ANATOMICAL AND BIOCHEMICAL INVESTIGATIONS ON *PHYTOPHTHORA* FOOT ROT DISEASE REACTION IN *Piper* spp.

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **'Anatomical and biochemical investigations on *Phytophthora* foot rot disease reaction in *Piper* spp.'** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship, associateship or other similar title of any other University or Society.

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We, the undersigned members of the Advisory Committee of **Mr.Suresh Baban Dagade**, a candidate for the degree of **Master of Science in Horticulture** with major in **Plantation Crops and Spices**, agree that the thesis entitled **'Anatomical and biochemical investigations on *Phytophthora* foot rot disease reaction in *Piper* spp.'** may be submitted by Mr.Suresh Baban Dagade in partial fulfilment of the requirement for the degree.

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In memory of my
beloved grand father

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ABBREVIATIONS

IAA	Indole-3 acetic acid
M	Molar
mM	Milli molar
nm	nano meter
rpm	revolutions per minute
µl	Micro litre
µm	Micro metre
µmol	Micro mole

Introduction

INTRODUCTION

Black pepper (*Piper nigrum* L.) originated in the Western Ghats of India, is an important spice crop commercially grown in India, Indonesia, Brazil, Malaysia, Madagascar, Srilanka, Thailand and in some parts of China. Popularly known as "black gold", it is the major export earner among spice crops in India. Of the total Rs.1352.15 crores received during 1997-98 through the export of spices, black pepper accounted for 35.47 per cent (Rs. 479.57 crores) of the total export.

Ravages due to diseases particularly, the most devastating *Phytophthora* foot rot disease caused by *Phytophthora capsici* (Leonin emend, Alizadeh and Tsao) is one of the major constraints in production all over the world. Sarma *et al.* (1994) estimated the annual crop loss to the tune of 4.5 to 7.5 million dollars on a global scale. Samraj and Jose (1966) and Nambiar and Sarma (1977) reported 20 to 30 per cent of vine death in Kannur and Kozhikode districts of Kerala. In India, the disease was known as early as 1902 when severe vine death was noticed in Wynad region of erstwhile Madras state (Menon, 1949). Detailed symptomatology of the disease have been described by Samraj and Jose, 1966; Alconero *et al.*, 1972; Mammootty, 1978; Sarma and Nambiar, 1982 and Sarma *et al.*, 1994. The fungus infects all parts of black pepper. Aerial infection includes rotting of green tender stem resulting in varying degree of defoliation leading to reduced canopy. Collar and root infection are the most fatal and the infected vine succumbs in 10 to 20 days. The root infection ultimately culminates into foot rot. Integrated disease management involving chemical, cultural and biological methods besides host resistance was reported to be the ideal strategy to combat the disease (Sarma *et al.*, 1988).

Disease screening studies conducted in black pepper showed that the cultivars like Narayakodi, Kalluvally, Balankotta, Uthirankotta and Cheriakanyakkadan were tolerant and cultivars like Panniyur 1 and Karimunda were highly susceptible to *Phytophthora* foot rot (Kueh and Khew, 1980 and Sarma and Nambiar, 1982). *Piper* *colubrinum* L. a wild species of *Piper* introduced from Amazone basins is reported to be immune to *Phytophthora capsici* (Sarma *et al.*, 1991). Electrolyte leakage studies conducted in leaves and calli with toxic metabolite(s) of *Phytophthora capsici* also showed lesser electrolyte leakage values and thereby greater resistance / tolerance in *P. colubrinum* and in cultivars like Kalluvally and Cheriakanyakkadan when compared to Karimunda and Panniyur 1 (Shylaja, 1996).

The nature of resistance reaction of the host is largely determined by the initial stimulus provided by the parasite and its secretions and the ability of the host to respond (Barnett, 1959). The resistant / tolerant varieties posses various physical and biochemical barriers to restrict the entry and growth of the pathogen in the host cell. The defence mechanisms which actually arrest pathogen penetration or development include the hypersensitive response, physical barriers, antimicrobial proteins and metabolites such as phytoanticipins and phytoalexins (Collinge *et al.*, 1996).

In this context an insight into the anatomical and biochemical bases of *Piper* spp. to immunity, tolerance and susceptibility to the disease will be useful in further breeding programmes. Comparison of the biochemical parameters in *P. colubrinum* L.and tolerant and susceptible cultivars of *P. nigrum* L.before and after inoculation with *Phytophthora capsici* will give added information on host pathogen interactions in the three different groups viz., immune, tolerant and susceptible.

The present study was thus aimed at analysing the reaction of *P. colubrinum* L. and *P. nigrum* L. to *Phytophthora* foot rot disease based on anatomical and biochemical investigations.

Review of Literature

REVIEW OF LITERATURE

Phytophthora foot rot disease incited by *Phytophthora capsici* is an ubiquitous disease of black pepper posing serious threat to it's cultivation. The disease was reported in India as early as 1902 (Menon, 1949) from Wynad region of Kerala. There is no effective control measure to tackle the disease and all the cultivated types are susceptible to the disease. But some of the wild species like *P. colubrinum* and *P. obliqum* were found to be immune. So a thorough understanding of the mechanism of disease reaction in *Piper* spp. will be helpful to get added informations on host-pathogen interactions. It is with this intention, an effort is made in this review to bring together facts, interpretations and theories of anatomical and biochemical defence mechanisms in host-pathogen interaction.

2.1 Symptomatology of *Phytophthora* foot rot disease

Symptomatological studies on *Phytophthora* foot rot disease of black pepper were reported by many authors.

Butler (1906) observed blackening of the diseased black pepper roots which subsequently extended acropetally into the base of the stem.

According to Muller (1936) leaves, stems and roots of the black pepper vine at all stages of growth were susceptible to disease, and he observed typical symptoms like leaf rot, collar rot and root rot in the field.

Holliday and Mowat (1963) found more number of infected leaves on the lower part of the vine with uniform spread of brown lesions with fimbricate edges.

Rapid development of symptoms within 36 to 48 h of leaf inoculation was observed by Turner (1969). Similarly, inoculation of roots with *Phytophthora palmivora* caused infection within a period of 24 to 48 h (Alconero *et al.*, 1971). They reported wilting and rapid defoliation of affected vines in Brazil and Puerto Rico.

Appearance of water soaked lesions on the stem of infected vines and brown discoloration of infected fine roots had been reported by Mammootty (1978).

Sarma and Nambiar (1982) noticed acropetal and basipetal advance of the pathogen in black pepper vines infected at the collar region. Discoloration of vascular bundles was also observed.

Sarma *et al.* (1988), treated root and stem infection of vine as most fatal as the infected vines succumbed in 10-20 days.

Sarma *et al.* (1994) reviewed symptomatology of black pepper on account of *P. capsici* infection. They reported that all parts of the plant viz., the tender runner shoots, leaves, spikes, aerial branches and roots were prone to the pathogen infection. Foliar phase of the infection was characterised by production of small dark brown leaf spots with fast advancing margin. In response to aerial infection, there was rotting of green tender stems which resulted in varying degrees of defoliation and finally led to reduced canopy. Being a soil borne pathogen, it gained entry to main roots through fine roots and reached the foot or collar of the vine and culminated into foot rot.Further, rotting of roots impeded transportation of water and minerals thus brought physiological drought in plants.

The toxic metabolite(s) of *Phytophthora capsici* was also found to inhibit the root growth in callus derived plantlets of black pepper (Shylaja *et al.*, 1997). The thickness of roots as well as the number of roots produced were found to be less. When the anatomy of the roots was compared, roots of the screened calli derived plantlets had poorly developed xylem vessels, less number of cortical cells with more aerenchyma and abundant starch grains.

2.1.2 Sources of resistance to *Phytophthora capsici*

The available genotypes of black pepper and related species of *Piper* were screened for resistance / tolerance to the fungus by many workers (Holliday and

Mowat, 1963; Ruppel and Almeyda, 1965; Leather, 1967; Alconero *et al.*, 1971; Turner, 1973; Kueh and Khew, 1980; Sarma *et al.*, 1982 and Vilasini, 1982). They found that none of the cultivated types were resistant to the disease but wild species like *P. colubrinum* and *P. obliqum* showed greater resistance.

Field tests for resistance to *P. palmivora* and graft compatibility of *P. colubrinum* with cultivated genotypes were conducted by Alconero *et al.* (1971) in Puerto Rico and Brazil. They found Kalluvally, P.I. 214301 and Balankotta as less susceptible and *P. colubrinum* as resistant. The tolerance of Indian varieties Kalluvally and Balankotta was also reported by Kueh and Khew (1980) from Malaysia. The immunity of *P. colubrinum*, a wild relative of *Piper* from Amazone basin was also reported by Sarma *et al.* (1991).

Tolerance of Balankotta, Cheriyakanyakkadan, Kalluvally, Narayakodi, Uthirankotta and susceptibility of Panniyur 1 and Karimunda to *Phytophthora* foot rot were confirmed by Sarma and Nambiar (1982).

The high susceptibility of Panniyur 1 and Karimunda and tolerance of Kalluvally, Cheriakanyakkadan and Balankotta were also reported by Shylaja *et al.* (1996a) from electrolyte leakage studies conducted in black pepper calli using toxic metabolite(s) of *Phytophthora capsici*. Shylaja *et al.* (1996b) further reported screening of calliclones of five black pepper cultivars regenerated with and without applying *in vitro* stress with toxic metabolite(s) of *Phytophthora capsici* for resistance / tolerance to '*Phytophthora* foot rot'. They could isolate highly tolerant plants showing lesion diameter less than 25 mm from the study.

2.2 Methods of artificial inoculation

Several artificial inoculation techniques were effective in distinguishing the reaction of *Piper* genotypes to *Phytophthora* diseases.

Holliday and Mowat (1963) made an attempt to study the degree of resistance of pepper clones to *Phytophthora palmivora*, using different screening techniques. They tried zoospore suspension for inoculating the roots and zoospore suspension and culture discs for inoculating the leaves. Detached, punctured and unpunctured leaves were used for the study. Dorsal sides of leaves were wounded by single needle puncturing. For immature leaves 60 µl of zoospore suspension was placed at each inoculation site and for mature leaves ten square mm. Oat Meal Agar (OMA) culture disc was placed. Resistance test in pots was conducted on rooted cuttings raised in partially sterilised sand mixture. The susceptible variety wilted within twelve days while resistant variety took nineteen days.

Following stem inoculation technique, Ruppel and Almeyda (1965) studied the suitability of *Piper* spp. as root stocks against 'collar rot' disease of black pepper. Stem inoculation technique consisted of, wounding stems with sterile scalpel, three to five cm above ground level and placing one week old PDA grown mycelium and finally covering with anhydrous lanolin to prevent dessication.

Sarma *et al.* (1991) also tried stem inoculation technique to assess the degree of tolerance / susceptibility of pepper types to *Phytophthora capsici*. The technique consisted of making pinpricks at the centre of the second internode from top, placing three day old inoculum discs of three mm size, covering with moist cotton and tying with a polythene strip.

Leather (1967) tested the resistance / tolerance of black pepper cultivars against *Phytophthora palmivora*. In roots, the fungus grown on OMA was placed after scratching the cortex while, in stems shallow slits were made into the cortex and OMA culture disc was placed. He found that, *P. nigrum* cultivars produced symptoms only when wounded, but when the pathogen was inoculated on unwounded portion, none of the cultivars produced symptoms.

Turner (1971) also conducted a resistance test with *Phytophthora palmivora* on *Piper* species by dipping roots in zoospore suspension and inoculating five mm culture disc of the pathogen on the excised leaves.

Since dorsal side of black pepper leaves possess numerous stomata than ventral side, Kueh and Khew (1980) selected lowerside of mature leaf for inoculation which inturn facilitated necessary avenues and congenial microclimate for germination, growth and entrance of the pathogen.

2.3 Anatomical investigations

Plants are endowed with a variety of defense mechanisms against microorganisms. Magnitude of host confering resistance to the assilant pathogen largely depends on genetic constitution of the host plant. Accordingly, internal structures and composition of susceptible, tolerant, immune and resistant types differ within a genus.

2.3.1 Anatomical defense mechanisms in leaves

2.3.1.1 Epiderm as defense barrier

The toughness of the skin imparted by the epidermis including culticle acts as a defensive barrier against the intruding pathogen.

Cuticle acts as a chemical and physical barrier to the germination and penetration of the fungi. The cuticle thickness was correlated with levels of resistance to fungi that penetrated directly into tissues (Bell, 1974; Wang and Pinckard, 1973).

Host-parasite interactions in a resistant and a susceptible cultivar of potato inoculated with *Phytophthora infestans* had been reported by Hohl and Suter (1976). The pathogen penetrated leaves of susceptible and resistant cultivar either through stomata or directly through the epidermis. In susceptible host, fungus spread throughout the tissues intercellularly and transcellularly. Whereas in resistant host it remained confined to the site of infection. Transcellular hyphae emerged from the host cell into intracellular spaces or directly into neighbouring host cells. During penetration by transcellular hyphae, host cell walls became more pliable and soft which resulted in deformed shape of the susceptible host cells.

Variation in resistance to infection in epidermal cells of barley was reported by Jorgenson and Mortenson (1977). Infection by *Erysiphae graminis* resulted in germination of 0.7 per cent conidia in the resistant cultivars compared to 60 per cent in the susceptible cultivars.

Agrios (1978) opined that penetration of a host cell by the pathogen depends on softness of cell wall. Soft walled cells could be penetrated easily whereas, in hard walled cells penetration could be achieved by enzymatic secretions of the pathogen at the site of infection.

Kueh and Khew (1980) examined reaction of leaf surface and age of the black pepper leaves to infection by *Phytophthora palmivora*. They observed stomata only in the lower epidermis. Lower epidermis of the inoculated leaves had the largest number of sporangia followed by mesophyll layer and the upper epidermis. Mature leaves had the largest number of sporangia in the lower epidermis and the mesophyll, while old and young leaves had less sporangia in these tissues.

Mayee and Apet (1995) studied the structural defense mechanisms of rust resistant groundnut genotypes. Thicker epidermis-cum-cuticle, more number of trichomes on abaxial surface of leaves, low frequency smaller stomata and compact palisade tissue were observed as the structural defense mechanisms. In addition, there were intense callose deposits around the site of infection in the resistant genotypes whereas susceptible genotypes had less intense callose deposits aloof the site of infection.

2.3.1.2 Mesophyll as defense barrier

The pathological histology of stored betel vine leaves infected by a bacterium has been reported by Nirula (1944). The bacterium gained entrance through the cut ends of the petiole and at the advanced stage of infection destroyed mesophyll cells and disorganised xylem vessels.

Asthana and Mahmud (1945) observed that in the early stage of infection of betel vine, the bacterial leaf spot pathogen was confined to the epidermis but subsequently, it invaded the spongy and palisade parenchyma cells. The attacked parenchyma cells then slightly enlarged, assumed dark yellow colour and disintigrated.

The fungus *Helminthosporum maydis* and *H. carbonum* causing southern leaf blight of corn, ramified rapidly through susceptible parenchyma without invading the vascular bundles, whereas in the resistant inbred growth of *H. maydis* was effectively empeded by the chloren- chymatous tissues (Jennings and Ullstrup, 1957).

Lazorovits and Higgins (1976) studied susceptibility of tomato varieties to *Cladosporum fulvum* race 1. The mode of penetration of the pathogen in the susceptible, resistant and immune varieties was the same. The leaf tissues of the susceptible variety were extensively colonised by the mycelium, but in the resistant variety mycelium developed slowly. The infected cells of the resistant variety became nerotic or exhibited changes like reduced starch content in the chloroplasts and accumulation of extracellular material in the cell wall. In immune variety fungal development was restricted to few cells of mesophylls. Host cells surrounding the fungus showed extensive deposition of callose like material.

The Sr6 temperature sensitive allele in wheat confers resistance to stem rust pathogen only in mesophyll cells, the epidermal cells being susceptible did not become necrotic (Rohinger *et al.*, 1979).

Akai and Fukutomi (1980) described the process of infection of host plant by the pathogen. Parenchymatous cells succumbed to destruction whereas, unmodified cells stood infection or colonisation by fungi unless and until the invading fungi did not exert enough physical force or secreted enzymes were weak to disjunct the cell walls.

Histopathological studies conducted in betel vine leaves by Jain *et al.* (1982) indicated that the bacterial leaf spot pathogen attacked xylem, collenchyma and parenchyma cells.

Histopathology of betelvine leaves infected with *Xanthomonas campestris* pv. *betlicola* were reported by Abraham(1986). He found the bacterium as parenchymatous pathogen, entering through the stomata and disintegrating the spongy and palisade parenchymatous cells, leaving the xylem vessels unaffected.

Kaur *et al.* (1992) investigated anatomical characteristics of groundnut varieties in relation to the tikka leaf spot pathogen. They found maximum thickness of spongy parenchyma tissue in the susceptible, when compared to moderately susceptible varieties. The resistant varieties had thicker epidermis, palisade tissues and thinner spongy parenchyma.

2.3.1.3 Vascular system as defense barrier

Kotwal (1978) noticed presence of *Xanthomonas* sp of bacterium in vascular bundles and parenchymatous cells of infected betel vine leaves.

Sherwood and Berg (1991) observed no correlation between vascular bundles or percentage of larger vascular bundles with mean leaf spot size in *Dactylis glomerata* an orchard grass inoculated with *Stagnospora arenaria*. The proportion of small vascular bundles with two girders was greater in leaves of resistant varieties than susceptible. Inoculated leaves had higher lignin content than control indicating that lignin like compounds were synthesized during infection process.

The histopathology of corn leaf blight incited by *Helminthosporium* spp had been investigated by Jennings and Ullstrup in 1957. The fungus *H. turcicum* entered the susceptible and resistant plants directly at the juncture of the epidermal cell. The epidermal cells of the susceptible plants were typically barrel shaped and larger than the resistant plants. The fungus became established early in the susceptible xylem tissue, with mycelial growth progressively filling vessels and tracheids. Distinct wilt symptoms and chlorenchyma necrosis followed extensive xylem plugging and hyphae moved out into parenchyma only when lesions were formed. Fourteen days after inoculation, xylem vessels of the smaller bundles were completely blocked and 80 to 90 per cent xylem area of the larger bundles was plugged with mycelium in susceptible inbreds. Conversly, in resistant inbreds plugging of water conducting elements by the fungus was observed only after 21 to 28 days after inoculation. They also observed rapid growth of *H. maydis* and *H. carbonum* through susceptible parenchyma, but the vascular tissues were not invaded. The growth of *H. maydis* was eventually blocked in the larger vascular bundles and parenchyma tissue of the resistant corn inbred.

Structural defence mechanisms of groundnut leaves in relation to *Phaeoisariopsis personata* were studied by Mayee and Suryawanshi (1995). Resistant genotype was characterised by smaller stomata with less frequency, compact palisade layer, thicker epidermis-cum-cuticle and presence of trichomes on abaxial surface of leaves. Prolonged incubation of pathogen in resistant leaves resulted in the late appearance of intracellular hyphae, occlusion of intercellular spaces, delayed rupture of the epidermal cells and accumulation of mycelial mass below the epidermis. Infection of pathogen in susceptible leaves was marked by thickening of palisade cells, formation of swollen cells in the vicinity of localised infection and complete discoloration of chloroplast.

2.3.2 Anatomical defense mechanisms in stems

2.3.2.1 Epidermis as defense barrier

Uchiyama and Ogasawara (1977) indicated that the absence of cuticle and wax in the outermost layer of leaf callus tissue of rice facilitated spore germination, penetration and saprophytic growth of the fungus *Aspergillus oryzae* in the tissue.

Resistance of wheat to straw breaker foot rot was correlated with hypoderm width and number of hypodermal cell layers, but not with the cell wall thickness, lumen or stem diameter. On account of infection, the epidermal cell walls became thickened and lignified in the resistant wheat varieties. Similarly in the resistant host, lignified cell wall apposition were larger and formed in greater profusion in the epidermal cells than in the susceptible host at infection site (Murray and Bruchi, 1983).

Kolattukudy (1984) reported that 'cutin' the structural component of plant cuticle is a biopolyster composed of hydroxy and hydroepoxy fatty acids. Also, natural and wound periderms and a variety of internal physical barrier layers contain analogus polymer to cuticle called 'suberin'. In order to break down cutinised layer, pathogenic fungi uses a hydrolytic enzyme 'cutinase'.

Stockwell and Hanchey (1984) reported that increased calcification of cell walls and increased cuticle thickness of hypocotyls in beans were resposible for resistance to *Rhizoctonia solani*.

2.3.2.2 Cortex as defense barrier

Hursh (1924) studied resistance of wheat varieties in relation to *Puccinia graminis tritici*. The resistant varieties had large proportion of sclerenchyma which divided the collenchyma into distinct areas and prevented spread of the pathogen and subsequent rupture of the epidermis but the susceptible variety had less conspicuous sclerenchyma.

The pathogen *Fusarium roseum* in infected stem of carnation spread through parenchymatous tissue and stimulated the proliferation of host cells in advance of the hyphae (Moreau, 1957). In response to pathogen intrusion, formation of a lignified ring of pericyclic tissue and lignification of xylem element took place in the internodal areas. But in nodal areas growth of the fungus was checked in the cortex by forming a sclerified layer of tissues.

Markose (1996) reported that the compact arrangement of the cells, thicker cell walls of the epiderm and cortical cells were responsible for resistance to bacterial wilt disease in chilli.

2.3.2.3 *Vascular system as defense barrier*

Walkar and Stahman (1955) reported that plugging of xylem elements took place in tomato plants upon infection by Fusarium wilt pathogen which in turn, affected the upward transportation of water.

Klarman and Corbett (1974) described the histopathology of resistant and susceptible soybean hypocotyls inoculated with *Phytophthora megasperma* var. *sojae*. All the tissues of the susceptible hypocotyls were ramified by hyphae but, the vascular tissues and cells with secondary wall remained intact. In inoculated resistant hypocotyls also vascular bundles remained undamaged but, cells surrounding inoculation wound were colonised. Host cells close to infected area were filled with granular dark staining cytoplasm which appeared to form structural barrier to further spread of the pathogen.

According to Yates *et al.* (1997) accelerated lignin deposition in *Fusarium moniliformae* infected corn shoots might account for increased rigidity and obstruction of fungus in the stem.

2.3.3 Anatomical defense mechanisms in roots

2.3.3.1 Epiderm as defense barrier

Though hyphae of *Theielavia basicola* infecting tobacco roots were in close connection with the outer cork or suberised epidermal layer, Conant (1927) could not observe the penetration of suberised cell walls or cork cells by the hyphae. The suberised and lignified walls slowly changed into pectin like substances and exhibited marked swellings. This might be due to accumulation of enzymes secreted by the mycelium in the vicinity of infection site.

Dickinson (1960) mentioned that internal physical structures inhibiting the spread of pathogens in tissues act by means of their impermeability, insolubility and inpenetrability to the pathogen.

Hohl and Stossel (1976) indicated that after 20 h of *Phytophthora infestans* infection, there were very few hyphae in the resistant cultivar restricted to the top cell layers of potato tubers. At the same time hyphae in the susceptible cultivar had penetrated the second cell layer and were more numerous. Fungal hyphae in resistant cultivar were small and were surrounded by an electron-dense extrahaustorial matrix and electron-transparent wall appositions. These wall appositions were callose like materials (glucanase) which were lacking in the susceptible host.

Beagle-Ristaino and Rissler (1983) histopathologically compared roots of the soybean plants inoculated with *Phytophthora megasperma* f. sp. *glycinea.*The fungal hyphae penetrated epidermis of both susceptible and resistant plants within 1.5 h of inoculation. Seventy two hours after inoculation hyphae grew fastly in the susceptible root tissues rather slowly in resistant one and within 192 h of inoculation, hyphal ramification reached to hypocotyls in the susceptible plants.

2.3.3.2 *Cortex as defense barrier*

The fungus *Ophiobolus graminis* penetrated cortex via epidermis but its advance was checked at the root endodermis in wheat plants (Fellows, 1928).

Tolboys (1968) indicated general thickening of cell walls in the cortex of the hop roots on account of penetration by *Verticillium*.

Pathological anatomy of alfalfa roots infected with *Phytophthora megasperma* was investigated by Marks and Mitchell (1971). On inoculation, infected cells appeared thicker compared to non infected ones. In infected roots the endodermis secluded the central stele from infected cortical tissues. Formation of wound periderm led to restricted lesion development on the tap roots but, there was no wound periderm formation around the infection court when vascular tissues were infected. Infact, minor damage was noticed when invasion was restricted to the cortex.

Philips and co-workers (1987) histologically examined *Phytophthora cinnamomi* infected avocado roots. They revealed two types of structural changes at the lesion boundary, formation of necrophytic periderm in the cortex and whorls of cells walling off infected phloem bundles in the stele. Periderm secluded necrotic tissues from non necrotic, uninfected cortical tissues from epidermis through endodermis which was coincided with disappearance of the infected cortical cells. After the formation of cell whorling and cell wall thickening, phloem bundles were devoid of hyphae, otherwise succumbed to intense colonisation.

2.3.3.3 *Stele as defense barrier*

Anderson and Walker (1935) reported that fungus causing cabbage yellows encountered difficulty in passing through the endodermis and pericycle than through cortical layer surrounding the endodermis. In the phloem tissues, fungal strands appeared much larger than in the xylem.

In extremely susceptible corn plants, the fungus *Gibberella zeae* entered stele frequently but its advance was checked at the endodermal region (Dickinson and Holbert, 1938). The pericycle of the corn seedlings was found to be well suberised and proved as structural barrier against invasion by the fungus.

