned to orange. After this washing the absorbed chlorogenic acid was eluted from the gel by washing the gel column with 4ml of 5N NaOH as bright red solution. The red colour solution of chlorogenic acid was collected, filtered and taken into a cuvette, the absorbance was read at 520nm on spectronic-20. The contents of chlorogenic acid were then calculated from the standard curve of chlorogenic acid.

3.2.4.5 Soluble Proteins

Soluble proteins in the plant parts were determined by the colourimetric method described by Lowry *et al.* (1951) using bovine serum albumin as standard protein.

Reagents

1. Reagent A (Alkaline sodium carbonate)

   Two grams of sodium carbonate were dissolved in 0.1M sodium hydroxide and volume was made to 100ml with 0.1M NaOH

2. Reagent B (Copper sulphate- sodium potassium tartarate solution ,0.5%)

   Five hundred milligrams of $CuSo_4$, $5H_2O$ were dissolved in 1% (w/v) sodium potassium tartarate solution and volume was made to 100ml with 1%Na-K tartarate solution.

3. Alkaline copper tartarate reagent

   Fresh alkaline copper tartarate reagent was prepared just before use by mixing 50ml of reagent 'A' with 1ml of reagent 'B'.

4. Folin-ciocalteau(phenol) reagent

   It was prepared diluting one part of commercial grade reagent with one part of distilled water on the day of use.

5. Stock solution of bovine serum albumin (BSA)

   The BSA was weighed 100 mg accurately and dissolved in distilled water and volume was made to 100ml with distilled water

6. Working standard solution of BSA

   Ten ml of stock solution was pipette in 100 ml of volumetric flask and volume was made with distilled water. The concentration of this solution was 100µg of BSA per ml.

Extraction of soluble proteins

One gram of finely powder sample was weighed and extracted in 20 ml distilled water on mechanical shaker for 60min. It was then centrifuged at 10000 Xg for 30 min. and supernatant was used for the estimation of soluble proteins.

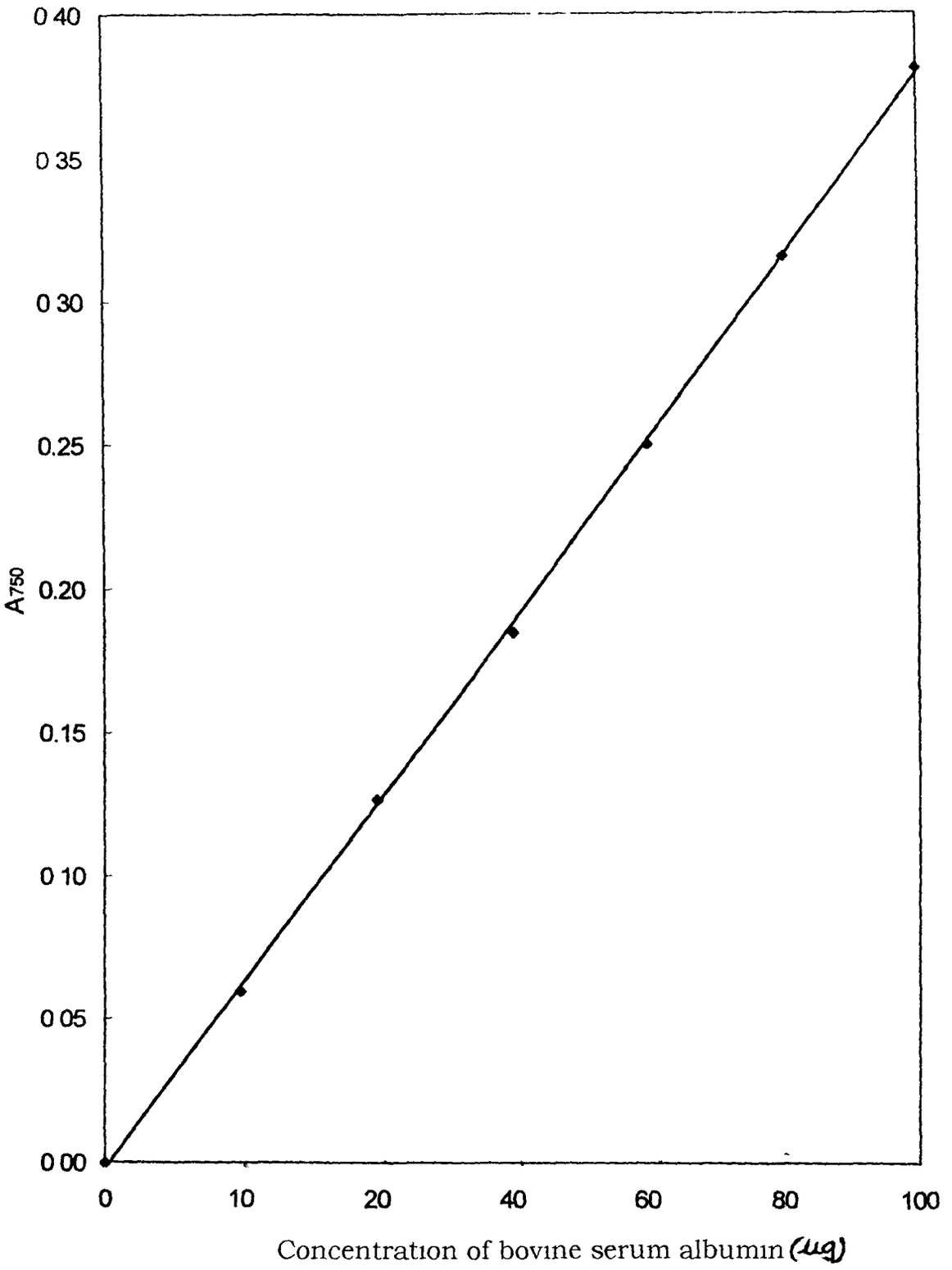
Colour development

One ml supernatant was pipetted in the test-tube mixed with 5ml of alkaline copper reagent and kept for 10 minutes at room temperature. To this, 0.5ml of diluted Folin ciocalteau reagent was rapidly with immediate mixing. The intensity of blue colour was measured after 30min at 660nm on Spectronic-20.

Calibration of standard curve

The 0, 0.1, 0.2, 0.3------1ml (i e 10, 20, 30-------100µg) of the working standard solution of BSA was pipetted in the series of test tube. The volume was made 1ml with distilled water and colour was developed as above. The absorbance was read at 750nm and standard curve was prepared by plotting absorbance against concentration of standard protein.

The soluble protein was calculated from standard graph and result was expressed as mg of soluble protein per mg dry weight of sample.



Standard graph for estimation of soluble proteins

RESULTS AND DISCUSSION

4. RESULT AND DISCUSSION

In several plant species, upon infection with viruses, viroid, fungi or bacteria the development of symptom is accompanied by the appearance of one or more new novel proteins. Occurrence of these new novel proteins is not pathogen specific, but are determined by the type of reaction of the host plant indicating that these proteins are of host origin. The defense proteins are drawing much attention of the plant breeders because defense related proteins usually provide a plant with resistance to stresses and the varieties that are apt to intensively induce such proteins are agriculturally valuable. PRs accumulate abundantly at the site of infection, but some also accumulate in the uninoculated part of an infected plant, but to a lesser degree in uninoculated parts. Although excellent plant sources are available in chickpea germplasm, success in breeding for resistance is limited, mainly due to region-specific races of *Fusarium oxysporum* f. sp. *ciceri* and gradual breakdown of resistance in chickpea cultivars. Till todate, 17 classes of PRs are characterised, identified and classified in different plant species. However, the characterization of PRs associated with *Fusarium* wilt resistance in chickpea is scanty. Therefore, the present investigation is an attempt to identify and characterize the PRs involved in *Fusarium* wilt resistance in chickpea.

4.1 Acidic PRs in the leaves of chickpea seedlings separated by native-PAGE

The acidic pathogenesis related protein were separated from the leaf portions of three resistant and three susceptible cultivars of chickpea by native polyacrylamide gel electrophoresis (native-PAGE) (Figure-1). It was observed that a total of 12 bands were synthesized in