During the development of *Phytophthora* root rot in safflower, the resistance to water movement in roots and stems increased progressively, but resistance to water movement was unaltered in leaves. Increased root and stem resistance to water movement accounted for the depression of water potential and wilt symptoms that occured in the infected plants (Duniway, 1977).

Cahill *et al.* (1989) examined histological changes in susceptible and resistant species of *Acacia, Eucalyptus, Gahnia, Juncus, zea, Triticum* and *Xanthorrhoea* due to infection by *Phytophthora cinnamoni*. Following inoculation, root growth of resistant species ceased within 24 h and resumed again after 48 h. While in susceptible species lesion extended up to the hypocotyls which resulted in root death. However, deposition of phenolic materials, granulation of cytoplasm, protoplast shrinkage, cell disruption and distortion was common in all the species. But lignification of cell walls, deposition of phenolics and formation of callosic papillae were more common in resistant species.

The fungus *Pythium diamorphum* ramified the root cortex and stele of *Picea abies* within a day (Bojra *et al.*, 1995). In response to inoculation, within 72 h the concentration of lignin in inoculated roots increased two fold than the control. The authors concluded that accumulation of lignin in inoculated plants was too late to check the spread of the pathogen.

Inoculation of the root systems of avocado plants with *Phytophthora citricola* caused infection, but pathogen did not move upward to the stems even after a month (El-Hamalawi *et al.*, 1995). There was no direct transmission of the fungus through phloem by grafting.

The fungus *Phytophthora megasperma* extensively colonised the cells of susceptible alfalfa plant when compared to resistant ones (Miller and Maxwell, 1984). In both the susceptible and resistant interactions, root cells in contact with hyphae were plasmolysed or became necrotic.

Markose (1996) reported that the bacterial wilt resistant variety of chilli had well developed secondary xylem with compactly arranged, thick celled epiderm layer and cortical cells in roots as compared to the susceptible one.

2.2 Biochemical investigations

Walker and Stahmann (1955) indicated that, in many histological studies, resistance generally was not associated with anatomical differences among the host plants; inturn more directly to the biochemical or physiological differences which might explain in part or at least the basis of resistance.

Disease results whenever the vital functions of plants are disrupted by a biotic stimulus and several quite often changes takes place in the primary metabolism of the affected cells (Swain, 1977). Further, interactions between host and pathogen determines biochemical dynamics of parasitism and pathogenesis (Cruishank, 1980).

The chemical factors, that are toxic to pathogen inhibit its growth and activity in the host. Similarly, pathogens may produce certain compounds against the host for example, *Helminthosporium maydis* produces phytotoxic compound, "*Helminthosporin*" (Angara-Sharma and Sharma, 1994) also *Phytophthora* produces "*Glucanases*" (Holten and Bartnicki-Garcia, 1972) or "*Mycolaminarins*" (Marcan *et al.*, 1979). Therefore the biochemical bases of infection becomes increasingly important, especially in characterising the host resistance mechanism.

2.4.1 Phenols in defense mechanism

A wide range of chemicals possesing an aromatic ring bearing a hydroxyl substituent called phenolic substances shows antifungal, antibacterial and antiviral activities. Phenolics in high concentrations are toxic to plant cell themselves (Tepper and Anderson, 1984). Hence, phenolics will normally be present in small quantities only in plants and these quantities may not be sufficient to supress the development of pathogens. But, in many plant-pathogen interactions, the synthesis of phenolics is activated after infection and high amount of phenolics are synthesised which rapidly supressed the pathogen development (Vidhyasekaran, 1990). Phenolic compounds acts as hydrogen acceptor / donar in oxidation-reduction reaction to form quinones which are involved in resistance of plants due to their higher toxicity to microorganisms (Barnett, 1959).

2.4.1.1 Total phenols

Protective role of phenolics against *Phytophthora* diseases had been reported by many authors.

Marten and Kneusel (1988) explained two way defense strategy of phenols in plants viz., rapid accumulation of phenols at the infection site and activation of *de novo* synthesis of specific defenses such as phytotoxins or other stress related compounds.

Phenols in plants may act as an allelopathic compound or plant growth regulators (Siqueria *et al.*, 1991), or as an antibiotic (Nicholson and Hammerschmidt, 1992).

Increase in levels of total phenols were associated with resistance response of potato to *Phytophthora infestans* (Bobes *et al.*, 1987), tomato to *Sclerotium rolfsii* (Prasada *et al.*, 1987), pigeon pea to *Phytophthora* blight (Kaur and Mehrotra, 1990), capsicum to *Phytophthora capsici* (Jeun and Hwang, 1991 and Lizzi *et al.*, 1995), Eucalyptus to *Phytophthora cinnamomi* (Cahill *et al.*, 1993), chilli to bacterial wilt (Markose, 1996), cocoa to *Phytophthora megakarya* (Ndoumou *et al.*, 1996) and chilli and brinjal to *Ralstonia solanacearum* (Paul, 1998).

However, Sindhan *et al.* (1996) reported pre-infection higher levels of total phenols in flag smut resistant wheat varieties as compared to susceptible ones. Reduction in post-infection concentration was observed in the both susceptible and resistant varieties, maximum being in susceptible ones.

2.4.1.2 Ortho dihydric phenol

The monomeric dihydroxy phenols have some antibiotic and enzyme denaturing activity (Mukherjee and Kundu, 1973).

The enzymes namely polyphenol oxidase and peroxidase, oxidise the colourless dihydroxy phenols to give the coloured *ortho* quinones, while certain dihydroxy phenols get conjugated with each other or glucose hydroxyl group to form tannins, both form constituent of plant melanins (Beckman *et al.*, 1974; Mayer and Harrel, 1979 and Bell, 1981). These tannins and *ortho* quinones have toxicity to microorganisms (Hunter, 1978).

Bell (1981) suggested that, some pathogenic fungi reduced plant dihydroxy phenols to tetralones as a mechanism of inhibiting melanisation and overcome resistance provided that higher levels of dihydroxyphenols must be released and oxidised effectively for resistance expression. Also, leaching of dihydroxy phenols from vacuoles into cytoplasm and intercellular spaces determine the level of resistance.

Increase in dihydroxy phenols and decrease in ascorbic acid content were observed in rice inoculated with a virulent strain of *Piricularia oryzae* (Sridhar and Ou, 1974).

Addy (1976)reported that, resistant varieties of apple to *Erwinia amylovora* leached dihydroxy phenols more rapidly than susceptible varieties.

Abraham (1986) noticed pronounced increase in OD phenol, total phenols and leucoanthocynin in resistant *P. betle* cultivar inoculated with bacterial leaf spot pathogen.

Levels of OD phenol and total phenols were higher in *Alternaria solani* inoculated plants than in healthy plants (Veermohan *et al.*, 1994).

Flag smut resistant wheat varieties had higher levels of OD phenols, but on inoculation with fungi its level decreased (Sindhan *et al.*, 1996).

Similar increase in the content of OD phenols was also reported in resistant varieties of chilli by Markose (1996) and tomato by Paul (1998).

2.4.2 Sugars in defense mechanism

When plant tissues are low in sugar they become more susceptible to some diseases and less susceptible to others (Horsfall and Diamond, 1957). Reduction in sugar content in infected plants might account for synthesis of polyphenols (Niesh, 1964).

Easwaran (1967) observed a high quantity of reducing sugar in sorghum variety susceptible to bacterial wilt disease than in the moderately susceptible varieties. Inoculation of pathogen led to reduction of sugars in both varieties.

Bacterial leaf blight resistant variety of rice had lesser quantities of reducing, non reducing and total sugars as compared to susceptible and moderately susceptible varieties (Prasada *et al.*, 1972). Inoculation with the pathogen caused reduction in reducing and total sugars, but increase in non reducing sugar.

Reddy and Sridhar (1975) noticed higher levels of reducing and non reducing sugars in leaves of bacterial leaf blight susceptible rice variety than tolerant one.

Significant decrease in levels of sugars and total phenols in leaves and fruits of capsicum plants susceptible to *Phytophthora nicotianae* var. *nicotianae* was reported by Bhardwaj *et al.* (1986).

Abraham (1986) attributed the resistance of betelvine cultivars to bacterial leaf spot pathogen as the higher levels of reducing, non reducing and total sugars. On inoculation, the non reducing sugar content increased in susceptible cultivars.

Veermohan *et al.* (1994) reported decreased photosynthetic efficiency and content of chlorophylls, reducing, non reducing and total sugars and starch content in capsicum leaves infected with *Alternaria solani*.

Sindhan *et al.* (1996) found higher quantities of total and reducing sugars in flag smut resistant varieties of wheat as compared to susceptible ones. On inoculation there was reduction in sugar content.

Paul (1998) reported higher levels of soluble sugars in bacterial wilt resistant varieties of chilli and tomato.

2.4.3 Amino acids in defense mechanism

The amino acids are building blocks of protein and during diseased condition the free amino acid composition in the plants exhibits changes.

The amino acid content of infected wheat cells was found to increase on inoculation by the fungus *Puccinia graminis tritici* (Rohinger, 1957). There was pronounced increase of glutamine content in the infected leaves of wheat plants. The uredospores of the host pathogen also had higher amount of glutamine.

Farkas and Kiraly (1961) also found increased amino acid content in wheat leaves, infected with stem rust fungus. They attributed that increase in amino acid content in infected leaves might be due to decomposition of host protein or decreased protein synthesis.

The amino acid content in wheat leaves either prevented parasitic development or reduced the pathogenicity of the rust fungus (Samborski and Forsyth, 1960).

Tuveson and Garber (1960) suggested a correlation between higher content of amino acid and resistance to *Fusarium oxysporum* f. sp. *pisi* in pea.

Moustafa *et al.* (1961) opined that not only the content but also the rate of secretion of amino acids decide the pathogenicity of *Rhizoctonia solani* in tomato.

Shaw and Colotedo (1961) found progressive reduction of amino acid content in the infected wheat leaves by rust fungus.

Prasada *et al.* (1987) reported increased levels of total, reducing and non reducing sugars, total amino acids and phenols in green and matured tomato fruits infected with *Sclerotium rolfsii*.

The amino acid content in leaves of *P. betle* was greater in the less susceptible cultivar than highly susceptible cultivar to *Phytophthora* leaf rot (Chile and Vyas, 1989). Infection by the fungus caused consistent depletion in free amino acid content.

Marginal differences among susceptible and resistant varieties for amino acid content in *Phytophthora capsici* infected pepper plants had been reported by Jeun and Hwang (1991).

Phukan (1994) reported variable concentrations of amino acids in potato plants due to *Phytophthora infestans* infection.

2.6 Enzymes in defense mechanism

Enzymes are large protein molecules which catalyse all the interrelated reactions in a living cell. Some of the host enzyme like β-1, 3-glucanase, peroxidase and polyphenol oxidase (Goy *et al.*, 1992) play key role in disease resistance.

2.6.1 Glucanase

Bartinicki-Garcia (1969) reported that β-1, 3-glucanase are reported to hydrolyse the β-1, 3D glucan component in cell walls of pathogenic fungi. Various plants (Abeles *et al.*, 1970) as well as phytopathogenic fungi (Holten and Bartinicki-Garcia, 1972 and Bell, 1981) produce glucanases.

Robenantoandro *et al.* (1976) reported that increased endo β-1, 3-glucanase activity in seedlings was involved in the infective mechanism of melon to the *Colletotrichum lagenarium*.

When the cultivars of *Lotus corniculatus* containing β-1, 3-glucanase and cyanogenic glycosides were wounded, glycosides hydrolysed to produce hydrocyanic acid; whereas in the cultivars lacking β-1, 3-glucanase there was no production of hydrocyanic acid (Miller and Hemphill, 1978). Mycolaminarins, the mycelial component of the fungus *Phytophthora infestans,* supressed hypersensitive reaction in potato tubers (Garas *et al.*, 1979).

Activity of β-1, 3-glucanase in fusarium wilt resistant and susceptible near isogenic lines of muskmelon had been reported by Netzer *et al.* (1979). The study revealed that the activity of β-1, 3-glucanase was higher and increased rapidly in resistant lines than in susceptible ones. Pre-treatment of susceptible muskmelon with laminarin prior to inoculation increased the enzyme activity and markedly reduced the disease.

Pathogenesis related proteins contain β-1, 3-glucanase or chitinase (Legrand *et al.*, 1987) which got accumulated in the intercellular spaces of infected tobacco leaves. These PR proteins play an important role in disease resistance (Mauch *et al.*, 1988).

Matta *et al.* (1988) inoculated the roots of tomato with a non pathogenic form of *Fusarium oxysporum* fungi. Inoculation resulted in an increase of β-1, 3-glucanase and chitinase activity. Activity of these enzymes was correlated with variations in peroxidase and polyphenol oxidase activity, concentration of phenols and released ethylene. Inoculation with pathogenic fungi, *F.oxysporum* f.sp. *lycopersici* caused further increase of enzyme activity in previously stressed plants. The death of rotted cells was attributed to increased glucosidase activity.

Similarly, Pan *et al.* (1991) found higher activity of β-1, 3-glucanase in tobacco leaves inoculated with *Peronospore tabacina* and Tobacco Mosaic Virus (TMV) compared to control. Upon subsequent challenge with *Peronospore tabacina*, the enzyme activity continued to increase in the plants inoculated by both TMV and *P. tabacina*. However, one day after mock inoculation enzyme activity increased in the uninoculated plants, but was lower in already induced plants. The authors concluded that, the higher activity of β-1, 3-glucanase in the control plants six day after challenge inoculation might be too late to prevent the development of lesions and sporulation of *P. tabacina*.

Rouhier *et al.* (1995) isolated β-D, glucans from cell walls of *Phytophthora capsici*. Structural analysis revealed that β-D-glucans had common β-1,3 linkage (β-(1→3). Constitutive expression of β-1,3-glucanase C DNA coding for PR-N isoform in tobacco plants, increased the foliar resistance to *Peronospore tabacina* and

Phytophthora parasitica var. *nicotiana*. Conversely, foliar resistance was not observed when tobacco plants were challenged with tobbaco mosaic tobamovirus, tobacco etch potyvirus or tobacco vein mottling potyvirus. Thus foliar resistance was attributed to the higher levels of β-1, 3-glucanase.

2.6.2 *Indole-3 acetic acid oxidase*

Like plants, pathogens also produce growth regulators in new or different form which might stimulate or retard the production of similar compounds by the plants.

According to Hare (1964) the rate of oxidation of IAA by IAA oxidase depends on its cofactor *P*-coumaric acid.

Indole-3 acetic acid is involved in resistance by affecting the cell wall properties.

Agrios (1978) indicated that the fungi causing late blight of potato (*Phytophthora infestans*), corn smut (*Ustilago maydis*), cedar apple rust etc. produce IAA through inhibition of IAA oxidase.

Comai and Kosuge (1984) pointed out that tumor formation in olive and oleander trees was due to the abnormal plant cell multiplication at the site of infection by *Pseudomonas syringae* pv. *savastanoi* and excess production of IAA or cytokinin by the bacterium.

Phenolic substances such as caffeic acid, chlorogenic acid, 2,2' azino-bis diammonium salt inhibited the oxidation of IAA in tobacco transgenic plants (Gazaryan and Lagrimini, 1996).

2.6.3 *Peroxidase*

Many investigators have noticed a close correlation between disease resistance and peroxidase activity. Peroxidase takes part in synthesis of lignin, catalyses the oxidation of phenolics into more toxic quinones (Kosuge, 1969).

Ten fold increase in peroxidase activity was reported by Mace (1964) in rust resistant varieties of wheat.

Grangeon and Coulomb (1977) reported increased activities of hydrolase and peroxidase in *Phytophthora capsici* inoculated stems of capsicum plants.

Inoculation of capsicum leaves with *Phytophthora capsici* caused a considerable increase in peroxidase activity which was more marked in the resistant cultivar than in susceptible (Coulomb and Coulomb, 1984). In both cultivars, enzyme was aggregated in the intercellular spaces invaded by the fungus and on the residual material of the necrotic host cells.

Lizzi and Coulomb (1991) evaluated the activities of peroxidase and catalase in the leaves of *capsicum annum* infected with *Phytophthora capsici* and TMV. They observed higher activity of the enzyme in resistant varieties when compared to susceptible. However, infection with TMV did not significantly modify peroxidase activity. Infection with *Phytophthora capsici*, induced catalase activity, but infection with TMV reduced its activity. Following infection, peroxidase activity was found to be increased and catalase activity decreased in the intercellular spaces, and their higher concentration led to the dilapidation of the pathogen.

Defense response of capsicum plants to *Phytophthora capsici* was studied by Alcazar *et al.* (1995). In the intercellular fluid, peroxidase activity of susceptible cultivar was lesser than moderately susceptible and resistant cultivars. Histochemical analysis revealed peroxidase activity in intercellular spaces of the resistant cultivar that had not invaded by the fungus. But the activity was detected neither in the other cultivars nor in the intercellular spaces of infected stems of the moderately susceptible and resistant cultivars.

Higher activity of peroxidase in conjunction with polyphenol oxidase and OD phenol was attributed to resistance in chilli (Markose, 1996) and brinjal (Paul, 1998) to the bacterial wilt pathogens.

2.6.4 Polyphenol oxidase

Polyphenol oxidase in combination with peroxidase and *ortho* dihydroxy phenol forms quinones that are toxic to the microorganisms.

Muller and Beckman (1976) indicated that activities of polyphenol oxidase and peroxidase were more important since the former could add hydroxyl group during synthesis of dihydroxy phenols in plastids and the later was responsible for the oxidation of phenols to melanin in the cell walls of roots and hypocotyls of cotton seedlings.

In response to injury activities of polyphenol oxidase and peroxidase were increased in the fusarium wilt resistant tomato plants (Gentile *et al.*, 1988).

Bobes *et al.* (1987) observed variation in the activities of depolymerase, cellulase, catalase, peroxidase, polyphenoloxidase and β-glucosidase in susceptible and resistant potato plants. Differences in enzyme activities were also noticed in leaves and tubers when infected with *Phytophthora infestans* and *Erwinia carotovora* var. *atroseptica*.

The resistance observed in tobacco hybrid *Nicotina glutinosa x N. debneyi* against TMV, tobacco necrosis necrovirus, *Pseudomonas syringae* pv. *syringae, P.s.* var. *tabaci, Pernospore tabacina, Pernospore parasitica* and *Thielaviopsis basicola* was due to higher levels of polyphenol oxidase, peroxidase, chitanase and β-1, 3-glucanase (Goy *et al.*, 1992).

Changes in leaf peroxidase, polyphenol oxidase, catalase due to alternaria leaf blight infection in Brassica species were reported by Gupta *et al.* (1995). The specific activity of polyphenol oxidase remained higher while that of peroxidase remained lower in tolerant *Brassica carinata* and *B. napus* when compared with susceptible *B. juncea* and *B. campestris*. In response to infection activity of both the enzymes increased comparatively at much faster rate in the susceptible species. Polyphenol oxidase activity was considerably higher at initial stages of plant growth in all species which dropped markedly at later stages.

Materials and Methods

MATERIALS AND METHODS

The present investigations were carried out at the Department of Plantation Crops and Spices and Biochemistry Laboratory of the College of Horticulture, Kerala Agricultural University, Vellanikkara, during 1996-1998. The study was aimed at analysing the reaction of *Piper nigrum* (L.) and *Piper colubrinum* (L.) to *Phytophthora* foot rot disease based on anatomical and biochemical investigations. Three groups of plants viz., *Piper colubrinum,* a species reported to be immune to *Phytophthora capsici*, *P. nigrum* - variety Panniyur 1, reported to be susceptible to *Phytophthora capsici* and *P. nigrum* cultivar Kalluvally, reported to be tolerant to *Phytophthora capsici* were subjected to intense investigations. The details regarding the experimental materials and methodology adopted for conducting various aspects of the study are presented in this chapter.

3.1 Raising of rooted cuttings

Runners for raising rooted cuttings of Kalluvally were collected from Pepper Research Station, Panniyur and that of Panniyur 1 from Pepper Research Scheme, Vellanikkara. Terminal cuttings of *P. colubrinum* were also collected from Pepper Research Scheme, Vellanikkara.

Two noded cuttings were dipped in IBA 1000 ppm for 45 sec and planted in 20 x 15 cm polybags filled with potting mixture prepared in equal proportions of sand, soil and cowdung. The polybags, after planting were placed inside a polythene chamber for maintaining high humidity and temperature. Watering of the polybags was done daily. Polythene sheet of the chamber was removed completely when 80 per cent of the cuttings were sprouted. The rooted cuttings were transferred to bigger polybags of size 30 x 22 cm and maintained in the glasshouse of the College of Horticulture (Plate I).

Plate I. Healthy plants of *Piper* spp.
Piper colubrinum L., *P. nigrum* L. (Panniyur 1) and *P. nigrum* L. (Kalluvally)


Panniyur-1
Kalluvally
P.colubrinum

3.2 Isolation of pure culture of *Phytophthora capsici*, maintenance of the culture and artificial inoculation

Pure culture of *Phytophthora capsici* was isolated from the infected leaves of black pepper. Small bits of infected portion of leaves were surface sterilised with 0.1 per cent mercuric chloride for one minute and washed free off the sterilant with sterile distilled water and inoculated to Potato Dextrose Agar (PDA) medium, the composition of which is given in appendix I . The fungal hyphae and sporangia were observed under microscope and the culture was purified by hyphal tip method. Stock culture was maintained by repeated subculturing to PDA medium.

3.2.1 Pathogenicity test

Pathogenicity test was conducted with five mm culture disc of the seven day old culture of *Phytophthora capsici* grown on PDA, by detached leaf puncture bioassay as reported by Kueh and Khew (1980). The pathogen was reisolated from the artificially inoculated leaves by the method already described. The reisolated culture was compared with the original isolates and was again subjected to purification and the pure culture was maintained in Carrot Agar medium the composition of which is given in Appendix I.

3.3 Anatomical investigations

Anatomical studies of the leaf, stem and root of the three different groups of plants viz., immune, tolerant and susceptible to *Phytophthora capsici* were conducted as per the procedure reported by Datta and Dasgupta (1977a and b) in *Piper* spp.

Transverse hand sections were taken, stained with safranin and temporary mounts were prepared and examined under microscope.

The three groups of plants were compared with respect to structural defense barriers and nature and extent of each layer of cells. Microscopic observations for various parameters were made with ocular and stage micrometer. Quantitative data were calculated from 20 readings and were indicated as minimum, mean and maximum.

For finding out stomatal index the lower side of the leaves was smeared with an adhesive, 'Quick fix' to facilitate easy removal of the peels and stomatal count was taken after preparing safranin stained mounts. The stomatal index was indicated as number of stomata per square mm.

3.4 Biochemical investigations

Biochemical investigations were carried out at the Biochemistry Laboratory of the College of Horticulture, Vellanikkara.

Leaf, stem and root portions of the plants of the three different groups of plants viz., immune, tolerant and susceptible to *Phytophthora capsici* were analysed on fresh weight basis for total and OD phenols, reducing and non reducing sugars, total free amino acids and activity of enzymes like glucanase, IAA oxidase, peroxidase and polyphenol oxidase as per standard procedures. Three replications were maintained for all the analyses.

The changes in the above biochemical parameters due to infection by the pathogen was also estimated. For this, artificial inoculation was carried out on leaves and stems using culture disc of *Phytophthora capsici.*

Artificial inoculation of *Phytophthora capsici* on leaves and stems was conducted as per the procedure reported by Kueh and Khew (1980) and Sarma *et al.* (1991) respectively. Mature leaf, third from the top was washed thoroughly and surface sterilised with 70 per cent ethyl alcohol. With sterile needle, minute pin pricks were made on the lower side of the leaf and five mm culture discs of the pathogen were inoculated.

Stem inoculation consists of making pin pricks with sterile needle at the centre of the second internode from the top, placing five mm culture disc of *Phytophthora capsici* and tying with a polythene strip of 150 guage thickness. High humidity was maintained in the bell jar.

Seventy two hours after inoculation, lesion development was observed and infected plant parts were taken for biochemical analyses

3.4.1 Estimation of total phenols and OD phenol

Methanol extracts of leaves, stems and roots were used for estimation. The samples were separately cut into small pieces and were crushed with methanol (ten ml for each g of tissues). The homogenised material was centrifuged at 3000 rpm for ten minutes. The total and OD phenol content was calculated from a standard curve of catechol and was expressed as mg^{-1} g of sample.

mg of phenols per g of sample =

$$\text{Factor} \times \frac{\text{Absorbance}}{\text{Volume made}} \times \frac{\text{Dilution (ml)}}{\text{Weight of sample (mg)}} \times \frac{1000}{1}$$

3.4.1.1 Total phenol

Total phenols were estimated with Folin-ciocalteu method suggested by Sadasivam and Manickam (1996). The intensity of blue colour developed was read at 650 nm in a spectrophotometer.

3.4.1.2 *Ortho dihydric phenol*

Arnow's Method was followed for the estimation of *ortho* dihydric phenols (Mahadevan and Sridhar, 1986). The absorbance of the pink solution was read in a spectrophotometer set at 515 nm.

3.4.2 Estimation of sugars

Reducing and non reducing sugars were determined in the samples following the method suggested by Mahadevan and Sridhar (1986). Dinitrosalicylic acid (DNS) reagent for estimation of sugars was prepared by dissolving one g of dinitrosalicylic acid, 200 mg of crystaline phenol and 50 mg sodium sulphite in one per cent solution of sodium hydroxide. Using glucose as a standard, values were expressed in mg per g of sample.

3.4.2.1 *Reducing sugars*

Methanol extracts of 500 mg samples were evaporated in a water bath. One ml of extract, two ml of distilled water and three ml of reagent were added to the test tube. Content was heated in boiling water bath, after adding one ml of 40 per cent potassium sodium tartarate allowed to cool. The intensity of dark red colour was measured at 510 nm.

3.4.2.2 *Non reducing sugar*

One ml of alcohol extract was taken in a test tube and evaporated the content to dryness in a water bath. After adding one ml of glass distilled water and one ml of 1N NH_2SO_4 it was hydrolysed for 30 min at 49°C. The tubes were then allowed to cool and two drops of methyl red indicator were added. By adding 1N NaOH, the content was neutralised and the volume was made up to ten ml. Six ml of the content was taken and by following DNS method with appropriate reagent blank as described for estimation of reducing sugar, the non reducing sugar present in the hydrolysate was estimated.

3.4.3 Estimation of total free amino acids

Procedure reported by Sadasivam and Manickam (1996) was followed for the estimation of total free amino acids in the sample.

One g of the sample was ground in ten ml of 80 per cent methanol. The homogenised mass was centrifuged and supernatent was evaporated to dryness. To one ml of the extract added one ml of Ninhydrin and heated for 20 minutes in a boiling water bath and added five ml of the diluent (50 ml of propanol-1 in 50 ml of distilled water). After fifteen minutes, the intensity of purple colour developed was measured at 570 nm in a spectrophotometer.

The percentage of amino acid content in the sample was calculated from the standard curve prepared using leucine.

3.4.4 Estimation of enzyme activities

3.4.4.1 Estimation of glucanase enzyme activity

Glucanase enzyme activity in the samples was estimated as per method suggested by Pan *et al.* (1991) with following modifications.

3.4.4.1.1 Preparation of enzyme extract

Extracted 500 mg of tissue with one ml of 0.05 M sodium acetate buffer (pH 5.0) at 4°C. The resulting homogenate was centrifuged at 10000 rpm for ten minutes in a refrigerated centrifuge.

3.4.4.1.2 Determination of β-1, 3 glucanase

The enzyme extract (62.5 µl) was mixed with the same quantity of Laminarin and incubated at 10°C for ten minutes. Then added 375 µl of DNS reagent and heated for five minutes in a boiling water bath and the final volume was made up to five ml with distilled water. The absorbance was measured using spectrophotometer set at 500 nm. Optical density of sample without incubation served as the blank.

One unit of enzyme activity is defined as the amount of enzyme that produces reducing sugar equivalent to one µ mol of glucose equivalent per ten minutes and is expressed as mg^{-1} g.

3.4.4.2 Estimation of IAA oxidase enzyme activity

Indole-3 acetic acid oxidase activity was assayed as method suggested by Sadasivam and Manickam (1996).

Frozen tissues (1.5 g) were blender homogenised in two successive ten ml aliquots of cold acetone. The homogenate was collected by Buchner filtration thorough Whatman No.1 filter paper and air dried till free of acetone odour. Freeze stored 0.2 g of acetone powder ground in two successive ten ml aliquots of 25 mM phosphate buffer with pH 6.2. The extract was collected by Buchner filtration through Whatman No.1 filter paper, after combining the filtrates and bringing up the volume to 25 ml with phosphate buffer.

To the test tube added two ml of 0.071 M phosphate buffer with pH 6.2, one ml *P*-Coumaric acid solution (25 mg of *P*-Coumaric acid dissolved in 50 ml water), one ml of manganese chloride solution (118 mg in 20 ml of water) and two ml of enzyme extract. The reaction was initiated by adding four ml of IAA solution (10 mg IAA dissolved in 40 ml water) and incubated the reaction mixture in the dark at 30°C with shaking. Without incubation and after 60 min of incubation one ml each of enzyme extracts were withdrawn and 5.2 ml of perchloric acid and half ml of 0.1N ferric nitrate solution were added and the volume was made up to ten ml with distilled water. After incubating the reaction mixture in the dark for 60 minutes, absorbance was measured at 535 nm in a spectrophotometer.

The enzyme activity was calculated as follows and expressed as mg^{-1} g.

Enzyme activity = Absorbance at 60 minutes - absorbance at zero minutes

3.4.4.3 *Estimation of peroxidase enzyme activity*

Peroxidase activity was assayed by the method suggested by Sadasivan and Manickam (1996). Guaiacol was used as the substrate and the rate of formation of guaiacol dehydrogenation product was assessed as a measure of the peroxidase activity.

$$\text{Guaiacol} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Oxidised guaiacol} + 2\,H_2O$$

3.4.4.3.1 *Preparation of enzyme extract*

Extracted one g tissue in three ml of 0.01 M phosphate buffer (pH 7.0) by grinding in chilled mortar and pestle. The homogenate was centrifuged at 18000 rpm for fifteen min in a refrigerated centrifuge at 5°C. The supernatant liquid collected was used as the enzyme source.

3.4.4.3.2 *Estimation of peroxidase activity*

Spectrophotometer was set at 436 nm with 3.1 ml phosphate buffer. Guaiacol (50 µl-240 mg of guaiacol in 100 ml of water), enzyme extract (20 µl) and hydrogen peroxide (30 µl-0.14 ml of 30 per cent hydrogen peroxide in 100 ml water) were taken in a cuvettee and mixed well and spectrophotometer was set at zero. When the absorbance of the sample had increased by 0.05, started the stop watch and noted the time required in minutes (Δt) to increase the absorbance by 0.1.

Enzyme activity in units per litre of extract was calculated as follows

$$\text{Peroxidase enzyme activity units / litre of extract} = \frac{3.18 \times 0.02 \times 1000}{6.39 \times 1 \times \Delta t \times 0.02} = \frac{498}{\Delta t}$$

3.4.4.4 Estimation of polyphenol oxidase enzyme activity

Polyphenol oxidase enzyme activity was assayed by the method suggested by Malik and Singh (1980).

$$\text{Catechol} \xrightarrow[\text{Oxidase}]{\text{Catechol}} \text{Quinone}$$

Buffer solutions used for the extraction and assay were 0.1 M monobasic and dibasic sodium phosphate.

Catechol 0.01 M dissolved in 100 ml of phosphate buffer, was used as the substrate.

3.4.4.4.1 Preparation of enzyme extract

Enzyme extract was prepared by grinding one g of tissue in mortar and pestle. Resulting supernatant liquid was used for the enzyme assay.

3.4.4.4.2 Estimation of polyphenol oxidase activity

To a clean cuvettee added three ml of buffered catechol. Inserted the cuvettee in the spectrophotometer and absorbance was adjusted to zero at 495 nm.

Then half ml of enzyme extract was added to the cuvettee, mixed immediately and noted the changes in absorbance for every 30 sec upto five min.

The changes in absorbance values per minute were plotted and linear phase of curve was drawn on the basis of OD value.

3.5 Statistical analysis

Statistical analysis of the data recorded was carried out as per the techniques described by Panse and Sukhatme (1985).

Results

RESULT

The results generated from anatomical and biochemical investigations on *Phytophthora* foot rot disease reaction in *Piper* spp. are presented in this chapter.

4.1 Anatomical investigations

Anatomical investigations on leaves, stems and roots of the three genotypes under present study revealed that there existed variation among genotypes with respect to size, shape and arrangement of the cells.

4.1.1 Leaf anatomy of *Piper* spp.

The various anatomical parametres observed are presented in Table 1 and Plates II and III.

4.1.1.2 Upper epiderm

The upper leaf epiderm consists of an outer layer of cuticle and epidermal cells with trichomes. The two species of the genus *Piper* and susceptible and tolerant genotypes of *P. nigrum* exhibited variation in anatomical features of upper epiderm.

4.1.1.2.1 Cuticle

The cuticle thickness varied in the two species studied. Thicker cuticle was observed in *P. nigrum* when compared with *P. colubrinum* (Plate II a-c). Of the *P. nigrum* genotypes, susceptible cultivar Panniyur 1 had a thicker cuticle (0.67 µm) as compared to tolerant cultivar Kalluvally (0.22 µm).

Table 1. Leaf anatomy of *Piper* spp.

Sl. No.	Parameters (Units in µm)	*Piper nigrum* L.							*Piper colubrinum* L.		
		Panniyur 1			Kalluvally						
		Min.	Mean	Max.	Min.	Mean	Max.	✡Mean	Min.	Mean	Max.
I	*Upper epiderm*										
1	Cuticle thickness	0.440	**0.67**	0.88	0.15	**0.22**	0.30	*0.45*	0.10	**0.40**	0.50
2	Epiderm thickness	4.40	**4.62**	4.80	3.00	**4.18**	5.00	*4.40*	3.00	**4.12**	5.50
*3	Epiderm cell size	19.92	**50.00**	18.30	14.94	**23.17**	133.20	*36.58*	9.96	**16.00**	20.75
4	Epidermal appendages	6.00	**8.00**	10.00	3.00	**4.00**	19.00	*6.00*	2.00	**6.00**	9.00
II	*Mesophyll*										
*1	Mesophyll cells size	40.00	**112.00**	131.0	27.00	**54.41**	133.00	*83.30*	49.00	**80.79**	116.00
2	Intercellular spaces	0.83	**4.60**	8.30	1.66	**3.04**	4.98	*3.82*	0.16	**0.28**	1.66
*3	Spongy parenchyma cell size	956.00	**1474.00**	4364.00	876.00	**1629.00**	1912.00	*152.00*	3.28	**342.00**	790.00
4	Palisade tissue thickness	8.30	**9.96**	11.62	6.64	**9.96**	13.28	*9.96*	9.96	**12.90**	16.60
5	Number of vascular bundles	1.00	**2.00**	3.00	2.00	**2.00**	3.00	*2.00*	2.00	**2.00**	3.00
*6	Vascular bundle size	1513.00	**1577.00**	1640.00	912.00	**2217.00**	2390.00	*1897.00*	2390.00	**2656.00**	3984.00
7	Distance between bundles	8.30	**9.20**	11.30	8.30	**9.12**	13.90	*9.16*	43.16	**54.80**	66.40
*8	Mucilage canal size	10.40	**12.33**	14.94	6.64	**49.80**	80.60	*31.05*	12.14	**51.03**	85.71
9	Collenchyma tissue thickness	8.30	**9.96**	11.62	9.96	**12.46**	14.94	*11.20*	8.30	**12.60**	15.30
III	*Lower epiderm*										
1	Lower epiderm thickness	2.00	**2.20**	2.90	0.70	**4.84**	8.30	*3.52*	3.30	**5.60**	11.62
*2	Lower epiderm cell size	13.90	**15.10**	16.60	41.50	**45.18**	49.80	*30.14*	16.70	**38.80**	49.80
3	Cuticle thickness	0.70	**0.91**	1.20	0.17	**1.03**	2.49	*0.97*	0.17	**0.83**	1.66
4	Stomata per microscopic field	72.00	**93.00**	130.00	52.00	**67.00**	90.00	*80.00*	41.00	**56.00**	71.00
5	Stomatal index per square mm	4.10	**5.06**	7.41	2.97	**3.76**	5.13	*4.41*	2.33	**3.31**	4.05
*6	Stomatal size	93.00	**175.00**	259.00	93.00	**181.00**	219.00	*178.00*	159.00	**365.00**	498.00
7	Stomatal pore width	1.50	**1.81**	2.00	0.83	**1.83**	2.50	*1.82*	1.66	**2.05**	3.32

*Measurement in μm^2 Min– Minimum   Max– Maximum

✡Worked out from the means of Panniyur 1 and Kalluvally.

4.1.1.2.2 Epiderm

The thickness of upper epiderm also varied in different genotypes studied. Thicker epidermis was observed in *P. nigrum* whereas *P. colubrinum* recorded thinner epidermis. When the susceptible and tolerant genotypes of *P. nigrum* were compared, Panniyur 1 had thicker epiderm (4.62 µm) as compared to Kalluvally (4.18 µm).

The size of the epidermal cells was also found to be smaller in *P. colubrinum* (16.00 µm^2) when compared to *P. nigrum* (36.58 µm^2). The tolerant cultivar Kalluvally recorded smaller epidermal cells (23.17 µm^2) as compared to Panniyur 1 (50.00 µm^2).

Trichomes arising from epidermal cells appeared unicellular or multicellular in all the genotypes.

Number of epidermal appendages appeared to be almost the same in the two species studied. However, Kalluvally had more number of trichomes (19.00) than Panniyur 1 (10.00).

4.1.1.3 Mesophyll

The mesophyll region comprises of round to ovoid mesophylls, spongy parenchyma and chlorenchymatous palisade tissues. Size and arrangement of cells of these tissues differed in the three genotypes studied.

Greater variation in mesophyll cell size was noticed in *P. nigrum* (27.00 to 133.00 µm^2) as compared to *P. colubrinum* (49.00 to 116.00 µm^2). The range of variation when compared, Kalluvally exhibited greater variation (27.00 to 133.00 µm^2) than Panniyur 1 (40.00 to 131.00 µm^2).

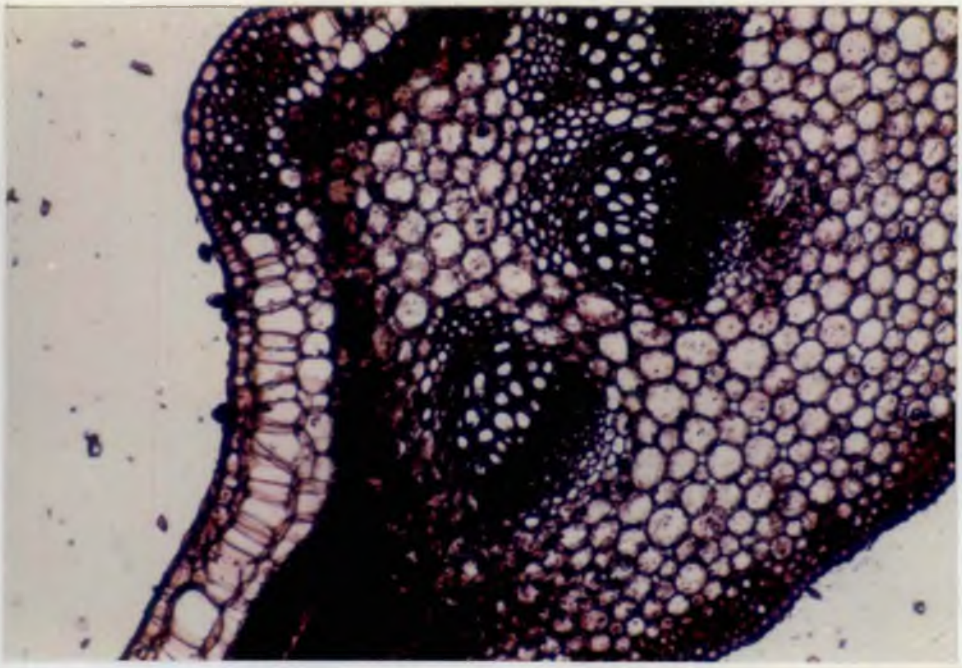
The cells of *P. colubrinum* were compactly arranged with less intercellular spaces (0.28 µm) as compared to *P. nigrum* (3.82 µm). Similarly, the cells of Kalluvally were compactly arranged (3.04 µm) as compared to Panniyur 1 (4.60 µm).

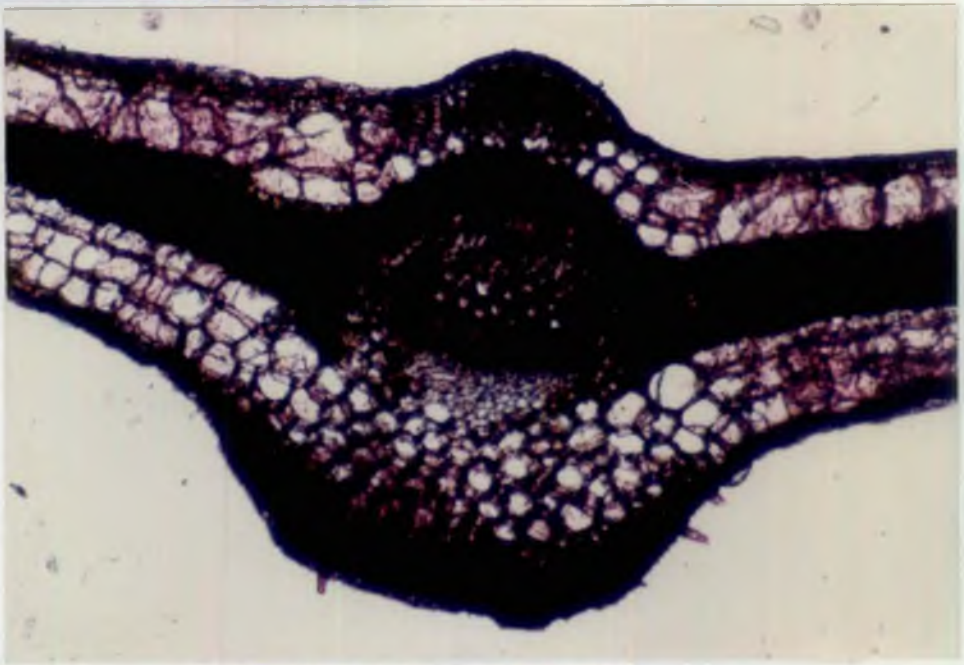
Plate II Leaf anatomy of *Piper* spp. (midrib region) showing trichomes, upper and lower epiderm, collenchyma and mesophylls (x = 500)

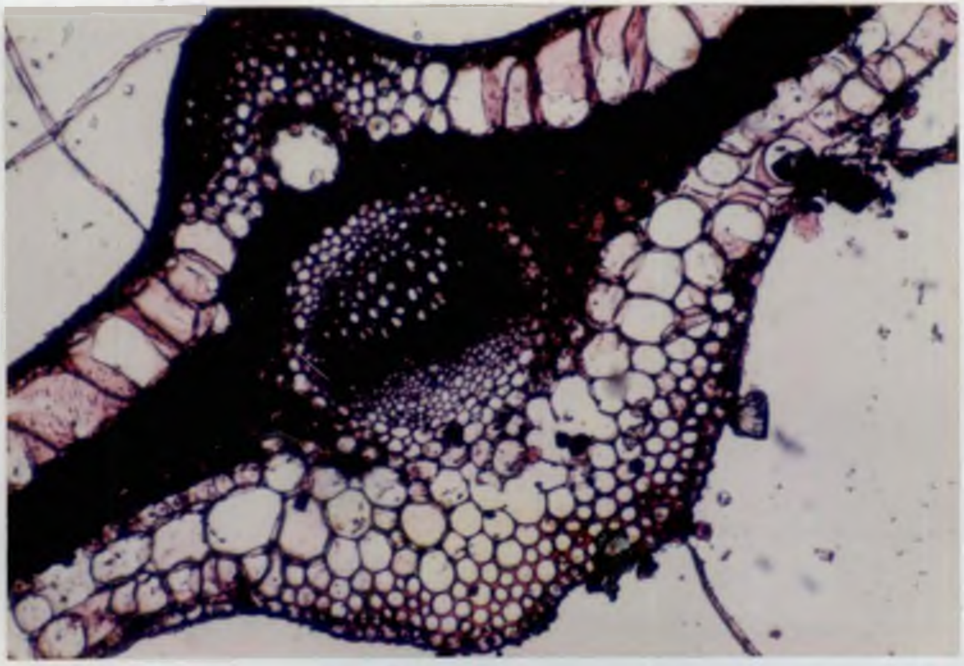
a) *Piper colubrinum* L.

b) *Piper nigrum* L. (Panniyur 1)

(c) *Piper nigrum* L. (Kalluvally)







Spongy parenchymatous cells were present in one or two layers on either sides of chlorenchymatous palisade tissues giving isobilateral orientation to the leaves. Size of these cells was 4.5 times larger in *P. nigrum* as compared to *P. colubrinum*. In the two genotypes of *P. nigrum*, spongy parenchymatous cells were bigger in Kalluvally than in Panniyur 1.

Palisade tissues of *P. colubrinum* were 30 per cent more thicker than that of *P. nigrum*. In the genotypes of *P. nigrum* studied no such variation was observed in the thickness of palisade tissues.

Beneath the vascular bundles and in the mesophyll region, round to oval shaped mucilage canals were seen, which were larger in *P. colubrinum* as compared to *P. nigrum*. Of the two varieties of *P. nigrum*, Kalluvally had large sized mucilage canals (49.80 μm^2) when compared to Panniyur 1 (12.33 μm^2).

Open, collateral, oval shaped vascular bundles with xylem, phloem and cambium were observed in the midrib region individually or in groups of two to three.

Vascular bundles and xylem vessels were larger in *P. colubrinum* as compared to *P. nigrum*. The tolerant genotype Kalluvally had bigger bundles (2217.00 μm^2) as compared to Panniyur 1 (1577.00 μm^2).

Average number of bundles remains the same in all the genotypes studied. The bundles of *P. colubrinum* were distantly placed than *P. nigrum* wherein corresponding distances between the bundles were 54.80 and 9.16 µm respectively. The bundles were separated by a maximum distance of 11.30 µm in Panniyur 1 and 13.90 µm in Kalluvally.

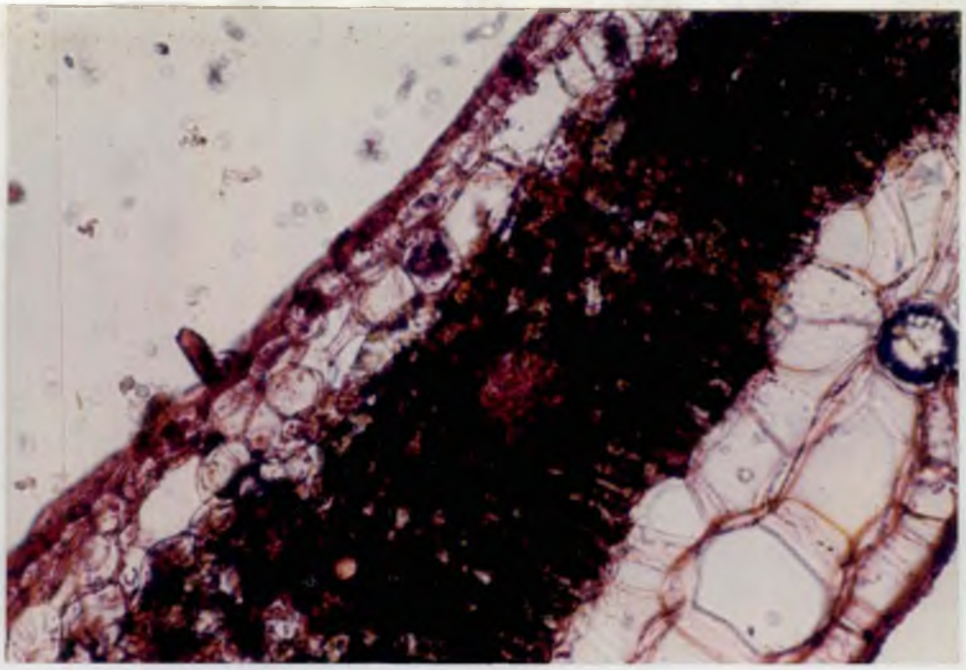
In the midrib underneath upper epidermis and aloof lower epidermis, five to six layered lacunar collenchymatous cells were observed. Thickness of these tissue was

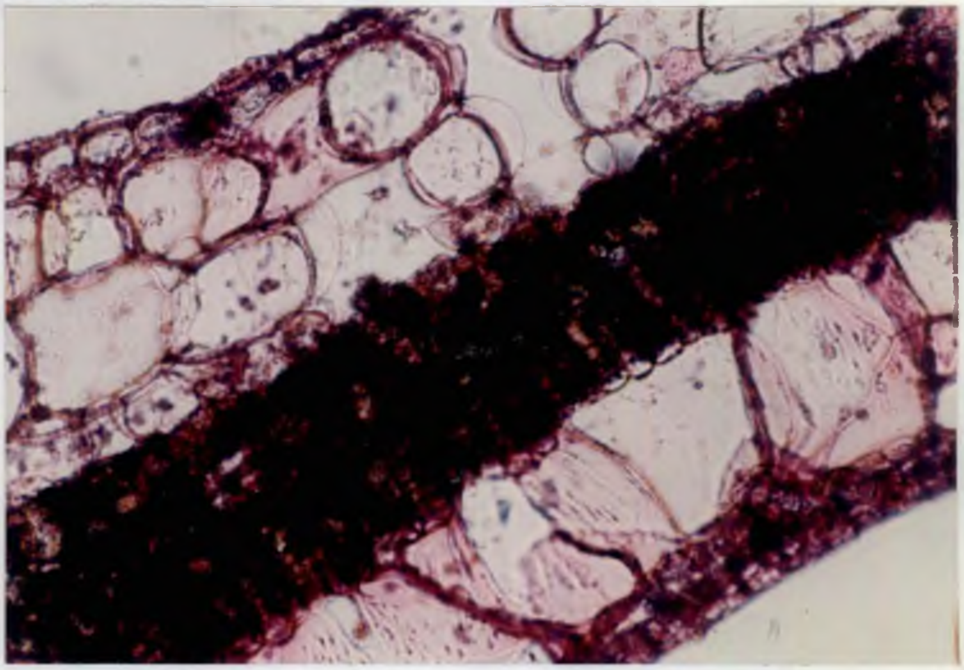
Plate III. Leaf anatomy of *Piper* spp. (lamina region) showing spongy parenchyma, palisade and mucilage canal (x = 1250)

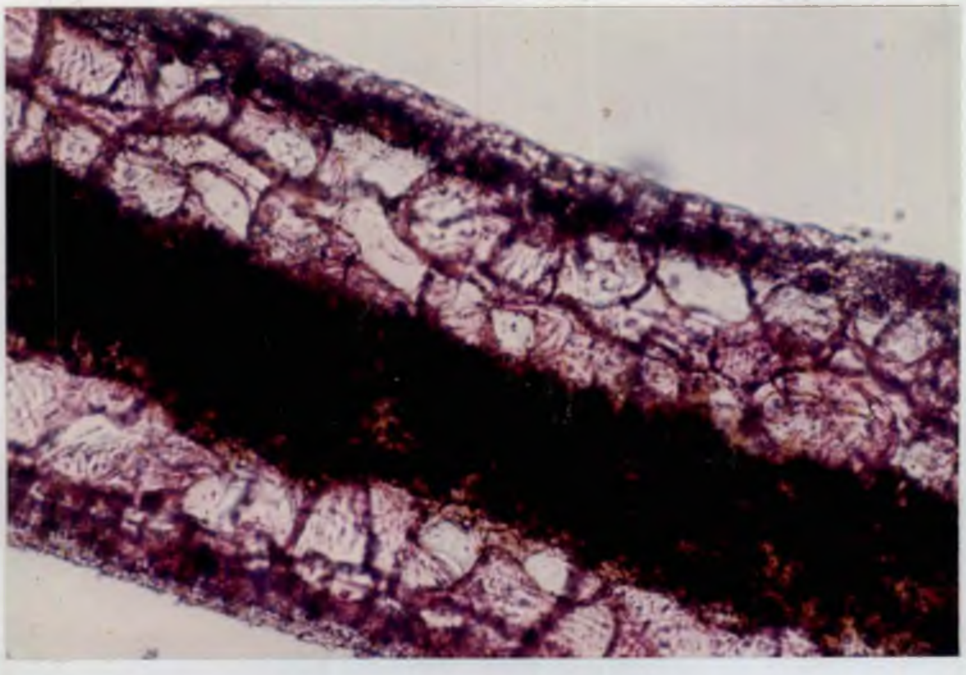
a) *Piper colubrinum* L.

b) *Piper nigrum* L. (Panniyur 1)

(c) *Piper nigrum* L. (Kalluvally)







more in *P. colubrinum* (12.60 μm) as compared to *P. nigrum* (11.20 μm). Similarly Kalluvally had higher thickness (12.46 μm) as compared to Panniyur 1 (9.96 μm).