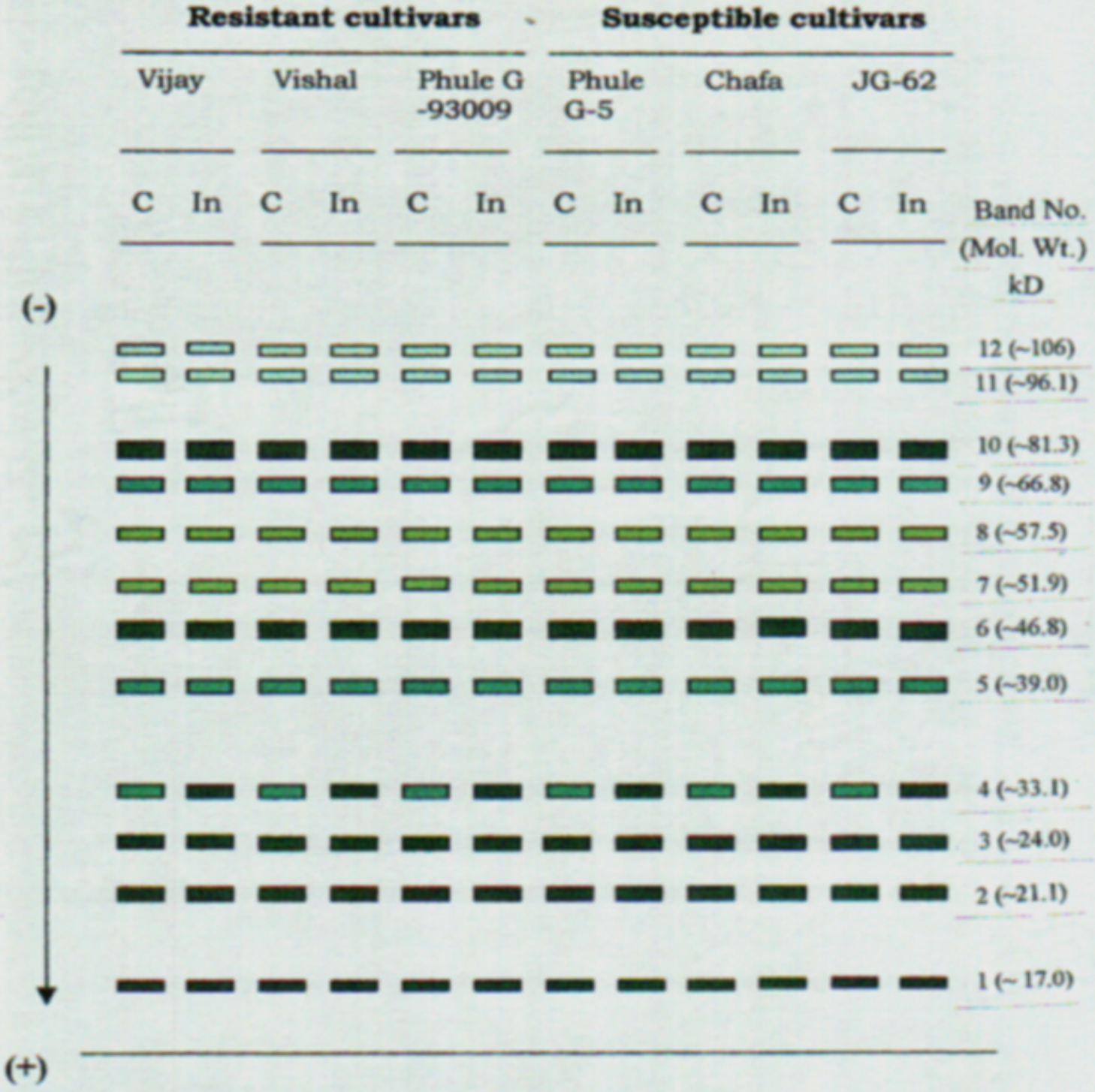


Fig.1 Acidic PRs in the leaves of chickpea seedlings of resistant and susceptible cultivars separated by native-PAGE.

each of the *Fusarium* wilt resistant and susceptible cultivars of chickpea under control and infected conditions. The type and intensity of these 12 bands *viz.*, 1, 2, 3, 5, 6, 7, 8, 9, 10, 11 and 12 in wilt resistant and susceptible cultivars under control and infected condition showed the similar intensity banding pattern. However, band number 4 which appeared in both resistant and susceptible cultivars under control and infected condition differed in respect of intensity of the banding pattern In control ~~condition~~ it appeared as dark band in all the cultivars, however, under infected condition it appeared as an intense one.

4.2 Acidic PRs in the stems of chickpea seedlings separated by native-PAGE

The acidic PRs synthesized in the stem portions of chickpea seedlings under control and infected condition (with *Fusarium oxysporum*) of three resistant and three susceptible cultivars is depicted in Figure 2. It was observed that only in resistant cultivars, 'Vijay' and 'Vishal' additional PR band of ~ 94 kD, (band number 7) was synthesized under infected condition which was absent under control condition of these cultivars. It is seen that band number 2 (~ 25.7 kD) appeared in resistant cultivars, 'Vijay' and 'Vishal' which was interestingly also observed in one of the susceptible cultivars, 'JG-62'. However, it appeared as an intense one in 'Vijay', 'Vishal' and in 'JG-62'. The intensity of band number 3, 4 , 5 and 6 increased under infected condition than under control condition. Similar intensity banding pattern was observed in respect of band numbers 1 and 8 in all the cultivars under control and infected conditions.

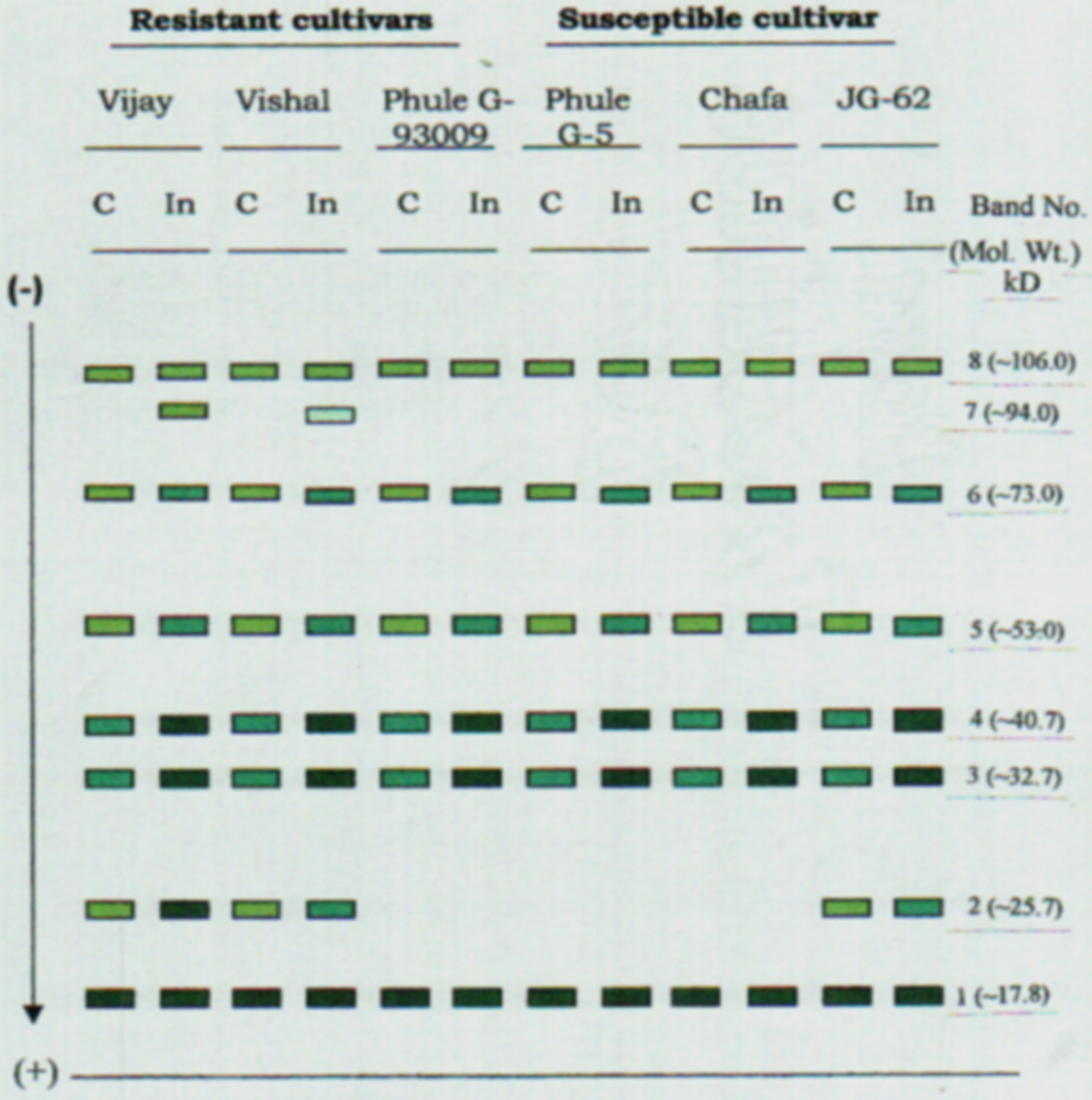


Fig 2. Acidic PRs in the stems of chickpea seedlings of resistant and susceptible cultivars separated by Native-PAGE.

4.3 Acidic PRs in the roots of chickpea seedlings separated by native-PAGE

The acidic PRs synthesized in the root portions of chickpea seedlings (inoculated with *Fusarium oxysporum*) of three resistant and three susceptible cultivars are shown in Figure 3. It is observed that in resistant cultivar, 'Phule G-93009' additional bands having molecular weights of ~ 21 kD (band number 2), ~ 29kD (band number 5) and ~ 72 kD (band number 9) were synthesized under control condition. The disappearance of these PR bands was noticed under the infected condition. The PR of ~ 25.7 kD (band number 3) was synthesized under infected condition only in the resistant cultivar, 'Vijay'. Interestingly, band number 4 of ~ 24 kD was observed only in a susceptible cultivar, 'JG-62' under infected condition which was absent under control condition and was also absent in all the cultivars under infected as well as control condition. The intensity of band numbers 6, 7 and 8 of ~ 33.5, 47.3, 64.0 kD were faint dark under control condition and dark under the infected condition in the resistant cultivars, 'Vijay' and 'Vishal'. However, in 'Phule G-93009' identified as resistant cultivar by the breeders, the reverse intensity banding pattern was observed than that of resistant cultivars, 'Vijay' and 'Vishal'. In this cultivar, band numbers 6, 7 and 8 appeared as intense and dark under control condition and faint dark under the infected condition. The susceptible cultivars did not show a particular intensity banding pattern. As regards the band number 11, it appeared as a faint band in all the cultivars under control and infected conditions. The band number 10 showed similar intensity banding pattern under control condition to the corresponding infected condition in each of the cultivar except 'JG-62', susceptible cultivar.