4.1.1.4 Lower epiderm

Histologically lower epiderm consists of an epiderm, stomata, and an outer layer of waxy cuticle.

Structure of upper and lower epidermis was the same except for the presence of stomata in lower epidermis. Compared to upper epidermis, lower epiderm was much thicker. The thickness of the lower epidermis was high in *P. colubrinum* (5.60 μm) as compared to *P. nigrum* (3.52 μm). The epidermal cells of *P. colubrinum* were also larger in size (38.80 μm²) when compared to *P. nigrum* (30.10 μm²). When the two cultivated varieties were compared, Kalluvally had large sized (45.18 μm²) epidermal cells than Panniyur 1 (15.10 μm²).

In the lower epidermis, stomata were abundant in *P. nigrum* as compared to *P. colubrinum.* In a microscopic field, stomata ranged from 52.00 to 130.00 in *P. nigrum* and 41.00 to 71.00 in *P. colubrinum*. Higher stomatal index was observed in *P. nigrum* which ranged from 2.97 to 7.41 per square mm. Of the two varieties studied, Panniyur 1 recorded higher stomatal number and stomatal index. In contrast to the stomatal number, stomatal size was two times larger in *P. colubrinum* than *P. nigrum* Similarly, pore width was also more in *P. colubrinum* (2.05 μm) than *P. nigrum* (1.82 μm). Among *P. nigrum* genotypes, Kalluvally had large sized (181 μm) stomata as compared to Panniyur 1 (175 μm). There was no variation in pore width in the two genotypes.

Cuticle was thicker in *P. nigrum* (0.97 μm) as compared to *P. colubrinum* (0.83 μm). In *P. nigrum,* Kalluvally had thicker cuticle (1.03 μm) than Panniyur 1 (0.91 μm).

The fundamental structure of the leaf was the same in all the genotypes but, the susceptible, tolerant and immune genotypes differed themselves in the size and arrangement of cells.

4.1.2 Stem anatomy of *Piper* spp.

4.1.2.1 Epiderm

The basic anatomical structure of stem in susceptible, tolerant and immune genotypes was the same. The cuticle, epiderm, hypoderm and epidermal appendages formed the basis of this region.

The data relating to the stem anatomy of *Piper* spp. are presented in Table 2 and Plates III and IV. Cuticle was thinner in *P. colubrinum* (1.90 µm) when compared to *P. nigrum* (2.67 µm). Among the *P. nigrum* genotypes, Kallluvally had less thickned cuticle (1.80 µm) when compared to Panniyur 1 (3.54 µm).

Epidermal cells of *P. colubrinum* were smaller in size (21.20 µm^2) as compared to *P. nigrum* (49.70 µm^2). Similarly, in *P. nigrum*, epidermal cells of Kalluvally were smaller (26.60 µm^2) as compared to Panniyur 1 (72.80 µm^2). When two varieties were compared, the cell size was larger in Panniyur 1 (72.80 µm^2) than Kalluvally (26.60 µm^2).

Smaller epidermal cells imparted thinner epidermis to *P. colubrinum*. Also cells of *P. colubrinum* were more uniform in size exhibiting less variation (9.96 to 33.20 µm^2) as compared to *P. nigrum* (25.00 to 83.00 µm^2). When the two varieties were compared, the range of variation in cell size was more in Panniyur 1 (34.86 to 83.00 µm^2) than Kalluvally (25.00 to 41.02 µm^2).

The epidermal appendages ranged from 12.00 to 31.00 in *P. nigrum* and 7.00 to 19.00 in *P. colubrinum*. Panniyur 1 had more appendages (25.00) as compared to Kalluvally (18.00).

Table 2. Stem anatomy of *Piper* spp.

Sl. No.	Parameters (Units in µm)	*Piper nigrum* L.							*Piper colubrinum* L.		
		Panniyur 1			Kalluvally						
		Min.	Mean	Max.	Min.	Mean	Max.	✡Mean	Min.	Mean	Max.
I	*Epiderm*										
1	Cuticle thickness	2.90	**3.54**	4.15	1.32	**1.80**	2.16	*2.67*	0.99	**1.90**	1.97
2	Epiderm thickness	5.00	**6.80**	8.30	6.70	**7.50**	9.96	*7.15*	6.00	**6.64**	8.30
*3	Epiderm cell size	34.86	**72.80**	83.00	25.00	**26.60**	41.02	*49.70*	9.96	**21.20**	33.20
4	Epidermal appendages	19.00	**25.00**	31.00	12.00	**18.00**	24.00	*21.50*	7.00	**12.00**	19.00
5	Hypoderm thickness	24.94	**33.20**	41.50	23.24	**25.45**	28.22	*29.33*	8.30	**10.60**	14.94
*6	Hypoderm cell size	6.64	**9.96**	19.00	9.96	**27.39**	49.64	*18.67*	4.00	**19.23**	23.00
II	*Cortex*										
1	Cortex thickness	20.00	**36.60**	69.00	60.00	**68.00**	76.00	*52.30*	66.00	**73.00**	99.60
*2	Cortical cell size	6.64	**31.42**	41.25	6.64	**28.22**	41.50	*29.82*	5.00	**39.47**	49.96
3	Intercellular spaces	0.16	**0.18**	0.33	0.16	**0.79**	0.90	*0.49*	0.90	**0.98**	1.50
4	Chlorenchyma tissue thickness	9.60	**14.00**	17.00	11.60	**12.31**	23.00	*13.20*	11.17	**14.33**	37.81
5	Sclerenchyma tissue thickness	9.96	**25.90**	26.50	16.66	**33.20**	66.40	*29.55*	22.13	**41.03**	47.30
III	*Medulla*										
1	Distance between cuticle and peripheral bundles	46.00	**61.10**	79.80	99.60	**101.00**	119.00	*81.05*	53.10	**73.00**	99.60
2	Peripheral vascular bundle number	12.00	**18.00**	30.00	18.00	**20.00**	32.00	*19.00*	24.00	**36.00**	44.00
*3	Peripheral vascular bundle size	79.68	**403.00**	876.00	278.00	**893.00**	1192.00	*648.00*	717.00	**1454.00**	1992.00
4	Distance between peripheral bundles	48.00	**119.60**	184.00	53.12	**132.80**	332.00	*126.20*	99.60	**287.00**	478.00
5	Distance between peripheral and medullary bundles	80.00	**107.00**	160.00	148.00	**206.00**	278.00	*157.00*	66.40	**73.00**	126.00
6	Medullary vascular bundle number	7.00	**9.00**	11.00	7.00	**10.00**	11.00	*9.50*	9.00	**11.00**	13.00
*7	Medullary vascular bundle size	876.00	**1096.00**	1394.00	372.00	**1096.00**	1593.00	*1096.00*	1275.00	**1483.00**	1674.00
8	Distance between medullary bundles	92.00	**120.00**	144.00	100.00	**143.00**	199.00	*131.50*	159.60	**278.00**	478.00
*9	Mucilage canal size	321.00	**597.00**	612.00	693.00	**792.00**	801.00	*694.50*	695.00	**934.00**	1003.00
*10	Pith size	1340.00	**1438.00**	1634.00	613.00	**796.00**	841.00	*1117.00*	485.00	**584.00**	619.00

*Measurement in μm^2 Min.- Minimum Max.- Maximum

✡Worked out from the means of Panniyur 1 and Kalluvally.

Hypodermal cells were comparatively smaller in size than the epidermal cells but its thickness was higher.

Tolerant and susceptible genotypes had thicker hypoderm as compared to the immune genotype (Plate III a-c). The thickness ranged from 8.30 to 14.94 µm in *P. colubrinum*, 24.94 to 41.50 µm in Panniyur 1 and 23.24 to 28.22 µm in Kalluvally. Hypodermal cells were larger in *P. colubrinum* (19.23 µm^2) as compared to *P. nigrum* (18.67 µm^2). The cells of Kalluvally were almost three times larger than Panniyur 1.

Pericyclic sclerchyma appeared continuous in a wavy ring in *P. nigrum*, but it formed a discontinuous ring around the peripheral vascular bundles in *P. colubrinum*. Sclenchymatous tissues were more sclerified in Panniyur 1 than Kalluvally.

4.1.2.3 Cortex

A well defined cortex with an outer chlorenchymatous zone and inner five to six cell deep sclerenchymatous zone were observed.

The thickness of the cortex was high in *P. colubrinum* (73.00 µm) when compared to *P. nigrum* (52.30 µm). In the genotypes of *P. nigrum*, Kalluvally had more thicker cortex (68.00 µm) as compared to Panniyur 1 (36.60 µm). The range of variation in cortical thickness appeared lesser in Kalluvally than Panniyur 1.

Thickness of sclerenchymatous tissue was maximum in *P. colubrinum* (41.03 µm) as compared to *P. nigrum* (29.55 µm^2). In Kalluvally, sclerenchyma was 1.3 times thicker than Panniyur 1.

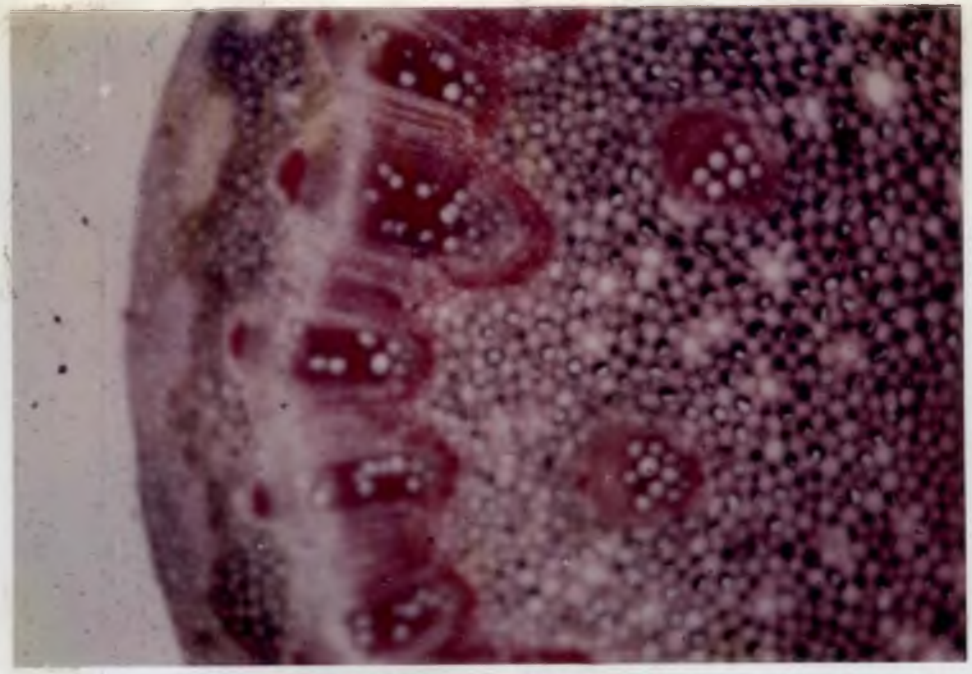
The cortical cells of *P. colubrinum* were larger in size (39.47 µm^2) and were arranged loosely with more intercellular spaces (0.98 µm). In *P. nigrum*, cortical cells

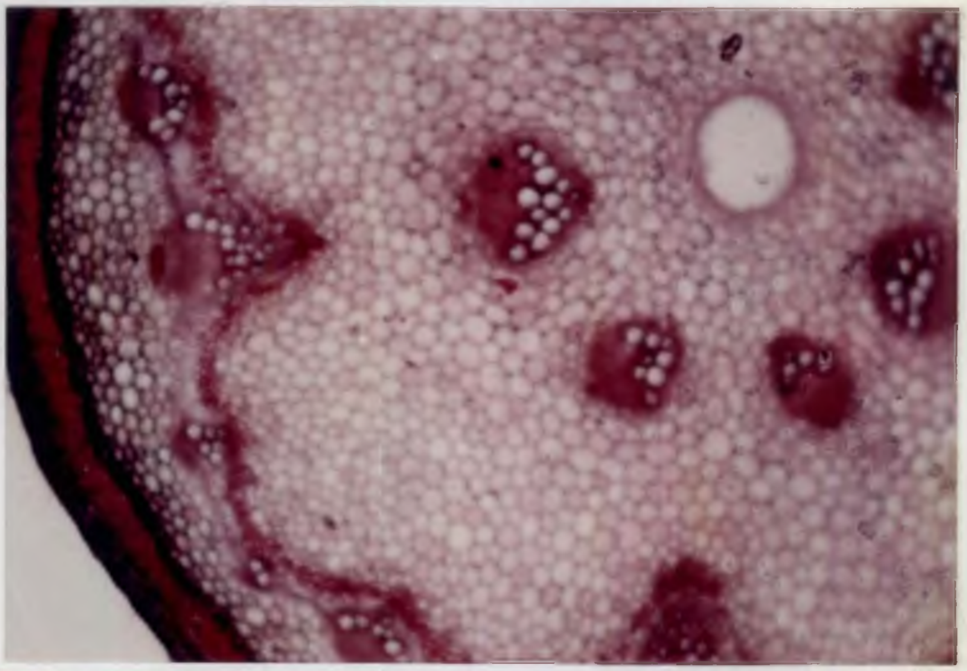
Plate IV. Stem anatomy of *Piper* spp. showing epiderm, hypoderm, pericyclic sclerenchyma and vascular bundles (x = 500)

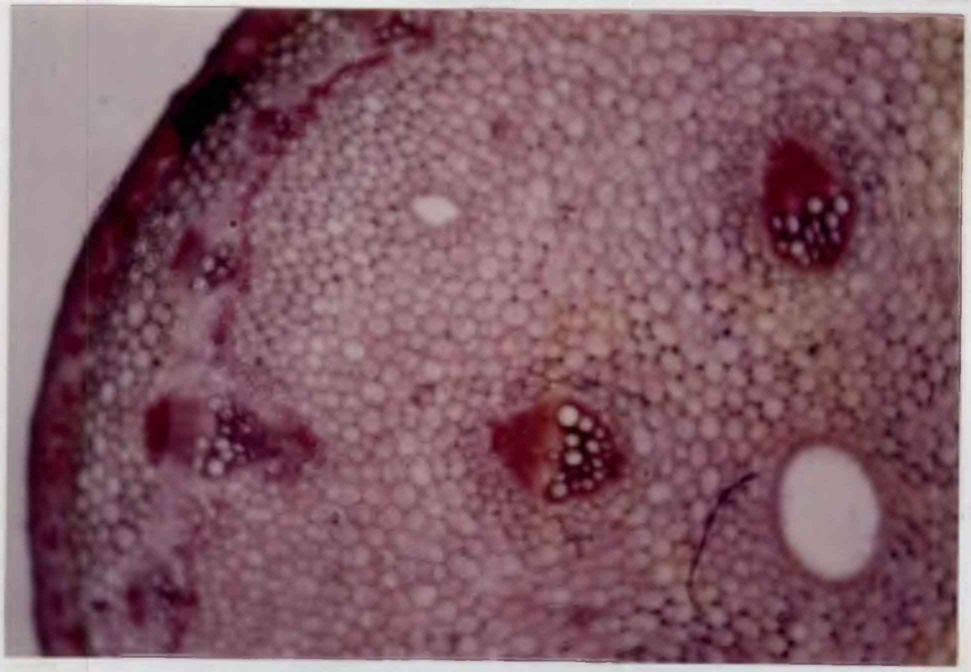
a) *Piper colubrinum* L.

b) *Piper nigrum* L. (Panniyur 1)

(c) *Piper nigrum* L. (Kalluvally)







of susceptible genotype were comparatively larger (31.42 μm²) with less intercellular spaces (0.18 μm) than the tolerant genotype.

4.1.2.3 *Medulla*

Medulla zone formed the major part of the stem and comprises two types of vascular bundles viz., peripheral and medullary vascular bundles, mucilage canals and pith.

Both peripheral and medullary vascular bundles were more in *P. colubrinum* than *P. nigrum*. Similarly, the tolerant genotype of *P. nigrum* had more bundles than the susceptible one. Bundles of *P. colubrinum* were larger than that of *P. nigrum*. Likewise, bundles of Kalluvally were larger than that of Panniyur 1. Both peripheral and medullary vascular bundles were distantly placed in *P. colubrinum* when compared to *P. nigrum*. Plate IV shows an enlarged view of medullary vascular bundles in the three genotypes.

The distance between peripheral and medullary bundle rings was found to be less (73.00 μm) in *P. colubrinum* compared to *P. nigrum* (157.00 μm). In *P. nigrum* genotypes, these two rings were closer in Panniyur 1 (107.00 μm) as compared to Kalluvally (206.00 μm).

In the medulla region, mucilage canals were seen, the size of which was larger in *P. colubrinum* (934.00 μm²) and smaller in *P. nigrum* (694.50 μm²). Among genotypes of *P. nigrum*, Kalluvally had larger canals (792.00 μm²) as compared to Panniyur 1 (597.00 μm²).

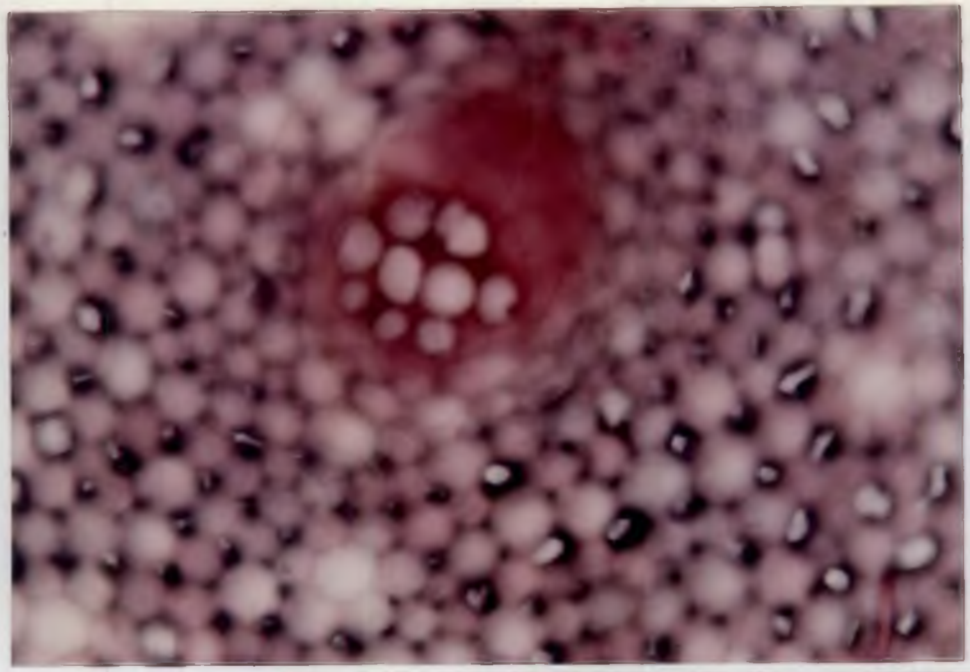
Central part of the stem was represented by the pith which was larger in *P. nigrum* (1117.00 μm²) and smaller in *P. colubrinum* (584.00 μm²). In *P. nigrum*, Kalluvally had smaller pith than Panniyur 1.

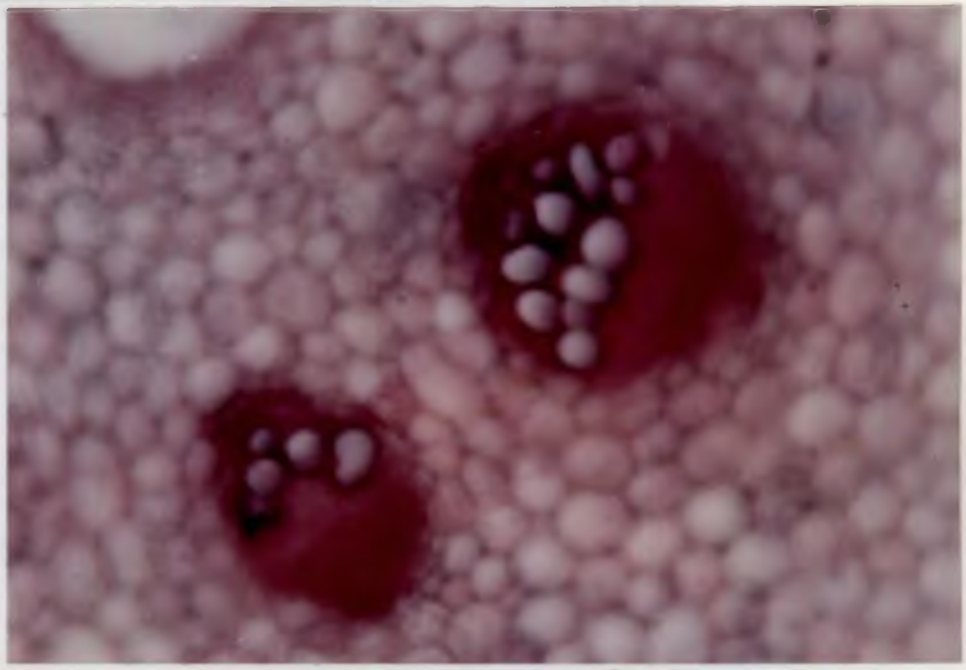
Plate V. Stem anatomy of *Piper* spp. - magnified view of medullary vascular bundles (x = 1250)

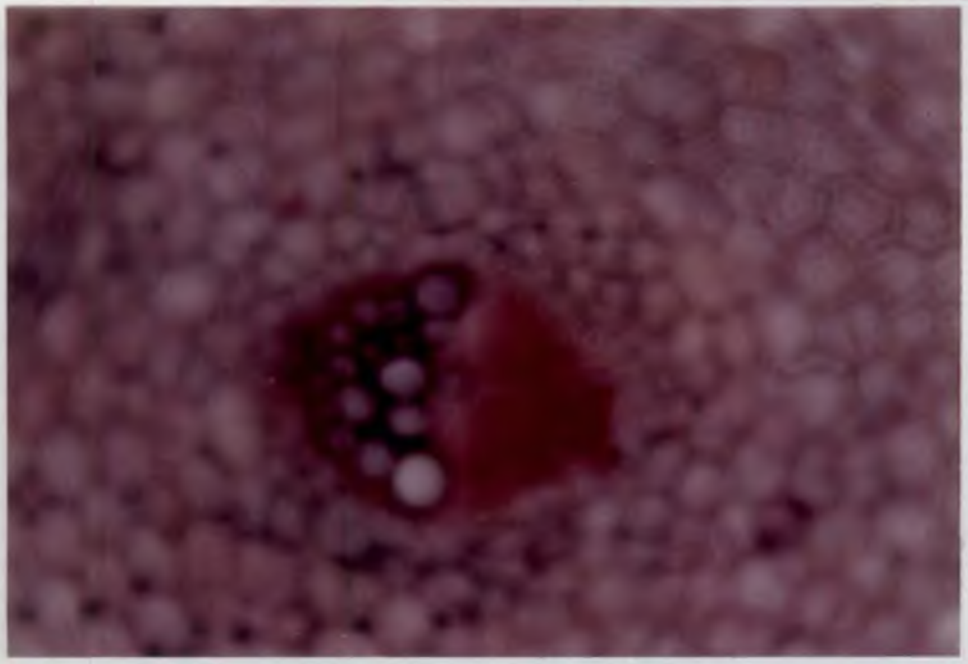
a) *Piper colubrinum* L.

b) *Piper nigrum* L. (Panniyur 1)

(c) *Piper nigrum* L. (Kalluvally)







Stem of *P. colubrinum* was characterised by less thickned cuticle, compact epidermis, hypodermis, larger and compact cortical cells, discontinuous sclerenchymatous pericyclic layer, more number of larger vascular bundles, larger mucilage canals and smaller pith.

4.1.3 Root anatomy of *Piper* spp.

Microscopically, internal structure of roots of both species was the same, but differed with respect to cell size and arrangement. Table 3 and Plates VI and VII describes the root anatomical features of *Piper* spp.

4.1.3.1 Epiderm

The epiderm comprised of an outer layer of piliferous epidermal cells and root hairs. The thickness of these cells was greater in *P. colubrinum* when compared to *P. nigrum*. Among *P. nigrum* genotypes, Kalluvally had thicker epiderm (18.28 µm) as compared to Panniyur 1 (12.80 µm).

Roots of *P. colubrinum* recorded lesser number of root hairs (27.00) when compared to *P. nigrum* (35.00). Similarly, Kalluvally had less number of root hairs (33.00) when compared to Panniyur 1 (37.00).

4.1.3.2 Cortex

The cortex region consists of parenchymatous and endodermal cells.

Cortical cells of *P. colubrinum* were closely packed and exhibited much variation in its size. The cell size varied from 39.80 to 332.00 µm^2 in *P. colubrinum* and 19.90 to 182.60 µm^2 in *P. nigrum*. However, the mean cell size was higher in *P. nigrum* (126.71 µm^2) than in *P. colubrium* (91.56 µm^2). The cortical cells were larger (157.70 µm^2) in Kalluvally than in Panniyur 1 (95.72 µm^2).

Table 3. Root anatomy of *Piper* spp.

		Piper nigrum L.							*Piper colubrinum* L.		
Sl. No.	Parameters	Panniyur 1			Kalluvally						
	(Units in µm)	Min.	Mean	Max.	Min.	Mean	Max.	✡Mean	Min.	Mean	Max.
I	*Epiderm*										
1	Epiderm thickness	12.40	**12.80**	14.11	16.16	**18.28**	19.64	*15.54*	16.16	**20.01**	24.97
2	Epidermal appendages	34.00	**37.00**	45.00	34.00	**33.00**	36.00	*35.00*	26.00	**27.00**	30.00
II	*Cortex*										
*1	Cortical cell size	19.92	**95.72**	157.70	119.50	**157.70**	182.60	*126.71*	39.81	**91.56**	332.00
2	Inter cellular spaces	1.33	**1.49**	1.67	1.50	**1.87**	2.10	*1.68*	1.50	**1.61**	1.66
3	Distance between epidermis and stele	114.10	**171.40**	199.20	212.50	**227.10**	239.40	*199.25*	199.20	**215.80**	232.40
4	Endoderm thickness	4.00	**6.06**	8.00	4.00	**5.20**	8.00	*5.63*	4.00	**6.60**	10.00
III	*Stele*										
*1	Stele size	10372.00	**10458.00**	11872.00	5845.00	**6374.00**	7139.00	*8416.00*	2790.00	**3818.00**	4012.00
2	Pericycle thickness	6.64	**8.22**	9.13	6.64	**9.88**	11.62	*9.05*	8.30	**10.60**	13.28
*3	Pericycle cell size	29.90	**43.30**	50.80	39.90	**52.60**	59.80	*47.95*	39.90	**54.70**	79.70
*4	Vascular bundle size	438.30	**471.40**	511.30	730.40	**1474.00**	2390.00	*973.00*	132.80	**202.50**	319.00
5	Distance between vascular bundles	6.64	**17.70**	33.20	9.96	**23.51**	33.20	*20.61*	13.28	**21.02**	33.20
6	Distance between pericycle and stele axis	225.80	**237.60**	245.70	99.60	**105.10**	112.90	*171.35*	79.68	**84.99**	89.64

*Measurement in μm^2 Min– Minimum Max– Maximum

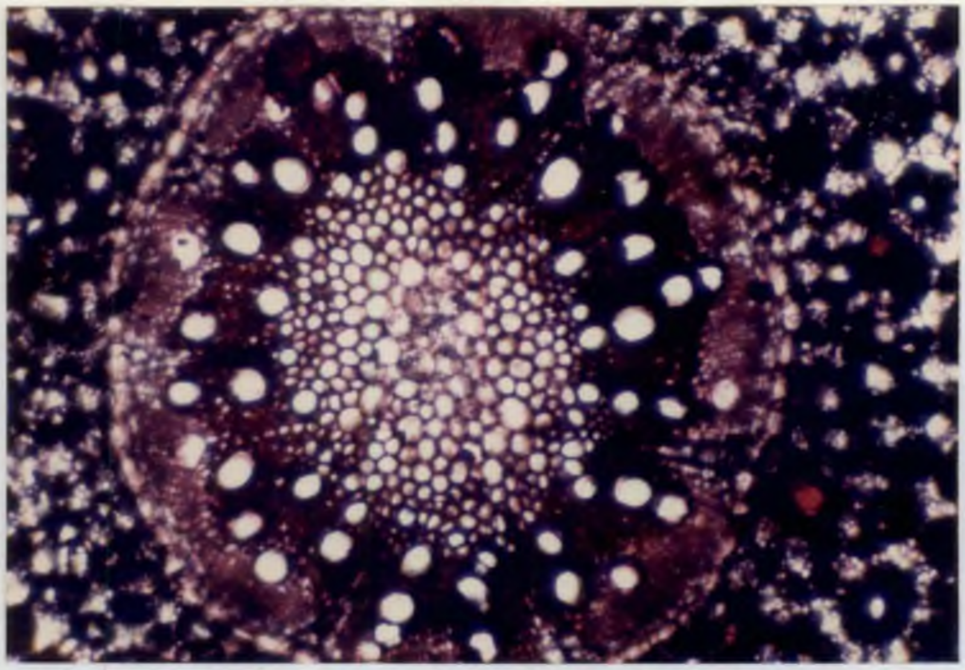
✡Worked out from the means of Panniyur 1 and Kalluvally

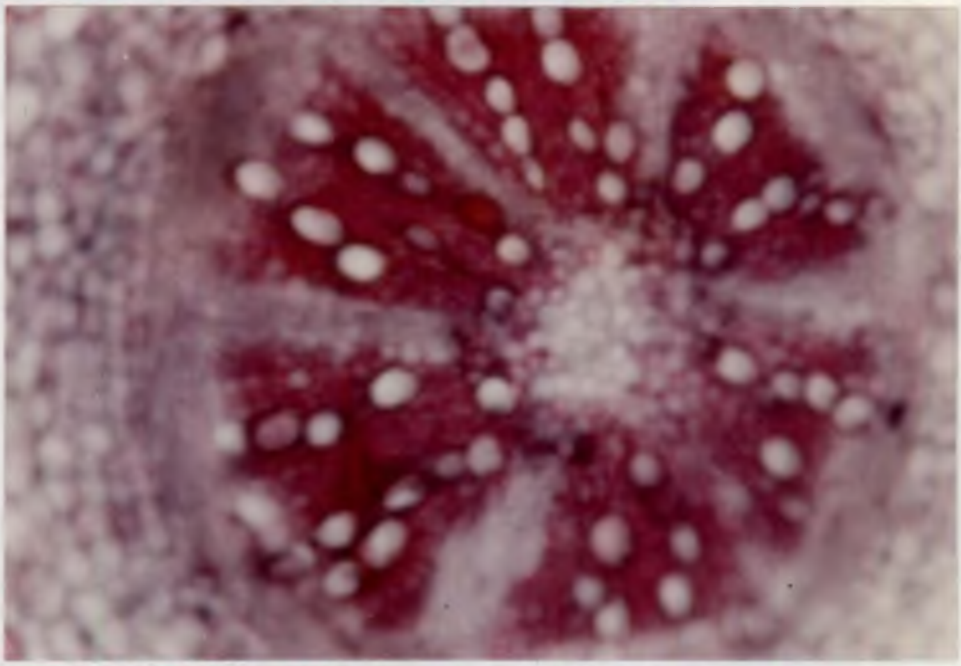
Plate VI. **Root anatomy of *Piper*** spp. showing endodermis, xylem vessels and pith (x = 1250)

a) *Piper colubrinum* L.

b) *Piper nigrum* L. (Panniyur 1)

(c) *Piper nigrum* L. (Kalluvally)





Intercellular spaces were comparatively smaller in *P. colubrinum* (1.61 μm) than *P. nigrum* (1.68 μm). Among *P. nigrum* genotypes, Kalluvally had larger intercellular spaces, the size of which ranged from 1.50 to 2.10 μm when compared to Panniyur 1.

Endodermis, the innermost layer of cortex was much thicker in *P. colubrinum* (6.60 μm) than in *P. nigrum* (5.63 μm). Among *P. nigrum* genotypes, tolerant cultivar Kalluvally had thinner endodermis (5.20 μm) as compared to the susceptible Panniyur 1 (6.06 μm).

4.1.3.3 Stele

A compact stele was formed by the assemblage of pericycle and vascular bundles.

Large sized stele was noticed in *P. nigrum* (8416.00 μm²). Among *P. nigrum* genotypes, stele of Panniyur 1 was larger (10458.00 μm²) than Kalluvally (6374.00 μm²). Inside the stele vascular bundles were arranged, the number of which was relatively the same in both the species studied. Comparatively smaller bundles were noticed in *P. nigrum*. The size of bundles in *P. nigrum* was 973.00 μm² and that of *P. colubrinum* was 202.50 μm². Among *P. nigrum* genotypes, Kalluvally had distantly placed larger bundles (1474.00 μm²) than Panniyur 1 (471.40 μm²).

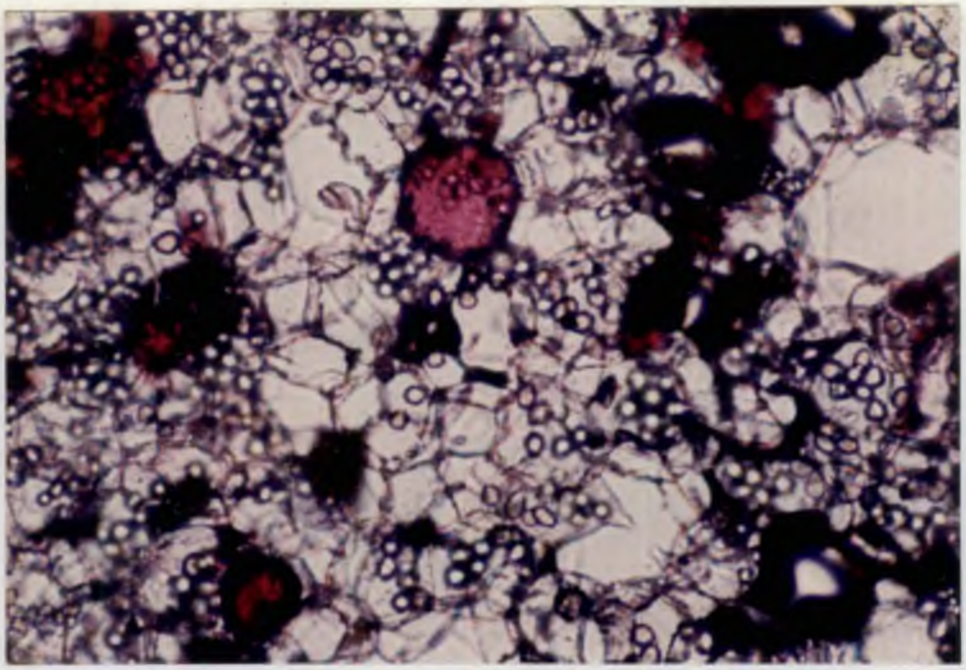
Secondary xylem vessels were small sized in *P. nigrum*. Secondary xylem thickening took place earlier in *P. colubrinum* which led to form a continuous solid cylinder. But in *P. nigrum*, the secondary xylem appeared discontinuous. The internal structures of *P. colubrinum* were compactly arranged and occupied less space in between pericycle and stele axis (84.99 μm) when compared to *P. nigrum* (171.35 μm). As in the case of immune genotype, the tolerant genotype also exhibited compact structure in the stele (Plate VI a-c).

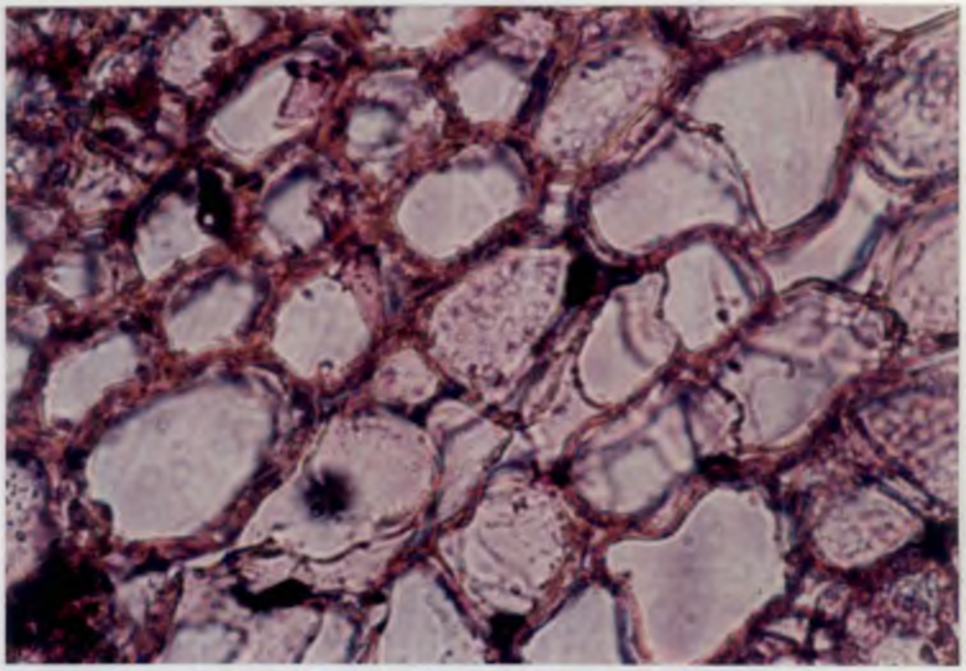
Plate VII. Root anatomy of *Piper* spp. showing the size, shape and arrangement of cortical cells, intercellular spaces and cell inclusions (x = 2500)

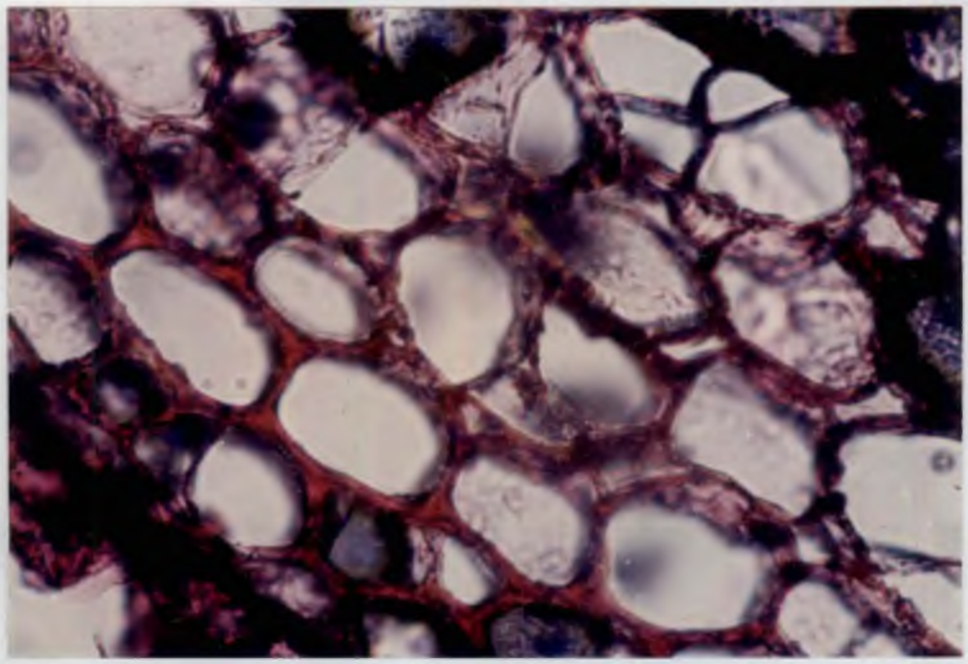
a) *Piper colubrinum* L.

b) *Piper nigrum* L. (Panniyur 1)

(c) *Piper nigrum* L. (Kalluvally)







The immune species *P. colubrinum* was characterised by the presence of fewer root hairs, smaller cortical cells with more cell inclusions less intercellular spaces, thicker endodermis and pericycle and compact smaller stele.

Essentially the fundamental anatomy of leaf, stem and roots remained the same in all the three genotypes studied, the differences being in the cell size, shape and their arrangement.

4.2 Biochemical investigations

4.2.1 Biochemical characterisation of *Piper* spp.

Biochemical constituents of the leaves, stems and roots of the susceptible, tolerant and immune genotypes were analysed and are described in this part.

4.2.1.1 Estimation of total and OD phenol in *Piper* spp..

4.2.1.1.1 Total phenols

Analysis of variance showed significant difference for total phenol content in the two species and the two genotypes of *P. nigrum* studied (Table 4 and Fig.1). The immune genotype, *P. colubrinum* exhibited 42 per cent higher phenol content than *P. nigrum*. Of the two genotypes of *P. nigrum*, Kalluvally recorded higher phenol content (3.802 mg/g) than Panniyur 1 (2.483 mg/g).

The different plant parts analysed also showed significant variation in the contents of total phenols. The content in roots was higher than in leaves and stem. When the plant part x genotype interaction was studied, roots of Kalluvally had the highest content (4.516 mg/g) which was 51 and 13 per cent higher than that of Panniyur 1 and *P. colubrinum* respectively.

Table 4. Total phenol content (mg g^{-1}) in different plant parts of *Piper* spp.

	Piper nigrum L.			*P. colubrinum* L.	
	Panniyur 1	Kalluvally	✡Mean		Mean
Leaf	2.123	3.245	*2.684*	5.539	3.636
Stem	2.239	3.645	*2.942*	3.437	3.140
Root	2.987	4.516	*3.752*	4.013	3.839
Mean	2.483	3.802	*3.143*	4.430	3.358
CD (0.05) for comparing genotypes				0.776	
CD (0.01) for comparing plant parts of the genotypes				1.345	

✡Worked out from means of Panniyur 1 and Kalluvally

Fig.1. Total Phenol content (mg/g) in different plant parts of *Piper* spp.

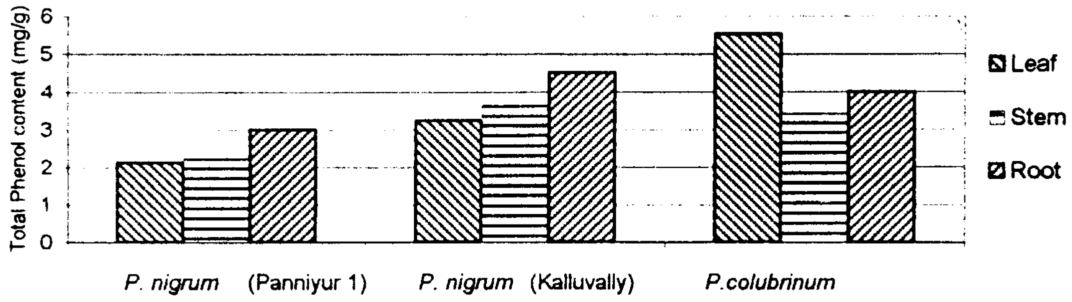
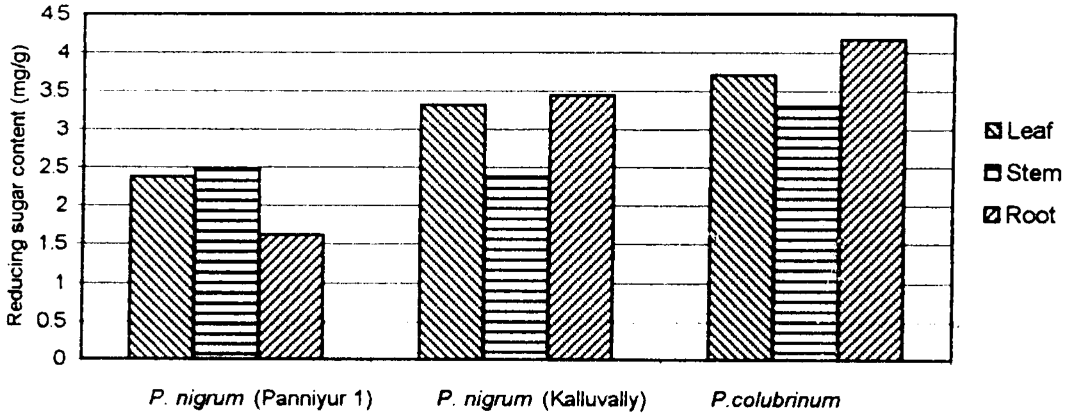


Fig.2. Reducing sugar content (mg/g) in different plant parts of *Piper* spp.



4.2.1.1.2 Ortho dihydric phenol

The contents of OD phenol differed significantly in two species studied. The immune genotype *P. colubrinum* recorded 12 per cent higher OD phenol than *P. nigrum* (Table 5). The tolerant genotype Kalluvally exhibited the highest content (6.632 mg/g) followed by the immune genotype *P. colubrinum* (6.176 mg/g) and susceptible genotype Panniyur 1 (4.386 mg/g).

Among the plant parts, leaves registered higher OD phenol content followed by stem and root. The leaves of *P. colubrinum* had the highest content (7.667 mg/g), whereas the roots and stems of Kalluvally recorded the highest contents. Stems of Kalluvally had 18 and 56 per cent higher content of OD phenol than *P. colubrinum* and Panniyur 1 respectively.

4.2.1.2 Estimation of sugars in *Piper* spp.

4.2.1.2.1 Reducing sugar

Statistically, the contents of reducing sugar differed significantly in all the genotypes (Table 6 and Fig.2). The immune genotype, had maximum content of reducing sugar (3.728 mg/g) which was 43 per cent higher than that of *P. nigrum*. The tolerant genotype Kalluvally had higher content of reducing sugar (3.048 mg/g) than Panniyur 1 (2.155 mg/g).

Among plant parts, leaves registered higher content followed by roots and stems. However, differences among them were statistically non significant.

4.2.1.2.2 Non reducing sugar

The data pertaining to non reducing sugar contents in the different genotypes studied are presented in Table 7. Irrespective of the genotypes studied, plant parts analysed showed significant differences in non reducing sugar content. The content was

Table 5. *Ortho* dihydric phenol content (mg g^{-1})in different plant parts of *Piper* spp.

	Piper nigrum L.			*P. colubrinum* L.	
	Panniyur 1	Kalluvally	✡Mean		Mean
Leaf	5.093	7.627	*6.360*	7.667	6.796
Stem	4.537	7.060	*5.799*	5.997	5.864
Root	3.527	5.210	*4.374*	4.863	4.533
Mean	4.386	6.632	*5.510*	6.176	5.731

CD (0.05) for comparing genotypes 1.2393
CD (0.05) for comparing plant parts of the genotypes 1.2393
✡Worked out from means of Panniyur 1 and Kalluvally

Table 6. Reducing sugar content (mg g^{-1})in different plants part of *Piper* spp.

	Piper nigrum L.			*P. colubrinum* L.	
	Panniyur 1	Kalluvally	✡Mean		Mean
Leaf	2.375	3.325	*2.850*	3.711	3.137
Stem	2.474	2.374	*2.424*	3.304	2.717
Root	1.615	3.444	*2.530*	4.170	3.076
Mean	2.155	3.048	*2.602*	3.728	2.977

CD (0 05) for comparing genotypes 0.940
✡Worked out from means of Panniyur 1 and Kalluvally

Table 7. Non reducing sugar content (mg g^{-1})in different plant parts of *Piper* spp.

	Piper nigrum L.			*P. colubrinum* L.	
	Panniyur 1	Kalluvally	✡Mean		Mean
Leaf	1.652	1.488	*1.570*	1.444	1.528
Stem	2.464	1.976	*2.220*	1.792	2.077
Root	1.716	1.444	*1.580*	1.350	1.503
Mean	1.944	1.636	*1.790*	1.529	1.703

CD (0.01) for comparing plant parts of the genotypes 0.491
✡Worked out from means of Panniyur 1 and Kalluvally

found to be high in *P. nigrum* (1.792 mg/g) as compared to *P. colubrinum* (1.529 mg/g). In the two varieties of *P. nigrum*, Panniyur 1 recorded higher content (1.944 mg/g) than Kalluvally (1.636 mg/g).

Irrespective of the genotypes studied, plant parts analysed showed significant differences in non reducing sugar content. Stems had higher content of non reducing sugar followed by leaves and roots. The stems of susceptible genotype had maximum content (2.464 mg/g) which was 25 and 38 per cent higher than the tolerant and immune genotypes. Similar trend was observed in leaves and roots of the genotypes studied. In roots, susceptible genotype Panniyur 1 had maximum content (1.716 mg/g) which was 19 and 27 per cent higher than that of Kalluvally and *P. colubrinum* respectively. In stems also the same trend was observed. Panniyur 1 had the highest content (2.464 mg/g) which was 25 per cent higher than that of Kalluvally and 38 per cent higher than that of *P. colubrinum*.

4.2.1.3 Estimation of total free amino acids in *Piper* spp.

The contents of total free amino acids differed in the two species studied (Table 8 and Fig.3). The immune genotype had the highest content (0.037%) which was 42 per cent higher than that of *P. nigrum*. Among the *P. nigrum* genotypes, Kalluvally had the higher content of amino acids as compared to Panniyur 1.