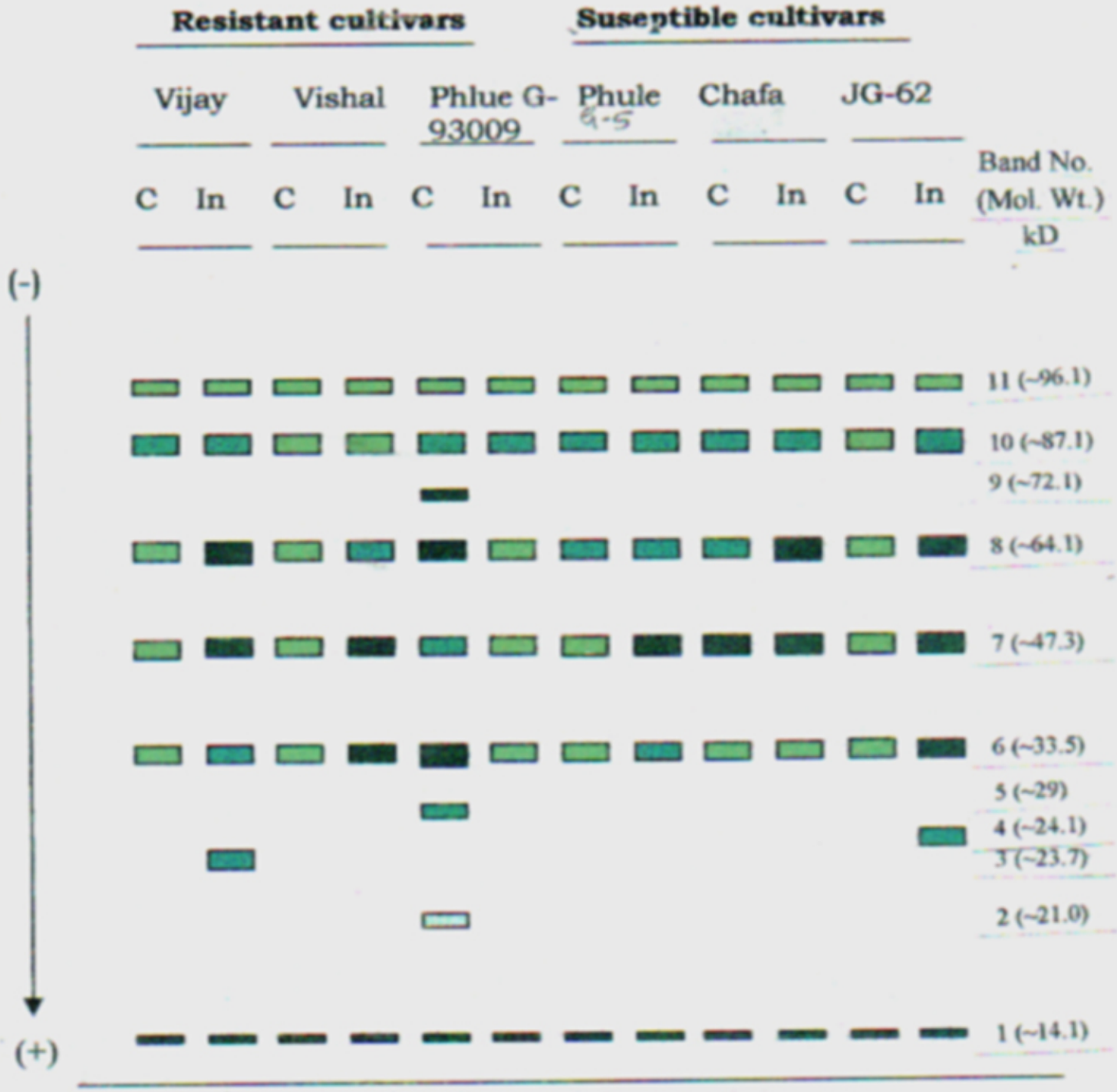


Fig 3. Acidic PRs in the roots of chickpea seedlings of resistant and susceptible cultivars separated by native-PAGE.

Faint Faint dark Dark Intense

C- Control In- Infected with *F. oxysporum* f. sp. *ciceri*

4.4 Acidic PRs in the leaves of chickpea seedlings separated by SDS-PAGE

The acidic PRs were separated from leaf portions of three resistant and three susceptible cultivars of chickpea by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 4). It was observed that a total of 9 bands appeared in each of the *Fusarium* wilt resistant and susceptible cultivars of chickpea under control and infected conditions. Similar intensity banding pattern was observed in respect of band numbers 1, 2, 6, 7 and 9 under control and infected conditions of wilt resistant and susceptible cultivars. In all the resistant cultivars, band number 3 appeared as an intense band under infected condition and a dark band under the control condition. In resistant cultivar, 'Vijay' and susceptible cultivars, 'Chafa' and 'JG-62', band number 4 and 5 were intense under infected condition than the control condition. However, no such intensity differences were found with band number 4 and 5 in resistant 'Vishal' and 'Phule G-93009' cultivars and susceptible 'Phule G-5' cultivar.

4.5 Acidic PRs in the stems of chickpea seedlings separated by SDS-PAGE

The acidic PRs synthesized in the stem portions of chickpea seedlings under control and infected conditions separated by SDS-PAGE of three resistant and three susceptible cultivars is shown in Figure 5. It is observed that in resistant cultivar 'Vishal', an additional band number 4 of ~ 32 kD appeared only under infected condition. Interestingly, it was observed that band number 2 of ~ 25.7 kD was synthesized in a susceptible cultivar, 'JG-62' under infected condition which was absent in control condition and was also absent in other cultivars under both control and infected conditions. A PR band of ~ 47 kD (band number 6) was newly synthesized under infected condition in resistant cultivars,

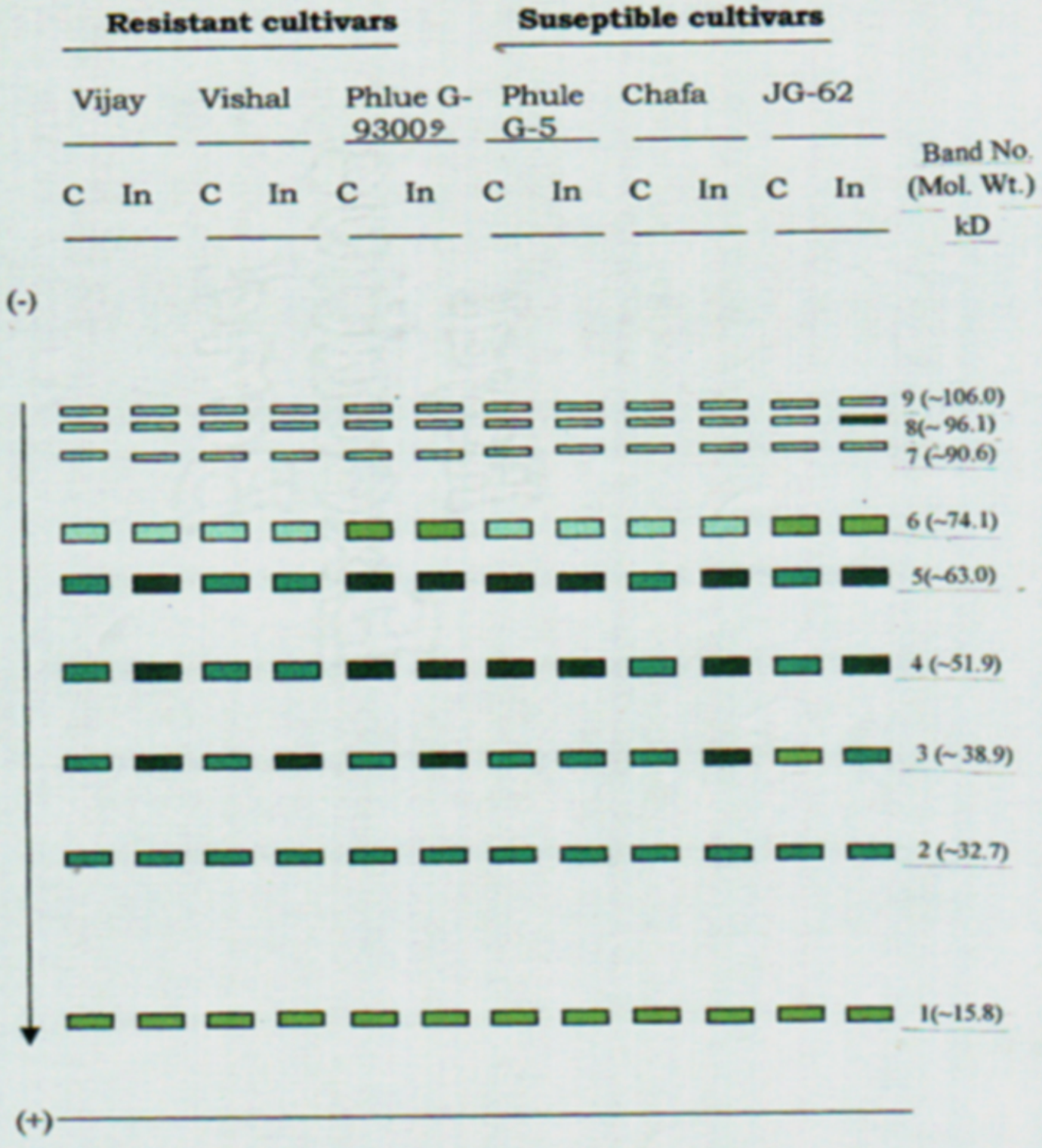


Fig.4 Acidic PRs in the leaves of chickpea seedlings of resistant and susceptible cultivars separated by SDS-PAGE.