In the plant parts analysed, leaves had higher content of total free amino acids followed by stems and roots. Content in leaves was 23 and 61 per cent higher than that of stems and roots respectively. The leaves of *P. colubrinum* exhibited higher content (0.047%) followed by Kalluvally (0.035%) and Panniyur 1 (0.030%). Similarly, contents of total free amino acids were also maximum in stems and roots of *P. colubrinum* as compared to tolerant and susceptible genotypes.

Table 8. Total free amino acid content (%) in different plants parts of *Piper* spp.

	Piper nigrum L.			*P. colubrinum* L.	
	Panniyur 1	Kalluvally	✡Mean		Mean
Leaf	0.030	0.035	*0.033*	0.047	0.037
Stem	0.017	0.032	*0.025*	0.039	0.030
Root	0.020	0.023	*0.022*	0.026	0.023
Mean	0.022	0.030	*0.026*	0.037	0.030

CD (0.05) for comparing genotypes 0.00594

CD (0.05) for comparing plant parts of the genotypes 0.00594

✡Worked out from means of Panniyur 1 and Kalluvally

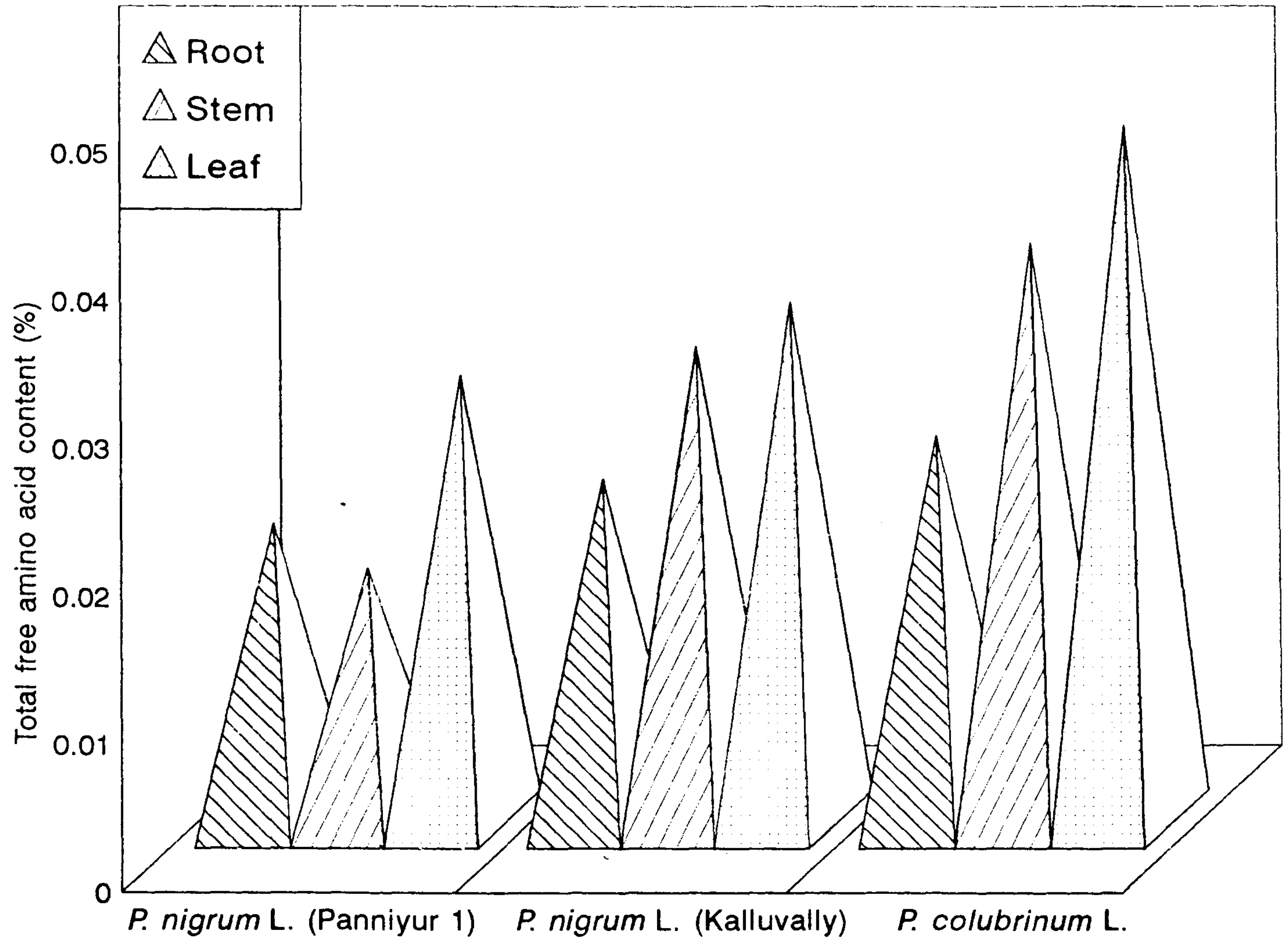


Fig.3. Total free amino acid content (%) in different plant parts of *Piper* spp.

4.2.1.3 Estimation of enzyme activities in *Piper* spp.

4.2.1.3.1 Glucanase

The activity of the enzyme glucanase differed significantly in the immune, susceptible and tolerant genotypes studied (Table 9 and Fig.4). Higher activity was noticed in *P. nigrum* as compared to *P. colubrinum*. When the two genotypes of *P. nigrum* were compared higher activity was observed in Panniyur 1.

Among the plant parts analysed, higher activity was noticed in leaves followed by stems and roots. Panniyur 1 exhibited higher activity in all the plant parts analysed.

4.2.1.3.2 Indole - 3 acetic acid oxidase

The genotypes studied showed no significant differences in IAA oxidase activity (Table 10 and Fig.5). However, higher activity was observed in *P. nigrum* (1.196 mg/g) as compared to *P. colubrinum* (1.148 mg/g). The susceptible genotype Panniyur 1 had the highest activity (1.268 mg/g) as compared to Kalluvally (1.124 mg/g).

The activity of the enzyme differed significantly in different plant parts analysed.

The highest activity was observed in roots (1.986 mg/g) followed by stems (1.047 mg/g), whereas the leaves recorded the lowest activity (0.507 mg/g). Roots and leaves of Panniyur 1 recorded higher activities as compared to other genotypes. However, in stem higher activity was recorded in *P. colubrinum*.

4.2.1.3.3 Peroxidase

Activity of peroxidase enzyme differed significantly among the genotypes and plant parts analysed (Table 11). The genotype x plant part interaction was also found to be significant.

Table 9. Glucanase enzyme activity (mg g^{-1}) in different plant parts of *Piper* spp.

	Piper nigrum L.			*P. colubrinum* L.	
	Panniyur 1	Kalluvally	✡Mean		Mean
Leaf	1.749	1.307	*1.530*	1.098	1.384
Stem	1.445	1.190	*1.320*	0.646	1.094
Root	0.967	0.857	*0.912*	0.731	0.852
Mean	1.387	1.118	*1.253*	0.825	1.110
CD (0.05) for comparing genotypes				0.225	
CD (0.05) for comparing plant parts of the genotypes				0.225	

✡Worked out from means of Panniyur 1 and Kalluvally

Table 10. Indole-3 acetic acid oxidase activity (mg g^{-1}) in different plant parts of *Piper* spp.

	Piper nigrum L.			*P. colubrinum* L.	
	Panniyur 1	Kalluvally	✡Mean		Mean
Leaf	0.794	0.476	*0.635*	0.253	0.507
Stem	0.936	0.953	*0.945*	1.251	1.047
Root	2.075	1.943	*2.010*	1.940	1.986
Mean	1.268	1.124	*1.196*	1.148	1.180
CD (0.05) for comparing plant parts				0.2463	

✡Worked out from means of Panniyur 1 and Kalluvally

Table 11. Peroxidase enzyme activity (units / litre) in different plant parts of *Piper* spp.

	Piper nigrum L.			*P. colubrinum* L.	
	Panniyur 1	Kalluvally	✡Mean		Mean
Leaf	147.181	422.414	*284.798*	430.175	333.257
Stem	200.000	280.591	*240.296*	368.548	283.046
Root	127.982	168.016	*147.100*	196.783	164.260
Mean	158.387	290.341	*224.364*	331.835	260.188
CD (0.05) for comparing genotypes				37.285	
CD (0.05) for comparing plant parts				37.285	
CD (0.05) for comparing plant parts of the genotypes				64.580	

✡Worked out from means of Panniyur 1 and Kalluvally

Fig.4. Glucanase enzyme activity (mg/g) in different plant parts of *Piper* spp.

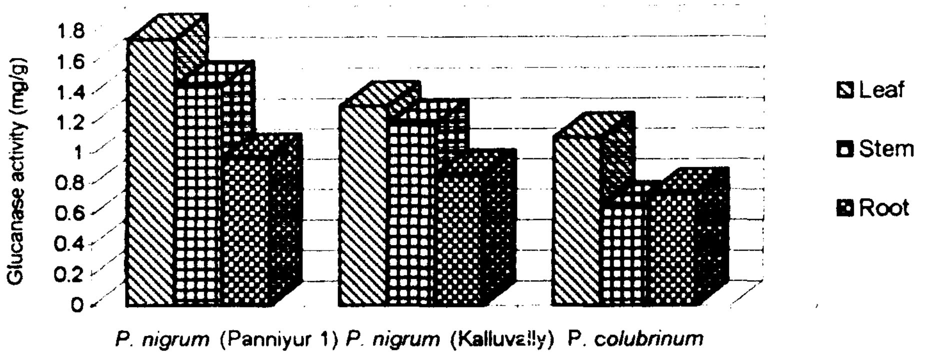
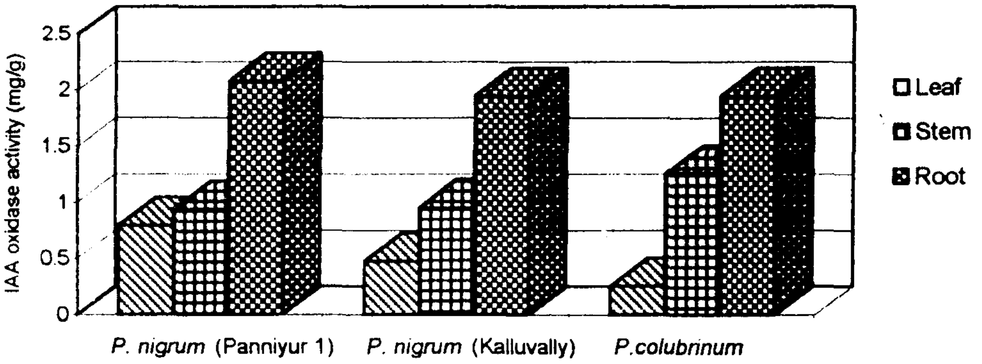


Fig.5. Indole - 3 acetic acid oxidase enzyme activity (mg/g) in different plant parts of *Piper* spp.



Piper colubrinum recorded the highest activity (331.835 units/litre) which was 48 per cent higher than that of *P. nigrum* (224.364 units/litre). Of the *P. nigrum* genotypes, Kalluvally had higher activity (290.34 units/litre) than Panniyur 1 (158.387 units/litre).

When the plant parts were compared, higher activity was observed in leaves (333.257 units/litre) followed by stems (283.046 units/litre) and roots (164.26 units/litre). *P. colubrinum* recorded the highest activity in all the plant parts analysed.

4.2.1.3.4 Polyphenol oxidase

The total activity of the enzyme polyphenol oxidase was found to be higher in *P. colubrinum* as compared to *P. nigrum* (Table 12 and Fig.6). When the two varieties of *P. nigrum* were compared, Kalluvally had higher activity compared to Panniyur 1.

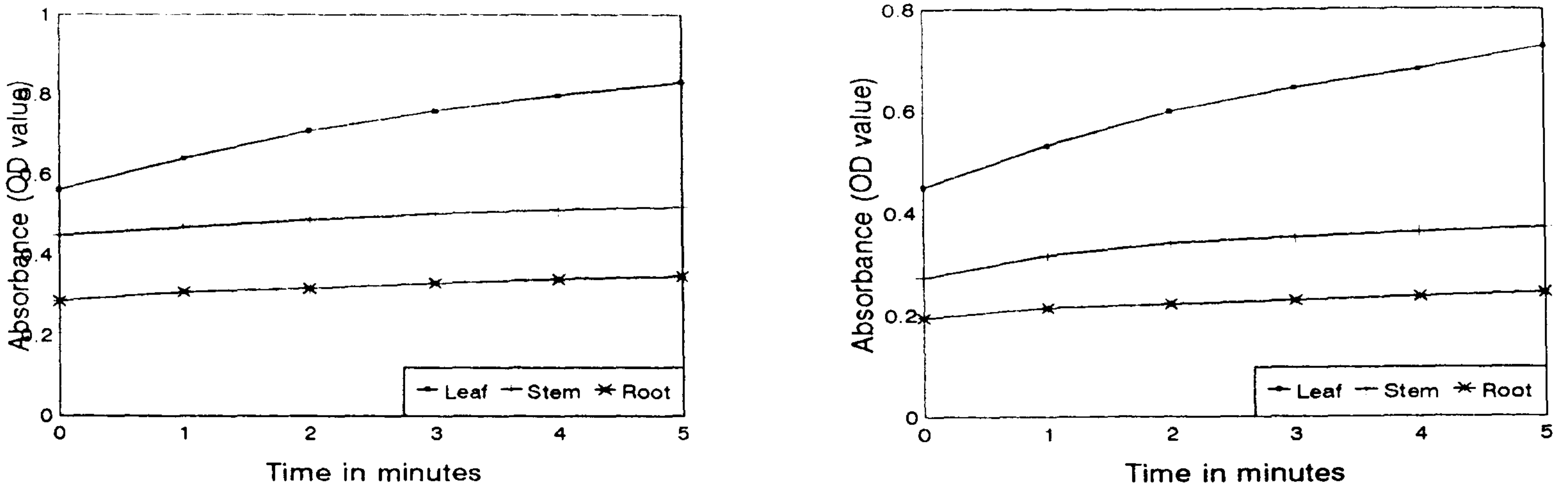
Irrespective of the genotype, leaves had higher activity followed by stems and roots. Leaves of Panniyur 1 had higher total enzyme activity (0.268 OD value) followed by Kalluvally (0.260 OD value) and *P. colubrinum* (0.260 OD value). But in stems and roots, higher activities were observed in *P. colubrinum* followed by Kalluvally and Panniyur 1.

From the linear phase of curve drawn it is apparent that all the genotypes exhibited steady increase in enzyme activity with time in all the plant parts, maximum being in *P. colubrinum*.

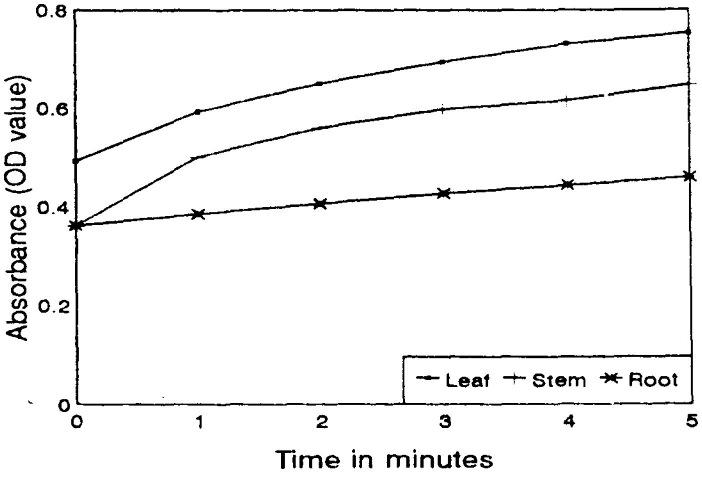
Table 12. Polyphenol oxidase total enzyme activity (OD value) in *Piper* spp.

	Piper nigrum L.			*P. colubrinum* L.	
	Panniyur 1	Kalluvally	✡Mean		Mean
Leaf	0.268	0.260	*0.264*	0.260	0.262
Stem	0.071	0.098	*0.085*	0.287	0.186
Root	0.061	0.049	*0.055*	0.098	0.077
Mean	0.133	0.136	*0.135*	0.215	0.173

✡Worked out from means of Panniyur 1 and Kalluvally



Piper nigrum L. (Panniyur 1)   *Piper nigrum* L. (Kalluvally)



Piper colubrinum L.

Fig.6. Polyphenol oxidase enzyme activity (OD value) in different plant parts of *Piper* spp.

4.2.2 Comparision of biochemical parameters in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

On artificial inoculation with culture disc of *Phytophthora capsici*, symptoms were observed in different plant parts after 24 h of inoculation. There were no advancement of lesions in *P. colubrinum* but the lesion size advanced fastly in Panniyur 1 when compared to Kalluvally. Plate VIII shows lesion development in *Piper* spp. after 72 h of inoculation.

4.2.2.1 Comparision of total and OD phenol in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

4.2.2.1.1 Total phenols

Following *Phytophthora capsici* inoculation, total phenol content in leaves and stems of *Piper* spp.. increased significantly in all the genotypes studied (Table 13 and Fig.7). Maximum increase in total phenols over the control was observed in *P. nigrum* which recorded 132 per cent increase over the control while *P. colubrinum* recorded only 39 per cent increase over the control. Of the genotypes of *P. nigrum* studied, Panniyur 1 recorded higher increase than Kalluvally.

Out of the plant parts analysed, leaves recorded higher increase in total phenols as compared to the stems in all the genotypes. On inoculation, the total phenol content in leaves increased to the maximum in Panniyur 1 (202%) followed by Kalluvally (126%) and *P. colubrinum* (35%) over the control.

4.2.2.1.2 Ortho dihydric phenols

Inoculation of *Phytophthora capcici* on leaves and stems resulted in significant decrease of OD phenol content in the three genotypes studied (Table 14 and Fig.8). The decrease was found to be more in *P.* colubrinum (266%) as compared to *P. nigrum* (109%) over the control. Similarly, the decrease was more in Kalluvally

Plate VIII. Lesion development in *Piper* spp. after 72 h of artificial inoculation with culture disc of *Phytophthora capsici*


Panniyur-1
Kalluvally
P.colubrinum

Table 13. Comparison of total phenol content ($mg\ g^{-1}$) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

	Piper nigrum L.						*P. colubrinum* L.			
	Panniyur 1		Kalluvally		✡Mean				Mean	
	UI	I	UI	I	UI	I	UI	I	UI	I
Leaf	2.123	6.413	3.245	7.328	*2.684*	*6.871*	5.539	7.504	3.636	7.082
Stem	2.239	5.628	3.645	6.926	*2.942*	*6.277*	3.437	4.973	3.140	5.842
Mean	2.231	6.021	3.445	7.127	*2.838*	*6.574*	4.488	6.239	3.388	6.462

CD (0.05) for comparing uninoculated and inoculated plants 0.948

✡Worked out from means of Panniyur 1 and Kalluvally

UI - Uninoculated I - Inoculated

Table 14. Comparison of OD phenol content ($mg\ g^{-1}$) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

	Piper nigrum L.						*P. colubrinum* L.			
	Panniyur 1		Kalluvally		✡Mean				Mean	
	UI	I	UI	I	UI	I	UI	I	UI	I
Leaf	5.093	4.973	7.627	3.306	*6.360*	*4.140*	7.667	1.303	6.796	3.194
Stem	4.537	2.223	7.060	1.157	*5.799*	*1.690*	5.997	2.433	5.864	1.938
Mean	4.815	3.598	7.343	2.231	*6.079*	*2.915*	6.832	1.868	6.330	2.566

CD (0.05) for comparing uninoculated and inoculated genotypes 1.489

CD (0.01) for comparing uninoculated and inoculated plant parts 2.105

CD (0.01) for comparing uninoculated and inoculated plants 0.859

✡Worked out from means of Panniyur 1 and Kalluvally

UI - Uninoculated I - Inoculated

Fig.7. Comparison of total phenol content (mg/g) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

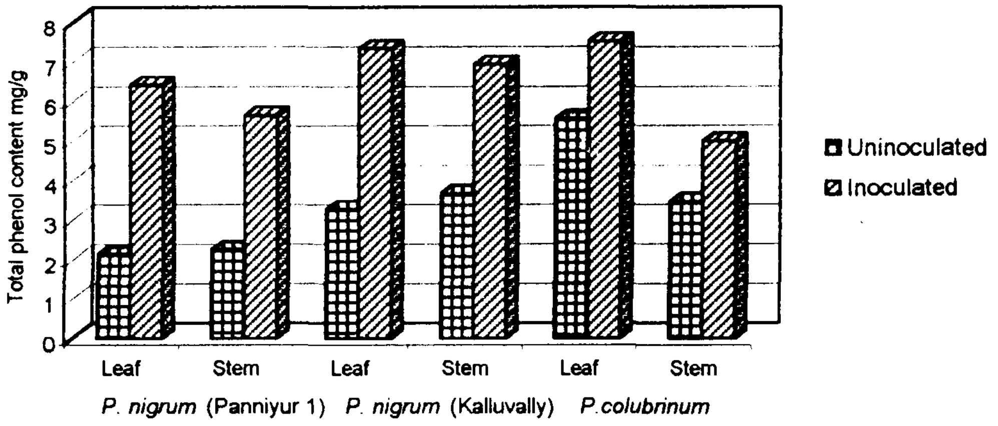
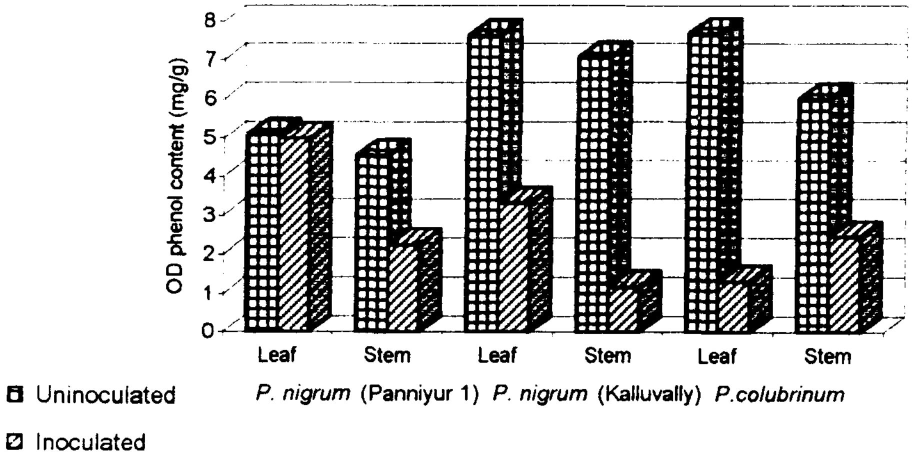


Fig.8. Comparison of *Ortho* dihydric phenol content (mg/g) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.



compared to *P. nigrum*. When the varieties were compared, Kalluvally registered more decrease in OD phenol content in stems and leaves on inoculation.

4.2.2.2 Comparision of sugars in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

4.2.2.2.1 Reducing sugars

Analysis of variance showed significant differences among the inoculated and uninoculated plant parts of the genotypes for the contents of reducing sugar (Table 15). On inoculation, the content of reducing sugars decreased in the two species studied. The decrease was found to be more in *P. colubrinum* (88%) as compared to *P. nigrum* (16%). When the two varieties were compared, the decrease in reducing sugar was observed only in Kalluvally while in Panniyur 1 increase over the control was noted Maximum decrease in content was observed in leaves as compared to stems. The decrease observed in the leaves of Kalluvally was more than that of Panniyur 1.

4.2.2.2.2 Non reducing sugar

Significant differences in non reducing sugar were noticed in inoculated and uninoculated plant parts of the three genotypes (Table 16 and Fig.9).

On inoculation there was depletion of non reducing sugar content in all the genotypes studied. The decrease was more in *P. colubrinum* as compared to *P. nigrum* The content decreased from 1.618 to 0.153 mg/g in *P. colubrinum* and from 1.895 to 1.289 mg/g in *P. nigrum*. In *P. nigrum* genotypes, Kalluvally recorded more decrease compared to Panniyur 1. Of the plant parts analysed, the decrease was more in stems as compared to leaves. The stems of *P. colubrinum* recorded maximum decrease followed by Kalluvally and Panniyur 1.