Faint Faint dark Dark Intense

C- Control In- Infected with *F. oxysporum* f. sp. *ciceri*

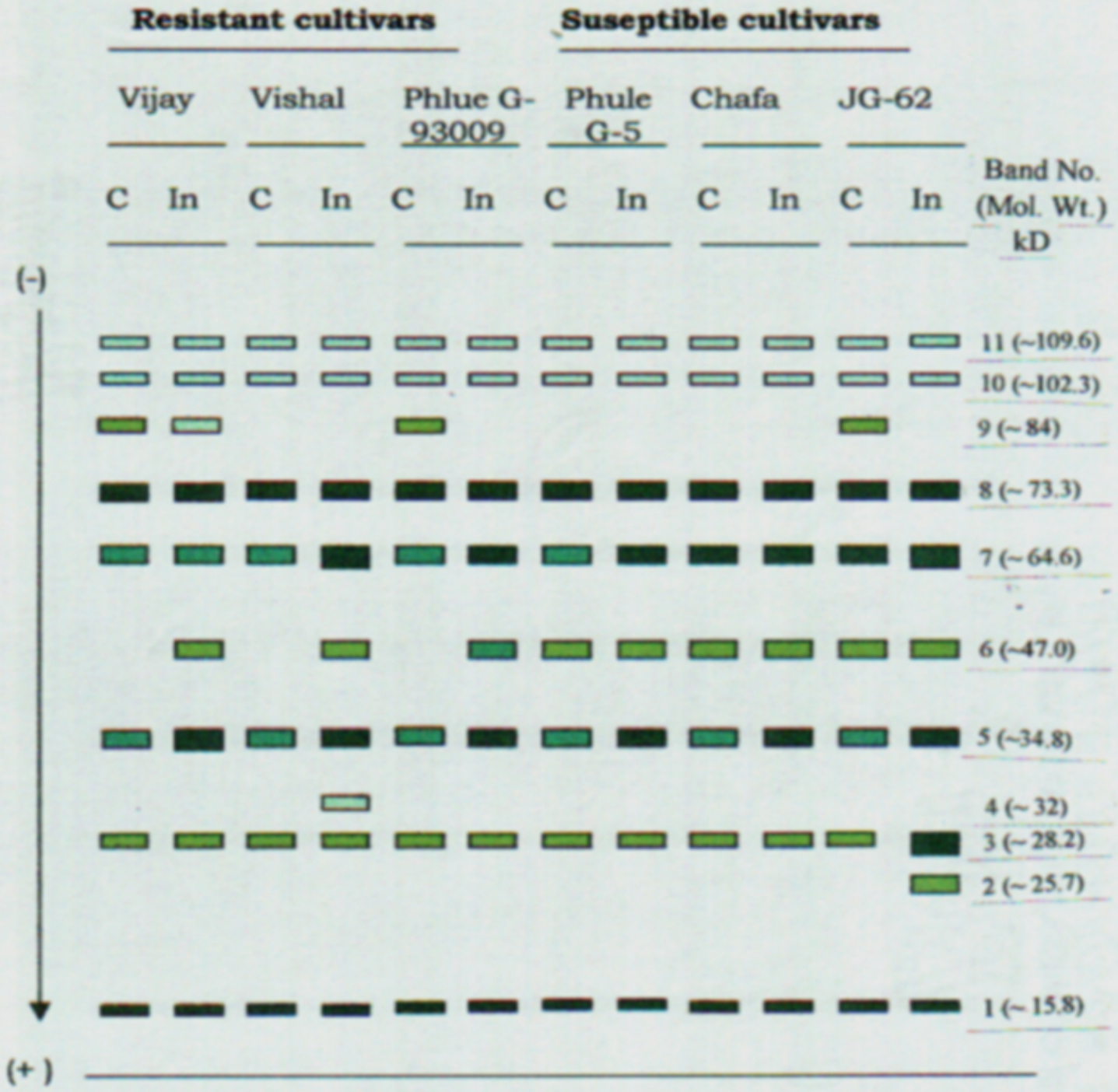


Fig.5 Acidic PRs in the stems of chickpea seedlings of resistant and susceptible cultivars separated by SDS-PAGE.

Faint Faint dark Dark Intense

C - Control In- Infected with *F. oxysporum* f. sp. *ciceri*

'Vijay', 'Vishal' and 'Phule G-93009'. However in susceptible cultivars, 'Phule G-5', 'Chafa' and 'JG-62' under both control and infected condition the band number 6 was clearly visible. The intensity of band number 7 was increased under infected condition in resistant cultivars, 'Vishal', 'Phule G-93009' and in susceptible cultivar 'Phule G-5'. However in a resistant cultivar 'Vishal', an increase in intensity alongwith its size was also noticed. In other cultivars the intensity of band number 7 was similar under control and infected condition.

As regard the band numbers 1, 8, 10 and 11 the similar intensity banding pattern was observed under control and infected conditions in all the cultivars. The PR band of ~ 84 kD (band number 9) was synthesized in a resistant cultivar 'Vijay' under both control and infected conditions. However, the intensity of band decreased under infected condition over the control. The band number 9 was also found in a resistant cultivar, 'Phule G-93009' and in susceptible cultivar 'JG-62' under control condition however this disappeared upon infection.

4.6 Acidic PRs in the roots of chickpea seedlings separated by SDS-PAGE

The acidic PRs synthesized in the root portions of chickpea seedlings of three resistant and three susceptible cultivars separated by SDS-PAGE is depicted in Figure-6. It is observed that under infected condition synthesis of new PR bands of ~ 23 kD (band number 4), ~ 33.5 kD (band number 6) and ~ 66 kD (band number 8) were noticed in a resistant cultivar, 'Vijay'. The PR band number 8 was also observed in a resistant cultivar, 'Vishal' under infected condition. The appearance of band numbers 1 and 5 with similar intensity was observed in all the cultivars. The appearance of band number 2 was observed in all resistant cultivars, 'Vijay', 'Vishal' and 'Phule G-93009' and also one of the susceptible cultivars, 'Phule G-5' under both control and infected