Table 15. Comparison of reducing sugar content (mg g^{-1}) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

	Piper nigrum L.						*P. colubrinum* L.			
	Panniyur 1		Kalluvally		✡Mean				Mean	
	UI	I	UI	I	UI	I	UI	I	UI	I
Leaf	2.375	1.753	3.325	1.690	*2.850*	*1.722*	3.711	1.516	3.137	1.653
Stem	2.474	3.654	2.374	1.963	*2.424*	*2.810*	3.304	2.224	2.717	2.614
Mean	2.425	2.704	2.849	1.827	*2.637*	*2.266*	3.508	1.870	2.927	2.134

CD (0.01) for comparing uninoculated and inoculated plant parts 0.682
CD (0.01) for comparing uninoculated and inoculated plants 0.965
✡Worked out from means of Panniyur 1 and Kalluvally
UI - Uninoculated I - Inoculated

Table 16. Comparison of non reducing sugar content (mg g^{-1}) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

	Piper nigrum L.						*P. colubrinum* L.			
	Panniyur 1		Kalluvally		✡Mean				Mean	
	UI	I	UI	I	UI	I	UI	I	UI	I
Leaf	1.652	1.787	1.488	1.440	*1.570*	*1.614*	1.440	0.232	1.528	1.154
Stem	2.464	1.595	1.976	0.330	*2.220*	*0.962*	1.792	0.074	2.077	0.066
Mean	2.058	1.691	1.732	0.887	*1.895*	*1.289*	1.618	0.153	1.803	0.910

CD (0.01) for comparing genotypes 0.401
CD (0.05) for comparing uninoculated and inoculated plants 0.401
CD (0.01) for comparing uninoculated and inoculated plant parts 0.567
✡Worked out from means of Panniyur 1 and Kalluvally
UI - Uninoculated I - Inoculated

Fig.9. Comparison of non reducing sugar content (mg/g) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

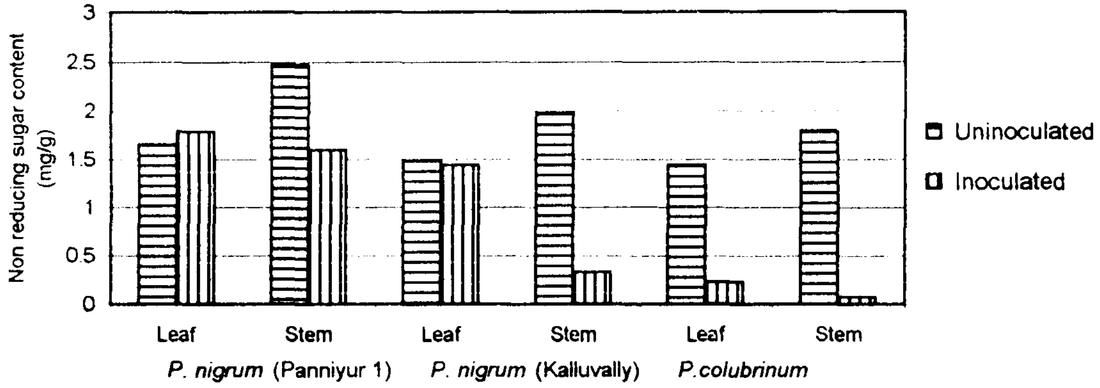
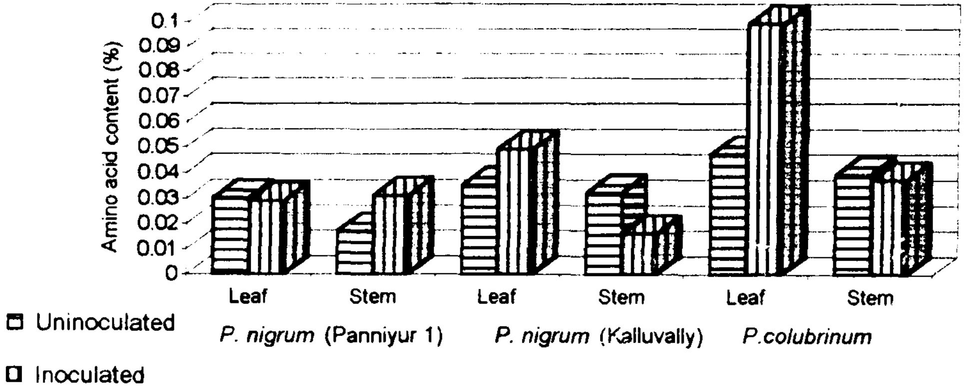


Fig.10. Comparison of total free amino acid content (%) in *Phytophthora capsici* inoculated and uninoculated plants parts of *Piper* spp.



4.2.2.3 Comparision of total free amino acids in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

On inoculation there was increase in amino acid content in the two species studied (Table 17 and Fig.10). *Piper colubrinum* recorded 111 per cent increase over the control as compared to 18 per cent increase in *P. nigrum.* In the two genotypes of *P. nigrum*, Panniyur 1 showed increase in content whereas a slight decrease was observed in Kalluvally. Irrespective of the genotypes, there was increase in amino acid content in both stems and leaves on inoculation. When genotype x plant part interaction was studied, the content was found to increase in leaves of Kalluvally and *P. colubrinum* and decrease in leaves of Panniyur 1. The reverse trend in amino acid content was observed in stems of the genotypes analysed.

4.2.2.4 Comparision of enzyme activities in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

4.2.2.4.1 Glucanase

Activity of glucanase enzyme differed significantly among inoculated and uninoculated plant parts of the immune, tolerant and susceptible genotypes. An interaction effect among healthy and diseased plant parts was also found significant (Table 18).

On inoculation, the glucanase enzyme activity increased in both the species. Maximum increase was observed in *P. nigrum* as compared to *P. colubrinum*, the percentage increase being 31 and 27 respectively. When the two varieties were compared, 60 per cent increase in enzyme activity in Panniyur 1 and 5 per cent increase in activity in Kalluvally were observed. In the plant parts analysed, irrespective of the genotype increase in activity was observed in leaves, whereas decrease was observed in stems. Increased activity in leaves was observed in Panniyur 1 and Kalluvally and

Table 17. Comparison of total free amino acid (%) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

	Piper nigrum L.						*P. colubrinum* L.			
	Panniyur 1		Kalluvally		✡Mean				Mean	
	UI	I	UI	I	UI	I	UI	I	UI	I
Leaf	0.030	0.029	0.035	0.049	*0.033*	*0.039*	0.047	0.099	0.037	0.059
Stem	0.017	0.031	0.032	0.016	*0.025*	*0.024*	0.039	0.037	0.030	0.043
Mean	0.024	0.030	0.034	0.032	*0.029*	*0.031*	0.043	0.068	0.034	0.038

CD (0.05) for comparing genotypes	0.012
CD (0.05) for comparing plant parts	0.00875
CD (0.01) for comparing uninoculated and inoculated plants	0.014
CD (0.01) for comparing uninoculated and inoculated plant parts	0.025

✡Worked out from means of Panniyur 1 and Kalluvally

UI - Uninoculated I - Inoculated

Table 18. Comparison of glucanase enzyme activity (mg g^{-1}) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

	Piper nigrum L.						*P. colubrinum* L.			
	Panniyur 1		Kalluvally		✡Mean				Mean	
	UI	I	UI	I	UI	I	UI	I	UI	I
Leaf	1.749	3.670	1.307	1.803	*1.528*	*2.737*	1.098	1.075	1.384	2.183
Stem	1.445	1.376	1.190	0.575	*1.318*	*0.973*	0.646	1.135	1.094	1.029
Mean	1.597	2.523	1.248	1.189	*1.423*	*1.856*	0.872	1.105	1.239	1.606

CD (0.05) for comparing genotypes	0.472
CD (0.05) for comparing plant parts	0.385
CD (0.01) for comparing uninoculated and inoculated plants	0.545
CD (0.01) for comparing uninoculated and inoculated plants parts of the genotypes	0.945

✡Worked out from means of Panniyur 1 and Kalluvally

UI - Uninoculated I - Inoculated

decreased activity was observed in *P. colubrinum*. However, the reverse trend in activity was seen in the stems of the genotypes.

4.2.2.4.2 Indole - 3 acetic acid oxidase

Significant differences were observed for IAA oxidase activity in the three genotypes studied (Table 19).

On inoculation, enzyme activity decreased in *P. nigrum* whereas increased in *P. colubrinum*. On analysis of *P. nigrum* genotypes, it was found that the activity got increased in Kalluvally and decreased in Panniyur 1.

Irrespective of the genotypes, leaves recorded increase in enzyme activity (89%) whereas, stems recorded decrease in activity. Increase in activity in the leaves was observed in Kalluvally and *P. colubrinum* whereas decrease in activity was observed in Panniyur 1. The activity got decreased in stems of all the genotypes.

4.2.2.4.3 Peroxidase

Data presented in Table 20 and Fig.11 show significant differences in peroxidase activity in the different genotypes studied and plant parts analysed.

Inoculation caused an increment in the peroxidase enzyme activity in all the genotypes studied. The increment in activity was higher in *P. colubrinum* (198%) as compared to *P. nigrum* (74%). The activity increased to the extent of 55 per cent in Kalluvally and 114 per cent in Panniyur 1, when the two varieties were compared. On inoculation, the activity increased in stems and leaves in all the genotypes. The increase in activity was more in leaves when compared to stems. *Piper colubrinum* recorded higher activity in stems and leaves followed by Kalluvally and Panniyur 1.

Table 19. Comparison of IAA oxidase enzyme activity (mg g^{-1}) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

	Piper nigrum L.						*P. colubrinum* L.			
	Panniyur 1		Kalluvally		✡Mean				Mean	
	UI	I	UI	I	UI	I	UI	I	UI	I
Leaf	0.794	0.139	0.476	1.327	*0.635*	*0.733*	0.253	1.411	0.507	0.959
Stem	0.936	0.513	0.953	0.446	*0.945*	*0.480*	1.251	0.713	1.047	0.557
Mean	0.865	0.326	0.714	0.886	*0.790*	*0.606*	0.752	1.062	0.777	0.758

CD (0.05) for comparing genotypes	0.167
CD (0.05) for comparing plant parts with genotypes	0.228
CD (0.01) for comparing uninoculated and inoculated plant	0.228
CD (0.05) for comparing uninoculated and inoculated plant parts	0.187
CD (0.05) for comparing uninoculated and inoculated plant parts of the genotypes	0.323

✡Worked out from means of Panniyur 1 and Kalluvally

UI - Uninoculated I - Inoculated

Table 20. Comparison of peroxidase enzyme activity (units / litre) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

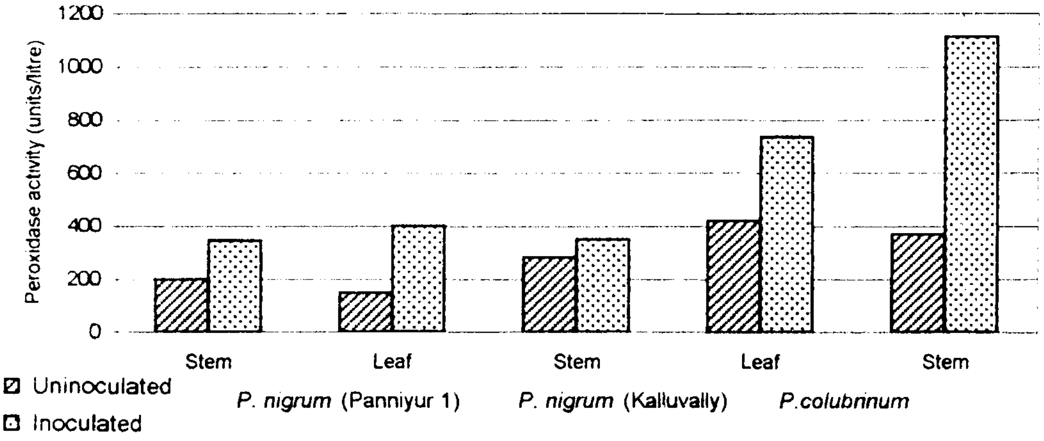
	Piper nigrum L						*P. colubrinum* L.			
	Panniyur 1		Kalluvally		✡Mean				Mean	
	UI	I	UI	I	UI	I	UI	I	UI	I
Leaf	147.181	399.415	422.414	735.608	*284.798*	*567.512*	430.175	1269.438	3332.57	801.487
Stem	200.000	344.699	280.591	351.153	*240.296*	*348.000*	368.548	1111.827	283.046	602.766
Mean	173.590	372.057	351.503	543.680	*262.547*	*457.869*	399.361	1190.643	308.152	702.127

CD (0.05) for comparing genotypes	188.121
CD (0.05) for comparing uninoculated and inoculated plants	153.600
CD (0.05) for comparing uninoculated and inoculated genotypes	266.044

✡Worked out from means of Panniyur 1 and Kalluvally

UI - Uninoculated I - Inoculated

Fig.11. Comparison of peroxidase enzyme activity (units / litre) in *Phytophthora capsici* inoculated and uninoculated plants parts of *Piper* spp.



4.2.2.4.4 Polyphenol oxidase

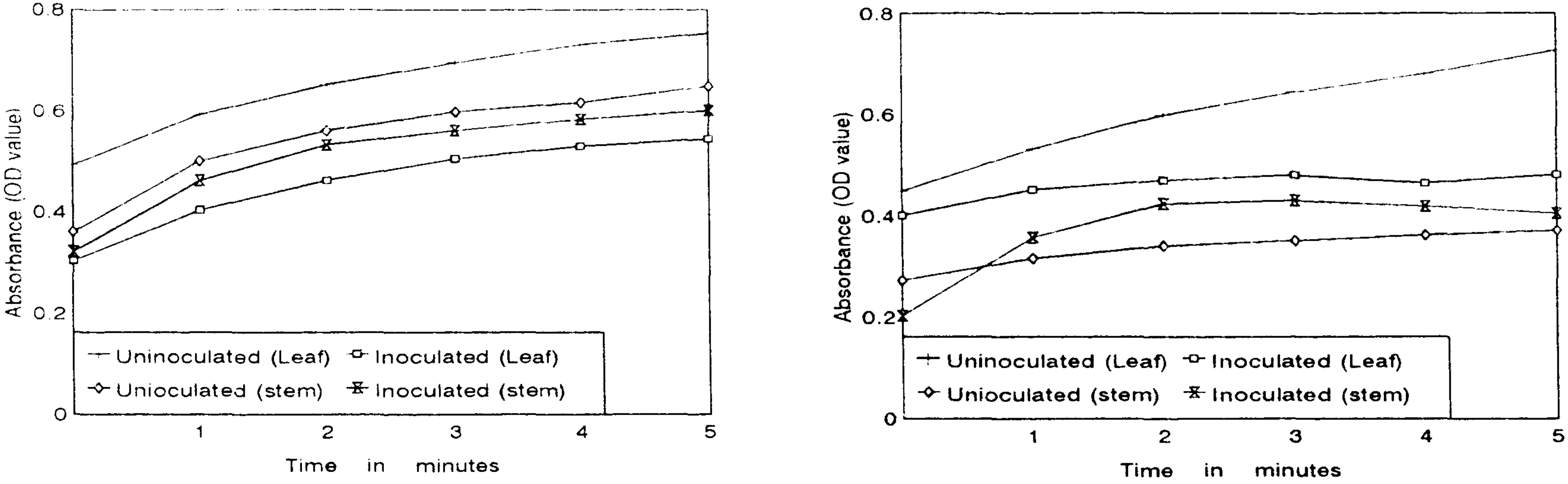
The OD values compiled in Table 21 show decrease in enzyme activity on inoculation in all the genotypes studied. Maximum decrease in enzyme activity was observed in *P. nigrum* when compared to *P. colubrinum*. When *P. nigrum* genotypes were considered, Kalluvally recorded higher decrease in enzyme activity than Panniyur 1. On inoculation, irrespective of the genotype, leaves recorded 30 per cent decrease in enzyme activity over control as compared to ten per cent decrease in stems. The inoculated leaves of *P. nigrum* genotypes recorded maximum decrease (64%) as compared to *P. colubrinum* (8%). Among the *P. nigrum* genotypes, the tolerant genotype Kalluvally exhibited higher decrease as compared to the susceptible genotype Panniyur 1. The activity of the enzyme in stem decreased to the maximum in Kalluvally followed by Panniyur 1 and *P. colubrinum*.

The activity of the enzyme in inoculated leaves and stems increased steadily in *P. colubrinum* and Panniyur 1 as evident from Fig. 12. But in Kalluvally, the activity of the enzyme in stems and leaves showed a steady increase up to third minute and thereafter it decreased.

Table 21. Polyphenol oxidase enzyme total activity (OD value) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

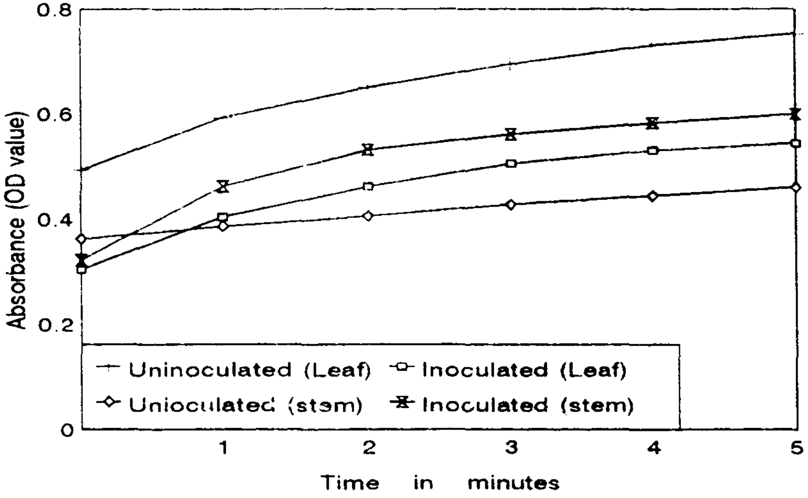
	Piper nigrum L						*P. colubrinum* L			
	Panniyur 1		Kalluvally		✡Mean				Mean	
	UI	I	UI	I	UI	I	UI	I	UI	I
Leaf	0.268	0.242	0.260	0.080	*0.264*	*0.161*	0.260	0.240	0.262	0.201
Stem	0.071	0.069	0.098	0.047	*0.085*	*0.058*	0.287	0.278	0.186	0.168
Mean	0.170	0.156	0.179	0.064	*0.175*	*0.110*	0.274	0.259	0.244	0.185

✡Worked out from means of Panniyur 1 and Kalluvally
UI - Uninoculated I - Inoculated



Piper nigrum L. (Panniyur 1)

Piper nigrum L. (Kalluvally)



P. colubrinum L.

Fig. 12 Comparison of Polyphenol oxidase enzyme activity (OD value) in *Phytophthora capsici* inoculated and uninoculated plant parts of *Piper* spp.

Discussion

DISCUSSION

Phytophthora foot rot disease incited by *Phytophthora capsici* is the pernicious disease of black pepper in India and abroad and there is no effective control measure to combat this malady. In view of the fatal nature of the disease, anatomical and biochemical investigations were carried out in *Piper* spp.. on *Phytophthora* foot rot disease reaction. Three pepper genotypes showing immunity / tolerance / susceptibility to *Phytophthora* foot rot were compared anatomically and biochemically. Changes in biochemical parameters on inoculation by *Phytophthora capsici* were also analysed. The results of the various studies conducted are discussed in this chapter.

5.1 Anatomical investigations

5.1.1 Anatomical investigations on leaves

Anatomical investigations on leaves of *Piper* spp.. revealed that there existed variation among *P. colubrinum* and *P. nigrum* in the various parameters studied (Table 1 and Plates II and III). The tolerant and susceptible genotypes of *P. nigrum* also showed differences in the various anatomical features.

The immune genotype *P. colubrinum* is characterised by compact arrangement of cells, small epidermal mesophyll and spongy parenchyma cells, thick palisade and collenchyma tissues, large vascular bundles and mucilage canals, thick lower epiderm with large cells and less number of stomata per unit area (Plates II a and III a).

The susceptible genotype Panniyur 1 differed much from *P. colubrinum* in all the anatomical parameters studied. Panniyur 1 exhibited greater variability in epidermal and spongy parenchyma cell size. The susceptible genotype was also characterised by thinner palisade and collenchyma tissues, smaller vascular bundles and mucilage canals, thinner lower epiderm with small cells (Plates II b and III b) and more number of stomata per unit area.

The tolerant genotype Kalluvally registered somewhat similar values to *P. colubrinum* in epiderm thickness, epidermal cell size, vascular bundle size, mucilage canal size, collencyma tissue thickness (Plates II c and III c), stomatal index per unit area etc.

Since the pathogen is making its entry through lower epidermis, the thickness of the lower epidermis, the size and arrangement of the cells and the stomatal number play a significant role in the type of disease reaction of the species. The thickness of palisade and collenchyma tissues, the arrangement of the cells and size and number of vascular bundles decide further spread of the pathogen in the leaf tissues after infection.

So it can be concluded that thicker lower epiderm with fewer stomata check the entry of pathogen in *P. colubrinum* whereas the thinner epiderm and more stomata make Panniyur 1 susceptible to the pathogen. The large vascular bundles and thicker collenchyma tissues and compact arrangement of cells are responsible for the immunity in *P. colubrinum* whereas smaller bundles and thinner collenchyma tissues favour the susceptibility in Panniyur 1. The tolerance observed in Kalluvally is because of its similarity with *P. colubrinum*.

Similar structural differences in the leaf anatomy and differential resistance to infection were reported by Wetzstein and Sparks (1983) in pecan against scab disease; Shaik (1985) in bean against *Uromyces appendiculates*; Kaur (1986) and Kaur *et al.* (1992) in groundnut against tikka leaf spot disease and Mayee and Apet (1995) against rust disease in groundnut.

As is observed in the present study, Hemigway (1957) and Brahmachari and Kolte (1983) also recorded thicker palisade tissues in resistant groundnut varieties to cercospora leaf spot. When the leaf anatomy of resistant, moderately susceptible and susceptible groundnut varieties were analysed by Kaur *et al.* (1992), they observed that thickness of epidermis including cuticle, palisade tissue and middle part of lamina were significantly more in resistant varieties as compared to susceptible varieties.

5.1.2 Anatomical investigations on stems

The three genotypes studied differed in the stem anatomical characters (Table 2 and Plates IV and V). When compared to *P. nigrum*, *P. colubrinum* was characterised by thin cuticle, epiderm, hypoderm and small sized epidermal cells. Cortex was found to be thick having large cortical cells with more intercellular spaces and with thick chlorenchyma and sclerenchymatic tissues. Medulla region of *P. colubrinum* was characterised by more number of large peripheral and medullary vascular bundles and larger mucilage canals and smaller indistinct pith. Pericyclic sclerenchyma appeared continuous in a wavy ring in *P. nigrum*, but it formed a discontinuous ring in *P. colubrinum* (Plate IV a-c).

The tolerant and susceptible genotypes of *P. nigrum* also varied in their anatomical features. The susceptible genotype exhibited thicker cuticle with larger epidermal cells and with more epidermal appendages. The hypoderm was thick with small cells. The cortex was thin, and made up of smaller sized cortical cells with less intercellular spaces. The thickness of sclerenchymatic tissues was found to be less (Plate IV b).

The tolerant genotype Kalluvally exhibited characters in between Panniyur 1 and *P. colubrinum*. Compared to Panniyur 1, Kalluvally was characterised by thinner cuticle, small epidermal cells with less number of trichomes, thinner hypoderm, thicker cortex, small cortical cells arranged with more intercellular spaces and with thinner chlorenchymatic tissues. In the medulla region, Kalluvally had more number of large peripheral vascular bundles, large mucilage canals and small pith (Plate IV c).

From the present study it is seen that the immunity observed in *P. colubrinum* might be due to small epidermal cells with less number of epidermal appendages, thick cortex and thick sclerenchymatous tissues. More number of large peripheral and medullary vascular bundles, large mucilage canals and small pith also decides the type of disease reaction. Despite the discontinuous pericyclic sclerenchymatous layer, more number of larger vascular bundles offers mechanical support to *P. colubrinum* stem.

Smaller sized epidermal cells observed in *P. colubrinum* may increase compactness of epiderm layer which may not allow free passage of pathogen. The susceptibility observed in Panniyur 1 might be due to the high number of trichomes which might secrete substances like salt, sugar,terpenes and gums as reported by Fahn (1989) that could attract the pathogen. Eventhough Panniyur 1 was characterised by thicker cuticle, the large sized epidermal cells, thinner cortex, thinner sclerenchymatic tissue and much more number of trichomes added to its susceptibility. Similar differences in disease reaction due to anatomical differences in stem characters were reported by Hursh (1924), Walker and Stahmann (1955), Moreau (1957), Jennings and Ullstrup (1957), Klarman and Corbett (1974), Murray and Bruchi (1983), Stockwell and Hanchey (1984) and Markose (1996) in various crop plants.

The thickness of sclerenchymatic tissues gives mechanical strength and rigidity to plant organ. The thickened and lignified cell wall present in sclerenchymatic tissue give mechanical support and check the advance of pathogen due to lignification of cell wall. The thicker sclerenchymatic tissue observed in *P. colubrinum* might be responsbile for its immunity to the pathogen. Restricted entry of the pathogen through sclerified tissue of carnation was observed by Moreau (1957) when they studied the reaction of carnation to *Fusarium roseum*.

Large vascular bundles observed in *P. colubrinum* and Kalluvally might check the growth of the fungus as reported by Jennings and Ullstrup (1957) in corn plants against *Helminthosporium maydis*.

The large mucilage canals observed in Kalluvally and *P. colubrinum* might involve in secretion of resin, gums, alkaloids, polyphenols, essential oils, alkaloids etc which restrict the growth and spread of the pathogen.

5.1.3 Anatomical investigations on roots

The three genotypes studied showed differences in epiderm, cortex and stele characters of roots (Table 3 and Plates VI and VII). *Piper colubrinum* had thicker epiderm and less number of root hairs compared to *P. nigrum*. The cortical cell size was found to be small with thicker endoderm with bigger sized stele, bigger pericycle cells, vascular bundles and greater distance between pericycle and stele axis (Plate VI a and VII a) .

When the two cultivars of the *P. nigrum* were compared, the epiderm was thicker in Kalluvally with less number of epidermal appendages and larger cortical cells. Pericycle was thick with bigger cells, small stele and larger vascular bundles which are distantly placed (Plate VI b and VII b).

The susceptible genotype Panniyur 1 was characterised by thinner epiderm, more number of epidermal appendages, smaller cortical cells with large stele size and thinner pericycle with smaller cells, smaller vascular bundles that are distantly placed when compared to Kalluvally (Plates VI c and VII c).

Anatomical features such as thicker epiderm, smaller number of epidermal appendages, smaller cortical cells, smaller stele, thicker pericycle, larger pericycle cells and smaller vascular bundles and compact arrangement of cells can be attributed to immunity of *P. colubrinum* to *Phytophthora capsici*. Since the pathogen is soil borne, thicker epiderm and compact arrangement of cells restrict the entry of pathogen to *P. colubrinum* roots.