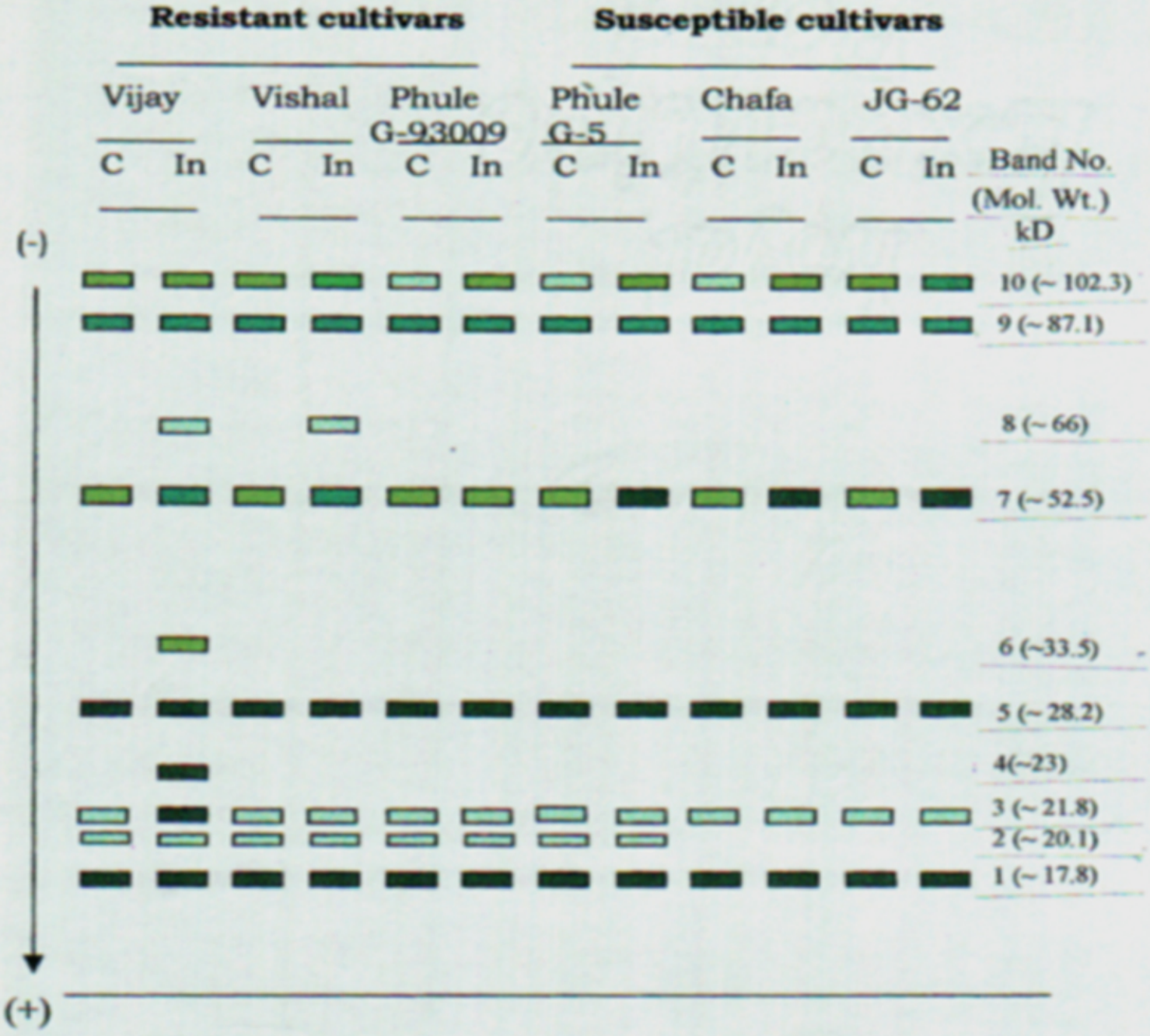


Fig.6 Acidic PRs in the roots of chickpea seedlings of resistant and susceptible cultivars separated by SDS-PAGE.

condition. The intensity of band 3 increased only in a resistant cultivar, 'Vijay' under infected than the control condition. However in other cultivars, the intensity of this band was similar under both control and infected conditions. The intensity of band number 7 increased under infected condition over the control in all the cultivars except the resistant 'Phule G-93009' cultivar. The intensity of band number 9 was same in all cultivars except 'JG-62', a susceptible cultivar. In this cultivar the intensity of band number 9 increased under infected condition. The intensity of band number 10 also increased in all the cultivars except resistant 'Vijay' cultivar upon infection.

4.7 Basic PRs in the leaves of chickpea seedlings separated by native-PAGE

The basic PRs synthesized in the leaves of chickpea seedlings of three resistant and three susceptible cultivars separated by native-PAGE is depicted in Figure 7. A total of 7 bands appeared in the leaves of all the cultivars. Similar banding pattern and band intensity observed in respect of band numbers 4, 6 and 7 in wilt resistant and wilt susceptible cultivars under both control and infected conditions.

With regard the band number 1, the intensity of band increased in a resistant cultivar, 'Vishal' and in susceptible cultivar, 'JG-62' under the infected condition than the control condition. However, rest of the cultivars showed similar banding pattern under control and infected conditions. Under the infected condition, the intensity of band number 2 increased in all the resistant cultivars except 'Phule G-93009'. The intensity of band number 3 increased in the resistant cultivar, 'Vishal' under infected condition than the control condition. However in susceptible cultivar 'Phule G-5', the intensity of band number 3 decreased under infected condition than control condition. The intensity of band number 5 appeared dark in resistant cultivars, while it

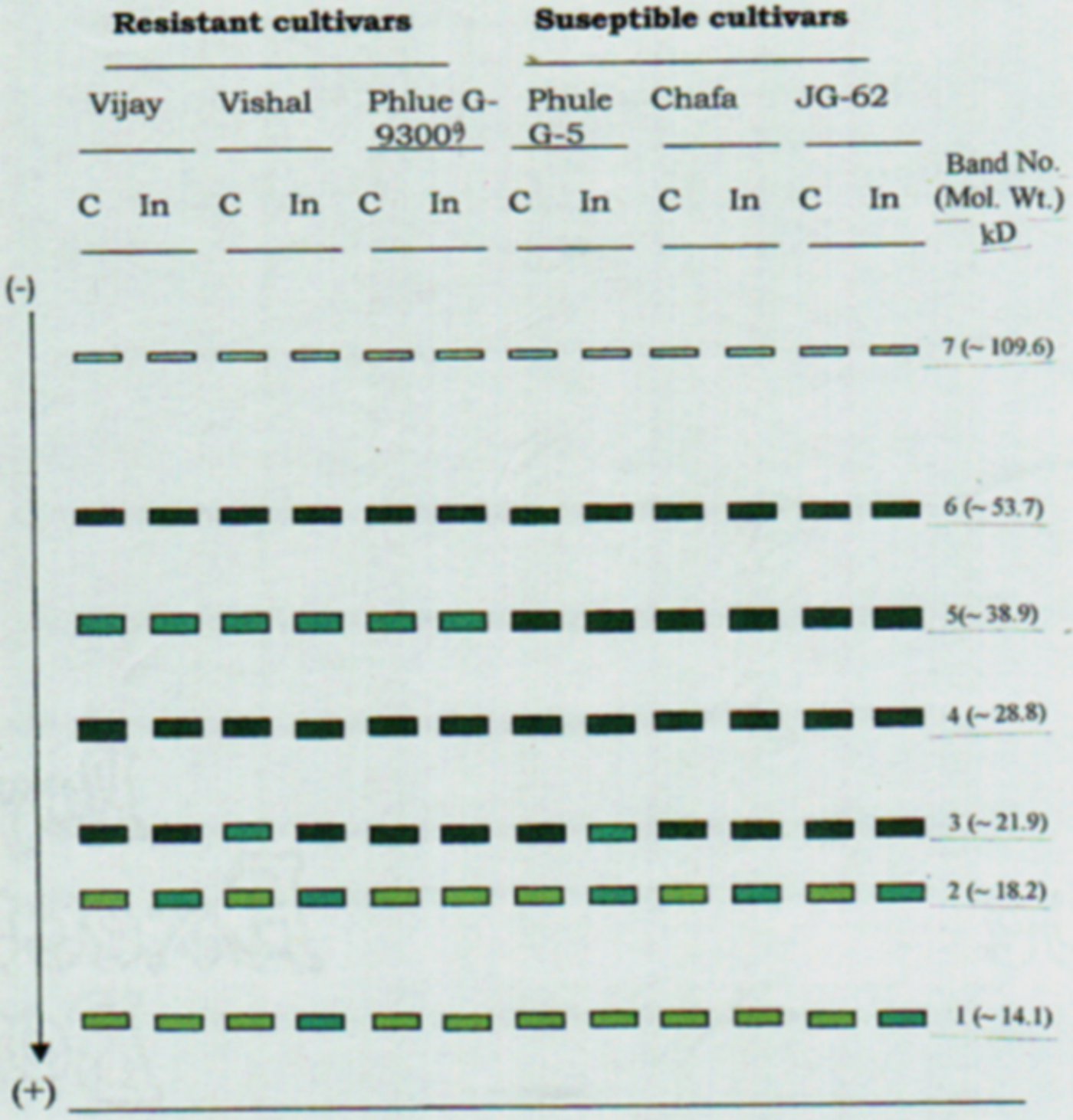


Fig.7 Basic PRs in the leaves of chickpea seedlings of resistant and susceptible cultivars separated by native-PAGE.

was intense one in susceptible cultivars under control and infected condition.

4.8 Basic PRs in the stems of chickpea seedlings separated by native-PAGE

The basic PRs synthesized in stem portions of chickpea seedlings separated by native polyacrylamide gel electrophoresis are depicted in Figure 8. It was observed that band numbers 1, 5 and 8 showed similar intensity banding patterns in all the cultivars, however, band number 5 and 8 appeared dark and intense respectively in susceptible 'JG-62' cultivar under infected condition. Under infected condition, the intensity of band number 7 increased over control condition in all the cultivars. The band number 2 appeared only in 'Vijay' and 'Chafa' under control and infected condition and in 'JG-62' under infected condition only. In resistant cultivar, 'Vijay' the intensity of band number 2 decreased under infected condition over the control condition. The intensity of band number 5 appeared similar in all cultivars under control and infected condition, however in susceptible 'JG-62' cultivar intensity was found to be increased.

4.9 Basic PRs in the roots of chickpea seedlings separated by native-PAGE

The basic PRs synthesized in root portions of chickpea seedlings are shown in Figure 9. It was observed that band numbers 1, 2, 5, 7, 8 and 9 showed similar banding pattern and band intensity under infected and control conditions in all the cultivars. However, in susceptible cultivar, 'Chafa' PR band number 2 was not synthesized under control condition. PR band number 4 and 6 appeared more intense under infected condition than the control condition in all the

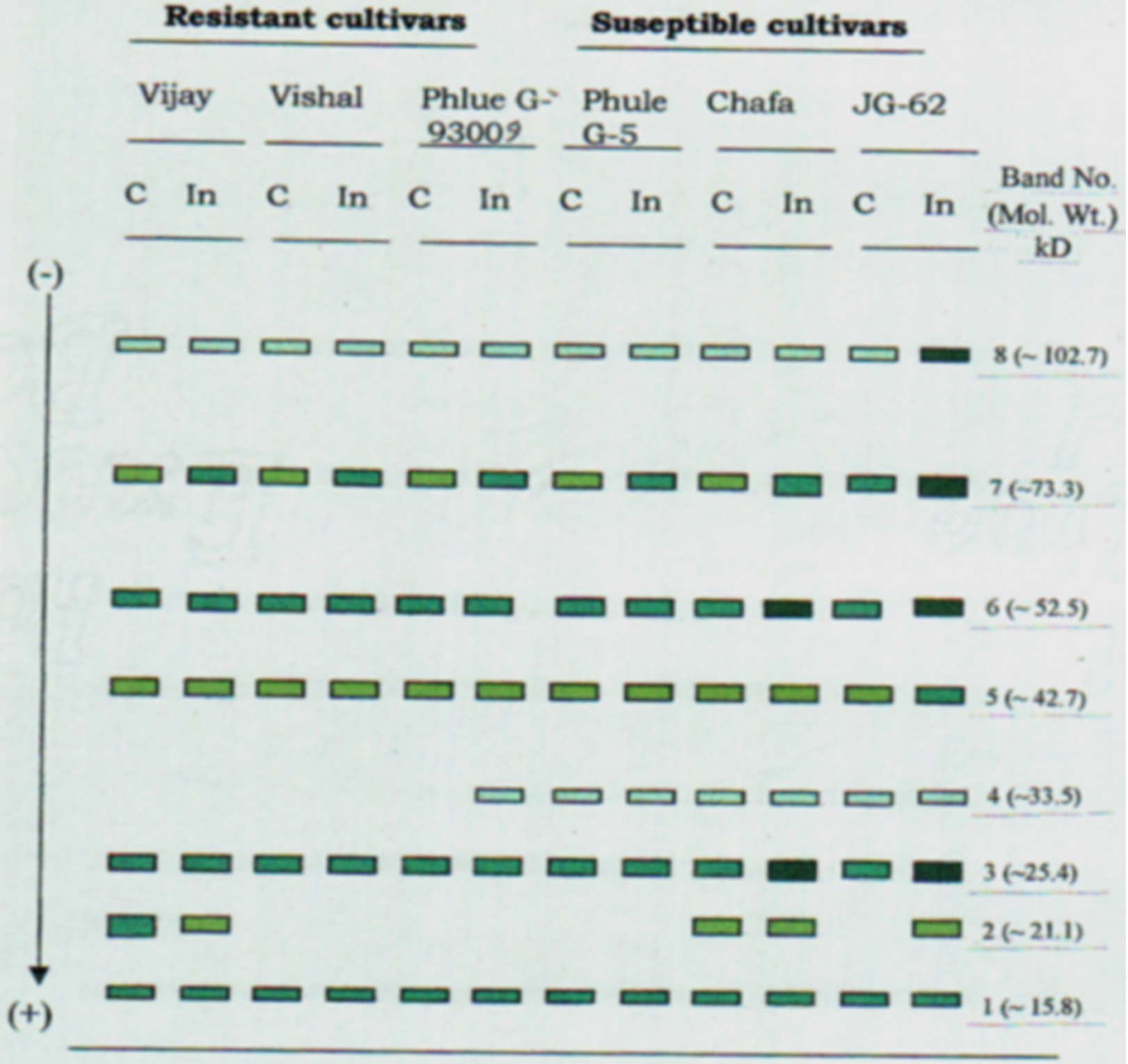


Fig.8 Basic PRs in the stems of chickpea seedlings of resistant and susceptible cultivars separated by native-PAGE.

Faint Faint dark Dark Intense

C - Control In - Infected with *F. oxysporum* f. sp. *ciceri*

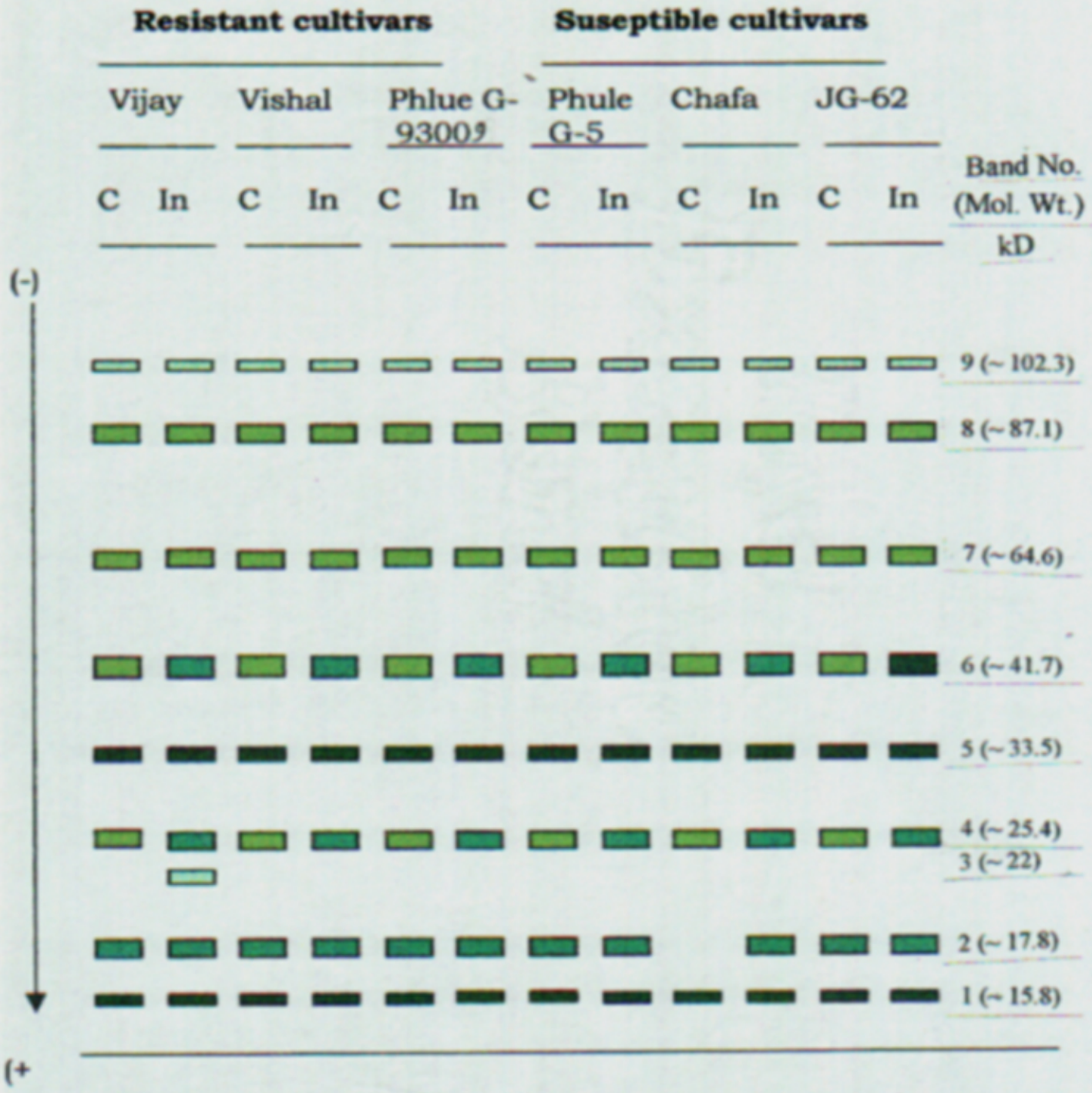


Fig.9 Basic PRs in the roots of chickpea seedlings of resistant and susceptible cultivars separated by native-PAGE.

cultivars. PR band of ~ 22 kD (band number 3) was synthesized only in 'Vijay' under infected condition.

It is thus noticed from these studies that the acidic and basic PRs separated from the leaves under control and infected conditions did not show the marked differences in banding patterns. In the stem portions acidic proteins of ~ 25.7 and 94.0 kD (bands 2 and 7, respectively, Figure 2) were synthesized only in the resistant cultivars, 'Vijay' and 'Vishal'. However, when these proteins were separated by SDS-PAGE, it was found that PRs of ~ 32.0 kD (band 4, Figure 5) were synthesized in resistant cultivar 'Vishal'. In root portions the acidic protein of ~ 25.7 kD (band 3, Figure 3) synthesized in the resistant 'Vijay' cultivar, was separated by native-PAGE. However, when these proteins separated by the SDS-PAGE, the PRs of ~ 23 kD, 33.5 kD and 66 kD (band 4, 6 and 8 respectively, Figure 6) were synthesized in the resistant cultivar, 'Vijay'. The PR band of ~ 66 kD was also synthesized in the roots of resistant cultivar, 'Vishal' (band 8, Figure 5). The basic PR band of ~ 22 kD was synthesized in the root portion of resistant cultivar, 'Vijay' (band 3, Figure 9).

Thus these novel proteins synthesized in the resistant cultivar may be associated with the defense response mechanism against the *Fusarium oxysporum* f.sp. *cicer* fungus. The cultivar, 'Vijay' appeared more tolerant followed by 'Vishal' against wilt disease whereas 'Phule G-93009' appeared to be least tolerant. Further, it was observed that most of the novel acidic and basic PRs were synthesized in the root portion followed by stem portion of the plants. This may be because of the fungus *Fusarium oxysporum* penetrating through the root system of the plant and therefore the plants may require immediate defense response reaction in this root portions. Then by signal transduction mechanism plants may synthesize PRs in stem portions of resistant

cultivars (Figure 10).This mechanism is confirmed in the present investigation by synthesizing the new novel proteins of ~ 32 kD (band 4, Figure 5) by the resistant cultivar 'Vishal'. Further, the intensity of PR bands were increased over the control conditions in leaf and stem portions because of the synthesis of higher quantity of PR proteins due to signal transduction mechanism. However, novel PRs were not synthesized in the leaves. Earlier, Bol *et al.* (1990) reported that PRs accumulate abundantly at the site of infection, but some may accumulate in the uninoculated parts of an infected plant, but to a lesser degree than in inoculated parts. Thus the results obtained in the present investigation are in agreement with the results reported by Bol *et al.* (1990 In the present study the synthesis of PRs in the root and stem portions of the resistant cultivar may limit the multiplication and /or spread of invading pathogen. PRs would thus be responsible for the resistance so called systemic acquired resistance (Van Loon, 1985). Giri *et al.* (2000) identified the ~ 70, 42, 34, 7 and 6 kD PRs in the root portions of resistant cultivars of chickpea upon *Fusarium oxysporum* infection. However in maize, Cordero *et al.* (1992) reported the induction of two acid soluble proteins of molecular weight of ~ 23 and 24 kD in seedlings infected with *Fusarium moniforme.* Thimmaiah and Ashoka (1999) indicated that the inoculated seedlings with the blast fungus (*Pyricularia oryzae*) induced the synthesis and expression of new acidic and basic PRs of ~ 14 to 20 kD. In the present investigation, PRs of ~ 66, 52.4, 33.5 and 23 kD were synthesized in the roots of resistant cultivars. The disappearance and appearance of newly synthesized acidic PRs in both the *Fusarium* wilt resistant and susceptible cultivars due to inoculation was observed. However, the resistant cultivars synthesized maximum number of both acidic and basic PRs as compared to the susceptible ones in response to pathogen attack. This may be ascribed to the resistant nature of 'Vijay' and 'Vishal' over the susceptible 'Phule

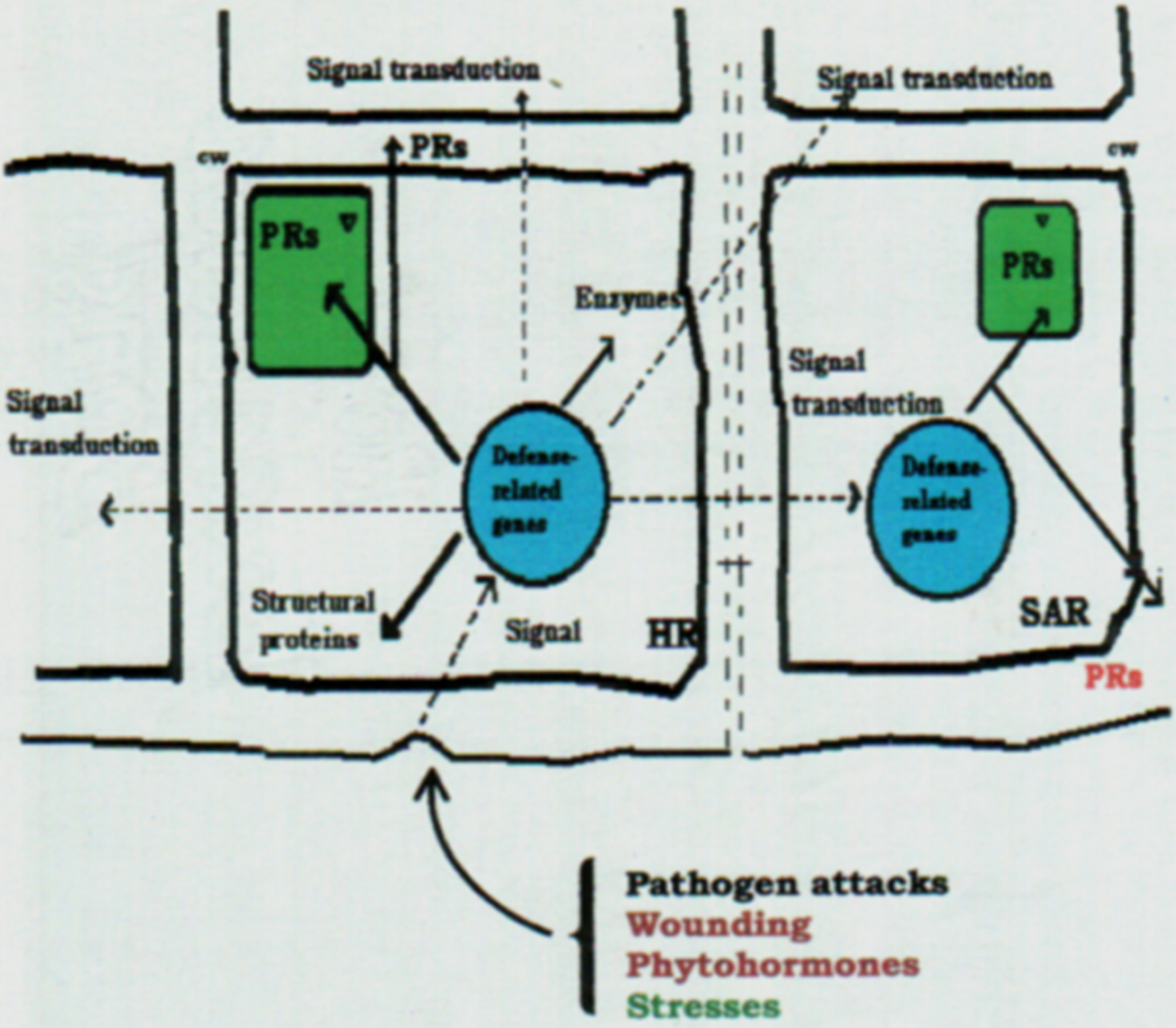


Fig.10 Signal transduction mechanism in relation to plant defense response

PRs: Pathogenesis-related proteins
HR: Hypersensitive response
SAR: Systemic acquired resistance
cw: Cell wall

G-5', 'Chafa' and 'JG-62'. Thimmaiah and Ashoka (1999) also reported the disappearance and appearance of newly synthesized acidic and basic PRs in both the blast resistant and susceptible rice varieties due to inoculation. Further, they noticed that resistant variety synthesized maximum number of both acidic and basic PRs as compared to susceptible ones in response to pathogen attack. The results obtained in the present investigation are concomitant with the results reported by Thimmaiah and Ashoka (1999). Earlier, Van Loon *et al.* (1985) reported that the production of PRs increases the resistance of plant to viruses. They further reported that the role of PRs *viz.*, chitinases or β-1, 3-glucanases can restrict the growth β-1, 3-glucans or chitin in their cell walls.

It was noticed that the intensity of ~ 25 kD PR band in stem was increased upon infection over the control in resistant cultivar 'Vijay' and 'Vishal' with the exception of 'JG-62' susceptible cultivar (band number 2, Figure 2). This suggests that the PRs synthesized in the susceptible cultivar may not have proper conformation or amino acid sequencing as that of protein synthesized in the resistant cultivar. It is therefore projected to assess the functional properties and amino acid sequencing of this PR.

4.10 Total phenolics and chlorogenic acid contents in the leaves, stems and roots of susceptible and resistant cultivars

The contents of total phenolics and chlorogenic acids ir leaves, stems and roots of resistant and susceptible cutivars grown in sterilized and wilt sick soil are presented in Table 2 and 3. Among the plant parts analysed, roots had higher total phenolics than leaves and stems of both resistant and susceptible cultivars. However the leaves, stems and roots of resistant chickpea cultivars 'Vijay', 'Vishal' and

Table 2. Total phenolics content (mg 100^{-g} fr. wt.)in leaves, stems and roots of susceptible and resistant cultivars of chickpea

Culti-vars	Plant parts								
	Leaves			Stems			Roots		
	Plants grown in		% variati-on over healthy stage	Plants grown ın		% variati-on over healthy stage	Plants grown in		% variati-on over healthy stage
	Sterilız-ed soil	Wilt-sick soıls		Sterılı-zed soıl	Wılt-sick soıls		Steril-ized soil	Wilt-sick soils	
ɛesistant									
ïjay	69.38	83.75	+20.72	63.01	76.99	+22.18	89.75	103.7	+15.63
ısha1	71.00	83.63	+17.78	64.18	75.83	+18.17	91.37	104.2	+14.12
hule 3009	67.75	79.75	+17.71	61.19	73.02	+19.33	88.25	100.8	+13.78
usceptible									
hule -5	57.37	60.63	+ 5.61	39.63	42.36	+ 6.91	65.12	67.96	+ 4.36
hafa	53.62	55.65	+ 3.97	38.50	40.38	+ 4.87	64.25	65.59	+ 4.17
G-62	57.88	61.13	+ 5.61	41.23	43.83	+ 6.58	66.50	69.35	+ 4.29
	S.E.	CD at 5%		S.E.	CD at 5%		S.E.	CD at 5%	
	1.5681	4.8344		1.3658	4.2109		1.2583	3.8793	

Table 3. Chlorogenic acid content (μg g^{-1} fr. wt.)in leaves, stems and roots of susceptible and resistant cultivars of chickpea

Cultivars	Plant parts								
	Leaves			Stems			Roots		
	Plants grown in		% variation over healthy stage	Plants grown in		% variation over healthy stage	Plants grown in		% variation over healthy stage
	Sterilized soil	Wilt-sick soils		Sterilized soil	Wilt-sick soils		Sterilized soil	Wilt-sick soils	
Resistant									
Vijay	309	389	+25.9	304	371	+22.0	336	437	+30.1
Vishal	301	388	+28.9	319	383	+20.1	317	401	+26.5
Phule G 93009	294	379	+28.9	309	373	+20.7	301	397	+31.3
Susceptible									
Phule G-5	240	261	+8.8	204	213	+4.4	197	210	+6.07
Chafa	218	234	+7.3	219	229	+4.6	214	227	+6.59
JG-62	213	224	+5.2	196	200	+2.0	186	204	+4.3
	S.E.	CD at 5%		S.E.	CD at 5%		S.E.	CD at 5%	
	1.2254	3.7779		1.6325	5.0330		1.4258	4.3957	

'Phule-G-93009' had significantly higher total phenols and chlorogenic acid than the susceptible cultivar 'JG-62', 'Phule G-5' and 'Chafa'. The total phenolics and chlorogenic acid contents increased during pathogenesis in both the cultivars and the increase was more pronounced in resistant cultivar than the susceptible one.

Results obtained in the present investigation are in good agreement with the earlier findings (Gupta and Kotasthane, 1984; Gupta and Khare, 1992; Stevenson *et al*, 1994; Mandavia *et al.*, 1997, 1999 and Patil, 2000). The higher concentration of phenols found in resistant cultivars may be responsible for the formation of stable complexes with proteins including enzymes and decreases the metabolic functions of infecting fungi (Salunkhe *et al.*, 1989). The reactive phenolics in wilt sick soil conditions were more pronounced in the resistant cultivar, which can be attributed to enhanced synthesis of certain phenolic compounds such as p-coumaric acid, catechol, chlorogenic acid, hydroquinone or umbelliferone. These compounds are intermediates in the path way of biosynthesis of lignins, quinines or phytoalexins, which offer resistance to host against fungal invasion (Taylor and Zucker, 1966). The rise in the levels of phenols under wilt sick soil condition in susceptible cultivar, may be ascribed to the release of phenolics from the cell wall structures during their destruction of host phenols by the infecting fungi for their metabolic activities (Prasad *et al*, 1988). Thus, it seems that higher level of phenols and chlorogenic acid in chickpea is a desirable character for resistance to *Fusarium* wilt.

4.11 Soluble protein contents in the leaves, stems and roots of susceptible and resistant cultivars.

The data obtained on soluble proteins in leaves, stems and roots of susceptible and resistant cultivars of chickpea grown in autoclaved and wilt sick soil are presented in Table 4. The susceptible and resistant

Table 4. Soluble protein content (mg g^{-1} dry wt.)in leaves, stems and roots of susceptible and resistant cultivars of chickpea

Culti-vars	Plant parts								
	Leaves			Stems			Roots		
	Plants grown in		% variation over healthy stage	Plants grown in		% variation over healthy stage	Plants grown in		% variation over healthy stage
	Sterilized soil	Wilt-sick soils		Sterilized soil	Wilt-sick soils		Sterilized soil	Wilt-sick soils	
Resistant									
Vijay	136.4	154.3	+13.2	108.0	122.2	+13.1	99.3	112.9	+12.5
Vishal	130.4	150.5	+15.5	112.9	129.3	+14.5	103.6	114.0	+10.0
Phule G 03009	145.3	163.7	+12.7	115.7	131.5	+15.8	102.0	114.5	+12 3
Susceptible									
Phule G-5	159.3	202.9	+27.4	151.6	206.7	+36.3	124.9	176.2	+43 2
Chafa	162.5	201.1	+23.8	143.5	201.2	+40.3	129.3	182.2	+40.9
JG-62	165.8	207.3	+25.0	148.4	204.0	+37.5	127.6	176.2	+38.0
	S.E.	CD at 5%		S.E.	CD at 5%		S.E.	CD at 5%	
	2.5689	7.9200		2.7863	8.5902		2.4893	7.6745	

cultivars significantly differed in their content of soluble proteins. Among the plant parts analyzed, highest soluble proteins were found in the leaves of both the cultivars, followed by stems and roots, grown in autoclaved and wilt-sick soil. The leaves, stems and roots of susceptible cultivar had higher amounts of soluble proteins than the resistant cultivar. After the infection, an increase in the soluble proteins was observed in both the cultivar. The results obtained in the present investigation agree with the reported values and trends by Gupta and Kotasthane (1982), Maravia *et al*, (1993) and Patil (2000).

Higher protein content in the susceptible cultivar, may give rise to higher amount of nitrate nitrogen, which in turn may induce succulency of the host tissues predisposing them to the attack of the pathogen (Saxena and Khare, 1998). Higher increase in the protein content of susceptible cultivar may be due to the *de novo* synthesis of the proteins in the form of certain carbohydrate degrading enzymes secreted by the infecting fungi (Prasad *et al.*, 1988). Increase in proteins (enzymes) such as glucose-6-phosphate and 6-phosphogluconate dehydrogenases in maize leaves infected with *Helminthosporium carbonum* was recorded by Malca *et al.* (1964). The possibility of release of bound protein during degradation of infected tissue of maize by *H. carbonum* was also indicated by Jennings *et al.* (1969). Thus, the high soluble protein content may be associated with the susceptibility of the crop plants for invasion of the pathogen.

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

Chickpea is one of the most important pulse crops in the world, including India. It is a rich source of proteins in human diet and animal feed. The yield of chickpea is affected by several factors such as cultural practices and biotic and aboitic stresses. The fungal disease, *Fusarium oxysporum* f. sp. *ciceri* is one of the most destructive causative agents affecting both the quality and quantity of the chickpea grains.

Among the various approaches made to overcome this problem, development of disease resistant cultivars is the most important one. Attempts have been made in several crop plants to identify resistant characters and utilize them in breeding programmes of crop varieties.

In this context, biochemical parameters such as pathogenesis-related proteins, phenols, chlorogenic acid, soluble proteins etc. have been widely implicated in imparting a degree of resistance to crop plants. The information on such biochemical constituents in relation to wilt resistance in chickpea will generate basic information useful for physiologists, plant breeders, pathologists and biochemists.

An experiment was conducted using resistant, 'Vijay', 'Vishal' and 'Phule G-93009' and susceptible cultivars, 'Phule G-5', 'Chafa' and 'JG-62' cultivars of chickpea to identify biochemical markers involved if any, in the resistance to *Fusarium* wilt. The plants were grown in sterilized and wilt-sick soils separately. Leaves, stems and roots were analyzed for various biochemical constituents, such as PRs, phenols,

chlorogenic acid and soluble proteins The results obtained are briefly summarized as follows :

1. Some novel PRs were synthesized in resistant cultivars mainly in root portions followed by stem portion.

2. The synthesis of a number of PRs were found to be of higher order (magnitude) in 'Vijay' than two other resistant cultivars, 'Vishal' and 'Phule G-93009'.

3. In the root portions of a resistant cultivar 'Vijay', the syntheses of acidic PRs of ~ 25.7kD, 23 kD, 33 5 kD and 66 kD and a basic PR of ~ 21.3 kD were observed

4 In root and stem portions of a resistant cultivar 'Vishal', acidic PRs of ~66 kD and 32 kD appeared to be synthesized.

5. The leaves, stems and roots of resistant cultivars contained higher levels of total phenolics and chlorogenic acid than the susceptible cultivars. The total phenolics and chlorogenic acid increased in leaves, stems and roots after the invasion of the fungi. The increase in the level of phenolics and chlorogenic acid was of higher magnitude in a resistant cultivar.

6. The leaves, stems and roots of susceptible cultivars had significantly higher levels of soluble proteins than the resistant types. The increase in the contents of soluble proteins, particularly in the susceptible cultivar, was observed after the invasion of pathogen, indicating a positive role of soluble protein in disposing the plants to *Fusarium* wilt infection.

In conclusion, the higher levels of novel PRs were synthesized in 'Vijay' imparting greater resistance than 'Vishal' and 'Phule G-93009'. Most of the PRs were found to be synthesized in root portions, because of the immediate defense response needs of the plants since the fungus *Fusarium oxysporum* enters the plant only through the root system. Some of the PRs were also synthesized in the stem and may be by signal cascade mechanism.Phenols and chlorogenic acid may have a significant role in imparting the resistance to cultivars while soluble protein may offer susceptibility to *Fusarium* wilt. Screening of a large number of germplasms for these biochemical parameters and their exploitations in chickpea breeding programmes will thus be of an immense use.

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6. LITERATURE CITED

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