The smaller vascular bundles present in *P. colubrinum* and early thickening of secondary xylem helps in transport of water and nutrients more efficiently. In contrast, the thinner epiderm as well as endoderm and more number of root hairs present in Panniyur 1 impart susceptibility. Pathogen gets its entry to the roots through the thinner epiderm. Root hairs may secrete root exudates that might be responsible for attracting the pathogen and may in turn increase infection. The bigger stele present in Panniyur 1 may expose greater area for pathogen invasion.

The results of the present study is in confirmation with the observations made by Markose (1996) that well developed secondary xylem with thick celled epidermal and cortical cells were present in the resistant genotypes of chilli to bacterial wilt pathogen compared to susceptible one.

Similar observations on the anatomical characters of the roots and resistance / susceptibility expression were reported by Marks and Mitchell (1971) and Miller and Maxwell (1984) in alfalfa against *Phytophthora megasperma;* Phillips *et al.* (1987) in avocado against *Phytophthora cinnamomi*, Cahill *et al.* (1989) in *Acacia, Eucalyptus, Triticum, zea* etc. also against *Phytophthora cinnamomi* and by El-Hamalavi *et al.* (1995) in avocado against *Phytophthora citricola.*

5.2 Biochemical investigations

5.2.1 Biochemical characterisation of *Piper* spp.

Disease resistance is the ability of a plant to prevent, restrict or retard disease development and occurs at high, moderate or low levels. The resistance of plants to pathogen is largely due to the presence of substances that are toxic to microorganisms and the distributions of these toxic substances varies in different plant species.

Several chemicals of plant origin act as defensive barrier against the pathogen. Therefore it is imperative to know the role of preformed biochemicals like phenols, sugars, amino acids and enzymes in disease reaction in different pepper genotypes. The results of the biochemical investigations on *Piper* spp. are ascribed in this part.

5.2.1.1 Phenols

5.2.1.1.1 Total phenols

The total phenol content of the two species of *Piper* viz., *P. coluburinum* and *P. nigrum* varied significantly (Table 4 and Fig.1). The tolerant genotype of *P. nigrum* had higher content than the susceptible genotype Panniyur 1. The higher content observed in immune genotype compared to tolerant and susceptible genotype suggest the role of total phenols in disease reaction.

The protective role of phenolics in host plants against pathogen have been reported by many authors. Phenolics are well known antifungal, antibacterial and antiviral compounds. Phenolics inhibit fungal spore germination and production of fungal enzymes (Vidhyasekaran, 1975), mycelial growth (Tourneau *et al.*, 1957) and toxin production by pathogens (Chet *et al.*, 1978). Some of the phenolics are known to detoxify the toxins produced by the pathogens (Tamari and Kaji, 1955).

In the present investigations the immunity observed in *P. colubrinum* may be due to the higher content of total phenols and the susceptibility of Panniyur 1 may be due to the lower content. The higher content of total phenol observed in Kalluvally may be responsible for its ability to survive the stress conditions.

Similarly, findings on the high levels of total phenols in resistant varieties had been reported in betelvine against bacterial leaf spot pathogen (Abraham, 1986), chilli against *Phytophthora capsici* (Lizzi *et al.*, 1995), wheat against flag smut (Sindhan *et al.*, 1996) and chilli against bacterial wilt pathogens (Markose, 1996 and Paul, 1998).

5.2.1.1.2 OD phenol

The monomeric dihydroxy phenols also have an antibiotic and enzyme denaturing activity. *Ortho* dihydroxy phenols are important in disease reactions and are easily oxidised by enzymes like polyphenol oxidase and peroxidase to form *ortho* quinones (Bell, 1981). Further, high levels of dihydroxy phenols are desirable for resistance but the phenols still must be released and oxidised effectively.

In the present study the higher content of OD phenol observed in *P. colubrinum* may be responsible for its immune disease reaction (Table 5). The higher content observed in stems and roots of Kalluvally may be due to its ability to withstand stress conditions. The susceptible genotype Panniyur 1 recorded lower content of OD phenol in all the plant parts.

As suggested by Bell (1981), Kalluvally and *P. colubrinum* might have released OD phenols in greater amounts and might have oxidised effectively to form the toxic quinones.

Higher level of OD phenols had been reported in resistant plants of betelvine against bacterial leaf spot (Abraham, 1986), in wheat against flag smut (Sindhan *et al.*, 1996), in chilli against bacterial wilt (Markose, 1996 and Paul, 1998) in tomato (Paul, 1998).

Co-existance of phenols and sugars results in glycolisation of phenols by sugar, forming phenolic glycosides which are more soluble in cell sap and thus are involved more efficiently in resistance expression (Walker, 1975).

5.2.1.2 Sugars

Sugars are the precursors for synthesis of phenolics, phytoalexins, lignin and callose. Hence they play an important role in the defence mechanism of plants (Vidhyasekaran, 1990).

5.2.1.3.1 Reducing sugars

The wild species *P. colubrinum* had significantly higher content of reducing sugar compared to *P. nigrum*. The tolerant genotype of *P. nigrum* also had higher content over Panniyur 1. The highest content of reducing sugar in *P. colubrinum* and Kalluvally particularly in roots determine its immunity / tolerance to disease reaction and hence the lower content in Panniyur 1 determines its susceptibility (Table 6 and Fig.2). The higher content of reducing sugars in *P. colubrinum* might have been used for synthesis of phenolic compounds.

The higher levels of differences in content of reducing sugar in resistant host had been reported by Abraham (1986) in betelvine against bacterial leaf spot, Sindhan *et al.* (1996) in wheat against flag smut pathogen.

5.2.1.3.2 Non reducing sugars

The levels of non reducing sugars differed significantly in *P. colubrinum* and *P. nigrum* (Table 7). The content was higher in *P. nigrum* compared to *P. colubrinum* whereas in Panniyur 1 the susceptible genotype had higher content compared to tolerant genotype Kalluvally. Higher levels were found in stems of all the genotypes studied. *P. colubrinum* roots had the lowest content.

Sugars especially non reducing sugars are the most preferrednutrients for the pathogens. In the present study since *P. colubrinum* had lower non reducing sugar content, chances of development of the pathogen was less compared to *P. nigrum*. The roots of both the immune and tolerant genotypes had lower contents. This implies that magnitude of pathogen multiplication would be less in these genotypes. In contrast, the susceptible Panniyur 1 had higher content of sugar in roots which made possible for the pathogen to build up its high level of inoculum.

5.2.1.4 Amino acids

Amino acids are the corner stones for the synthesis of proteins and some of them are essential for the synthesis of phenolics, phytoalexins and lignin (Vidhyasekharan, 1990) and may enhance the production of toxins (Tanaka, 1963). Amino acids in their capacity behave as methyl donars and thus change the properties of cell wall and thus interfere with auxin action (Andel, 1965).

The content of amino acid differed significantly in *P. colubrinum* and *P. nigrum* and in also susceptible and tolerant genotypes of *P. nigrum* (Table 8 and Fig.3). *Piper colubrinum* had higher content compared to *P. nigrum*. Similarly, Kalluvally had higher content than that of Panniyur 1. The levels of amino acids in leaves were higher compared to stems and roots irrespective of the genotype. Very same trend was observed in immune and tolerant genotypes compared to susceptible wherein higher content was found in leaves followed by roots and stems.

Considering the above facts, higher levels of amino acids in immune genotype can afford enough protection against the invasion by pathogen. Since the main site of synthesis of amino acids and phenol related compound seems to be in leaves as the content in leaf was found to be high in all the genotypes. The higher contents of total free amino acids in all plant parts of *P. colubrinum* and Kalluvally govern the immunity / tolerance expression whereas the lower content in Panniyur 1 decides its susceptibility reaction.

Higher levels of amino acids in the expression of resistance were reported by Phukan (1994) in potato against *Phytophthora infestans* and Paul (1998) in brinjal against bacterial wilt pathogen.

5.2.1.5 Enzymes

Cellular death in connection with disease infection may deprive nutrients for the pathogen or may lead to release of many host enzymes which may supress the growth of the pathogen. Glucanases, IAA oxidases, peroxidase and polyphenol oxidase are such enzymes which are frequently correlated with expression of disease resistance.

5.2.1.5.1 Glucanase

Significant differences were observed among *P. colubrinum* and *P. nigrum* species, as well as tolerant and susceptible genotypes of *P. nigrum* for glucanase activity (Table 9 and Fig.4). Activity of enzyme was found to be more in Panniyur 1 followed by Kalluvally and *P. colubrinum*. In *P. colubrinum*, accumulation of enzyme was more in leaves followed by roots and stems. Roots of Kalluvally and Panniyur 1 had shown lower activity. Present study indicate that lower the enzyme activity greater the resistance reaction.

Bartinicki-Garcia (1969) reported that β-1,3-glucanase hydrolyse the β-1,3D glucan component of cell walls of pathogenic fungi.

The higher activity of glucanase make Panniyur 1 susceptible to the disease and the lower activity in Kalluvally and *P. colubrinum* make it tolerant / immune to the disease.

The higher activity of glucanases had been reported by Netzer *et al.* (1979) in fusarium wilt resistant near isogenic lines of muskmelon. Matta *et al.* (1988) reported that levels of β-1, 3-glucanase in tomato plants were dependent on the levels of peroxidase and polyphenol oxidase, phenols and released ethylene.

5.2.1.5.2 Indole - 3 acetic acid oxidase

Plants as well as phytopathogens produces growth regulators in new or different form which might stimulate or retard the production of similar compounds by the plants. IAA takes part in disease resistance by affecting cell wall structure and cell multiplication.

The activity of IAA oxidase varied in *P. colubrinum* and also in both the genotypes and *P. nigrum* (Table 10 and Fig.5). The activity also differed in all plant parts analysed. High activity was noticed in *P. nigrum* compared to *P. colubrinum* and in *P. nigrum* it was higher in Panniyur 1 compared to Kalluvally. Of the plant parts analysed, higher activity was noticed in roots followed by stems and leaves irrespective of genotype. In leaves and roots, higher activity was noticed in Panniyur 1 and Kalluvally than in *P. colubrinum* whereas in stems it was higher in *P. colubrinum* compared to Panniyur 1 and Kalluvally.

Present result indicates that higher the enzyme activity higher will be the susceptibility.

According to Hare (1964) the rate of oxidation of IAA by IAA oxidase depends on its cofactor *P*-coumaric acid which is phenolic in nature. Thus in the present study, higher level of total phenols might have inhibited the production of IAA oxidase in *P. colubrinum* whereas in Panniyur 1 and Kalluvally, the content of total phenols was low and hence activity of IAA oxidase was higher. Thus it is evident that there exist a negative correlation between levels of total phenols and IAA oxidase activity.

Vidhyasekaran (1974) reported that IAA interferes with normal lignification of host tissue and there was reduction in lignin in the ragi leaves. This could lead to accumulation of certain phenolic constituents as the phenolics are the precursors of lignin. The resultant accumulation of phenolics would have resulted in accumulation of IAA by interferring with IAA oxidase.

5.2.1.5.3 Peroxidase

Peroxidase appears to be a key enzyme involved in the biochemical pathways. In many cases peroxidase was involved in phenol and lignin metabolism and it is the enzyme which oxidises phenolics into quinones that are more fungitoxic than phenolics thus may induce disease resistance.

In the present study higher activity of peroxidase enzyme was observed in *P.colubrinum* compared to *P.nigrum* genotypes, kalluvally and Panniyur 1. Irrespective of the genotypes studied leaves had highest activity followed by stems and roots (Table 11).

From the study it is evident that higher levels of peroxidase enzyme activity in all parts of *P. colubrinum* might be involved in defense mechanisms either by forming toxic compounds with polyphenol oxidase and OD phenols or by promoting process of lignification or by synthesis of melanin. Thus in the present investigations, correlation between higher levels of total phenols, peroxidase and lower levels of OD phenol and polyphenol oxidase were observed. Higher activity of peroxidase in resistant host plants had been reported by Mace (1964) in wheat against rust, Lizzi and Coulomb (1991) in chilli against *Phytophthora capisici*, Markose (1998) in chilli against bacterial wilt and by Paul (1998) in brinjal against bacterial wilt pathogen of brinjal.

5.2.1.5.3 Polyphenol oxidase

Polyphenol oxidase can catalyse the oxidative polymerization of phenolic substances into quinones and tannins (Vidhyasekaran, 1990). The total activity of polyphenol oxidase was found to be higher in *P. colubrinum* compared to *P. nigrum* (Table 12). Also Kalluvally had higher activity than Panniyur 1. The total enzyme activity was found to be higher in leaves followed by stems and roots irrespective of genotype studied.

A comparison of enzyme activities in leaf, stem and root of susceptible, tolerant and immune genotypes revealed that higher the polyphenol oxidase activity higher is the resistance. Considering the soil borne nature of the pathogen and main site of infection, activities of enzymes in roots and stems may play a greater role in disease incidence. In this context *P. colubrinum* possesses higher activities in roots and stems followed by Kalluvally and Panniyur 1 (Fig.6). The higher activity in immune genotype indicate that *P. colubrinum* is endowed with greater potentiality to activate polyphenol more rapidly so that quinone, melanin and tannin production may be increased.

Higher levels of polyphenol oxidase in resistant varieties had been reported by Gupta *et al.* (1995) in *Brassica* species against alternaria leaf blight, Markose (1996) in chilli against bacterial wilt, Paul (1998) in brinjal and tomato against bacterial wilt. Thus present study is in line with the above findings.

5.2.3 Comparison of biochemical parameters in *Phytophthora capsici* inoculated and uninoculated plants of *Piper* spp.

In many plant pathogen interactions phenomena involved in the defense action of plants to pathogen have been found and reported. Principal factors involved in the expression of resistance is based on genetic composition of the host and expression of the resistance genes may be influenced by many factors like aggressiveness of the pathogen, availability of specific nutrients, inhibitory substances at the infection site, metabolism of the host constituents including the activity of numerous enzyme systems, host nutrition and environment (Barnett, 1959).

5.2.3.1 Phenols

Diverse role of phenols is well known. In general phenols do accumulate within infection site and in zone surrounding to it. Thus accumulation of phenolics as an initial response to infection may reflect a general increase in host metabolism as well as an accumulation of relatively non toxic secondary metabolites, which could ultimately serve as precursors for compounds essential for the expression of resistance (Nicholson and Hammer- Schmidt, 1992).

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5.2.3.1.1 Total phenols

Following *Phytophthora capsici* inoculation total phenol content increased in all the genotypes irrespective of plant parts analysed (Table 13 and Fig.7). Higher increase was found in Panniyur 1 followed by Kalluvally and *P. colubrinum*. Of the plant parts, leaves had shown higher increase compared to stems. Tepper and Anderson (1984) reported that phenolics in high concentrations are toxic to plant cell themselves and hence will normally be present in small quantities. The higher content of total phenol observed in *P. colubrinum* might be responsible for checking lesion development after inoculation with Pathogen. But in Kalluvally and Panniyur 1 increased lesion development was observed after inoculation. The lower phenol content in plant part as well the increased production after inoculation might be responsible for increased lesion development in the two genotypes of *P. nigrum*. The increased production of phenols which are cytotoxic might have contributed to increased cell death in Panniyur 1 and Kalluvally and thereby its hypersensitive reaction to the disease.

Markose (1996) reported higher content of total phenols in susceptible chilli varieties to bacterial wilt.

5.2.3.1.2 Ortho dihydric phenol

The enzymes viz., polyphenol oxidase and peroxidase oxidise the colourless dihydroxyphenols to give the coloured *ortho* quinones. While certain dihydroxy phenols get conjugated with each other or glucose hydroxyl group to form tannins, both form constituent of plant melanins. (Beckman *et al.*, 1974; Mayer and Harrel, 1979 and Bell, 1981). These tannins and *ortho* quinones have toxicity to microorganisms (Hunter, 1978).

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Inoculation of Pathogen on leaves and stems resulted in significant decrease of OD phenol content within 72 h in the three genotypes studied (Table 14 and Fig.8). Maximum decrease was observed in *P. colubrinum* compared to *P. nigrum*. Similarly decrease was found to be more in Kalluvally compared to Panniyur 1.

As in the present investigation, Addy (1976) reported that on inoculation of *Malus pumila* with *Erwinia amylovora*, resistant varieties leached dihydroxy phenols more rapidly than the susceptible one. Similarly flag smut resistant wheat varieties had higher levels of OD phenols, but on inoculation with fungi its level decreased (Sindhan *et al.*, 1996).

5.2.3.2 Sugars

According to Vidhyasekaran (1990) sugars are the preferred nutrient for pathogens and they are precursors for the synthesis of phenolics.

5.2.3.2.1 Reducing sugars

There was significant reduction in quantity of reducing sugars in both species of *Piper* on inoculation (Table 15). More reduction in reducing sugar content was observed in *P. colubrinum* compared to *P. nigrum*. Among *P. nigrum* genotypes, decrease in reducing sugar was observed only in Kalluvally while in Panniyur 1, sugar content increased.

Sugars are the precursors for phenol synthesis. The higher content of sugar present in *P. colubrinum* may be responsible for higher content of total phenol and there by its immunity. On inoculation, the decrease in sugar content observed in *P. colubrinum* might be due to its conversion to polyphenols. Since the sugars are the preferred nutrient for the pathogen development, decrease in sugar content observed in *P. colubrinum* on inoculation might be responsible for its immunity. On the other hand, increase in sugar content observed in Panniyur 1 on inoculation might be responsible for its susceptibility by giving ample substrate for the pathogen. The decrease in sugar content observed in Kalluvally as in *P. colubrinum* might be responsible for its tolerance.

Easwaran (1967) observed reduction in quantity of reducing sugar in susceptible as well as in moderately susceptible varieties on inoculation with bacterial wilt pathogen. Prasada *et al.* (1972) also observed reduction in reducing and total sugar in bacterial leaf blight resistant rice varieties inoculated with pathogen. Reduction in sugar content had been reported by Sindhan *et al.* (1996) in flag smut resistant wheat varieties.

5.2.3.2.1 Non reducing sugar

Significant depletion of non reducing sugar was observed in inoculated plant parts of the three genotypes studied. The decrease was more in *P. colubrinum* compared to *P. nigrum*. Maximum decrease in non reducing sugar content was observed in *P. colubrinum* followed by Kalluvally and Panniyur 1 (Table 16 and Fig.9).

The co-existance of phenols and sugars results in glycolisation of phenols by sugars forming phenolic glycosides which are more soluble in cell sap and thus are involved more efficiently in resistance expression (Walker, 1975). The carbohydrates may also be utilised for meeting the energy requirement of host plants due to increased respiration. The reduction in sugar contents in stems of all genotypes and leaves of Kalluvally and *P. colubrinum* can be attributed to the fact that a major part of these sugars being shifted for polyphenol synthesis as reported by Niesh (1964).

Abraham (1986) attributed the resistance of betelvine cultivars to bacterial leaf spot pathogen as pre-inoculation higher levels of reducing, non reducing and total sugars and post-inoculation increase of non reducing sugar content in susceptible cultivars. Decreased photosynthetic efficiency and content of chlorophyll, reducing, non reducing and total sugars and starch content in capsicum leaves inoculated with

Alternaria solani was reported by Veermohan *et al.* (1994). Paul (1998) reported higher levels of soluble sugars in bacterial wilt resistant varieties of chilli and tomato.

5.2.3.3 Total free amino acids

On inoculation there was significant increase in amino acid content in *P. colubrinum* and *P. nigrum*. Maximum increase was found in *P. colubrinum* and Panniyur 1, whereas decrease was observed in Kalluvally (Table 17 and Fig.10). In general there was increase in content due to inoculation in stems as well as in leaves irrespective of the genotypes studied.

An increase in amino acid content may be due to decomposition of host protein or decreased protein synthesis. Similarly synthesis of amino acid by growing fungus would also cause an increase in the amino acid content, whereas decrease in content was attributed to the utilisation by pathogen (Andel, 1965).

The inhibitory effect of the phenols already present might be stimulated by an increase in the content of amino nitrogen. Also several natural amino acids in their capacity behave as methyl donars and thus change the properties of cell wall and there interference with auxin preferably antagonise the effect of IAA in defense response.

There was decrease in amino acid content in less susceptible cultivar of betelvine due to inoculation of *Phytophthora* leaf rot pathogen (Chile and Vyas, 1983). In brinjal the resistant genotype recorded higher content of amino acids under healthy condition and its level decreased under diseased environment of bacterial wilt pathogen (Paul, 1998).

5.2.3.4 Enzymes

Enzymes are large protein molecules which catalyse all the interrelated reactions in a living cell. Most of the plant pathogens secrete enzymes throughout their existance or upon contact with a substrate.

5.2.3.4.1 Glucanase

Fungal cell wall contains glucans and the β-1,3-glucanase enzyme can degrade them leading to lysis of mycelium. On inoculation there was no increase in lesion size in *P. colubrinum* but was maximum in Panniyur 1 as compared to Kalluvally (Plate VIII). The lower glucanase activity observed in *P. colubrinum* (Table 18) might be due to lower degradation of cell wall glucans present in the mycelium of *Phytophthora capsici*. Due to higher degradation of cell wall glucans of pathogen higher enzyme activity was observed in Panniyur 1.

Increased enzyme activity might be due to acceleration of host enzymes on account of wounding or pathogen attack and rapid accumulation of β-1,3-glucanase from surrounding cells containing pathogenesis related protein at the infection site.

The cultivar of *Lotus corniculatus* containing β-1,3-glucanase and cyanogenic glycosides were wounded, glycosides hydrolysed to produce hydrocyanic acid whereas in the cultivars lacking enzyme there was no production of hydrocyanic acid (Miller and Hemphill, 1978). In the present study, enzyme activity was maximum in leaves of Panniyur 1 which could be due to production of enzyme both by host and pathogen.

Increased activity of β-1,3-glucanase and chitinase upon infection with non pathogenic form of *Fusarium oxysporum* were observed in tomato plants. The activity of these enzymes was correlated with variations in peroxidase and polyphenol oxidase activity (Matta *et al.*, 1988). Thus there exist a positive correlation of this enzyme with increased peroxidase and decreased polyphenol oxidase activity.

5.2.3.4.2 Indole - 3 acetic acid oxidase

Growth regulators have repeateadly been shown to influence disease resistance. IAA may affect cell wall properties and there by resistance but on the other hand a primary change in cell wall composition might influence the response of plants to IAA or fungi.

On inoculation, activity of enzyme increased in *P. colubrinum* while it decreased in *P. nigrum* (Table 19). Similarly, in Kalluvally activity increased while that of decreased in Panniyur 1. Irrespective of the genotype, there was increase in enzyme activity in leaves while stems of all genotypes recorded decrease.

This fact is exemplified by decreased OD phenol levels in inoculated plants. Since IAA and phenols have common synthetic pathway (Kosuge, 1969), decrease in OD phenol showed increase in IAA oxidase activity.

Another factor to be taken into consideration is the rate of oxidation of IAA by IAA oxidase by its cofactor *P*-coumaric acid which is phenolic in nature (Hare, 1964). Phenolic based compounds like caffeic acid, chlorogenic acid inhibited the oxidation of IAA in tobacco transgenic plants (Gazaryan and Lagrimini, 1996). When taken into account these facts, higher levels of total phenolics in inoculated plants exemplifies the higher level of IAA oxidase in inoculated leaves of Kalluvally and *P. colubrinum.*

Comai and Kosuge (1984) reported abnormal multiplication of olive and oleander trees at infection site was due to excess production of IAA by *Pseudomonas syringae* pv. *Savastoni*. The higher activity observed in *P. colubrinum* might be due to excess production of IAA by pathogen at infection site.

5.2.3.4.3 Peroxidase

In the presence of hydrogen peroxide this enzyme carries out the oxidation of a variety of compounds, including aliphatic and aromatic amines and phenolic compounds. It also increases the growth promoting activity of IAA (Kosuge, 1969). Thus changes in peroxidase enzyme system after inoculation seems to be of undue importance. Peroxidase activity increased by 198 per cent over control in

P. colubrinum as against 74 per cent in *P. nigrum* (Table 20 and Fig.11). Similarly, in Kalluvally it increased by 114 and in Panniyur 1 by 55 per cent. Activity increased much fastly in stem and leaves of all genotypes.

The higher activity of *P. colubrinum* suggest the role of enzyme in imparting resistance against *Phytophthora capsici*. To the greater extent, many fold increased activity in stems of *P. colubrinum* might be responsible for restricted lesion development after 72 h of pathogen inoculation as compared to Kalluvally and Panniyur 1. Since peroxidase promotes IAA activity, possibility of cell wall thickening and cell multiplication can not be denied. Further, in conjunction with OD phenols and polyphenol oxidase peroxidase forms toxic compound like lignin and tannin and quinone which are more toxic to the pathogen. The very same fact can be exemplified by decreased levels of OD phenols and polyphenol oxidase in inoculated plants under present study.

Increase in peroxidase activity on inoculation in various host plants can substantiate present findings. Grangeon and Coulomb (1977) found increased activities of hydrolase and peroxidase in *Phytophthora capsici* inoculated stems of capsicum plants. Coulomb and Coulomb, (1984) observed more accumulation of enzyme in the intercellular spaces of resistant capsicum leaves infected with *Phytophthora capsici* than the susceptible one. Lizzi and Coulomb (1991) also reported higher activities of peroxidase and catalase in capsicum leaves inoculated with *P. capsici* and high concentration of accumulated enzyme could be laid to the destruction of pathogen. Alacazar *et al.* (1995) also reported higher enzyme activity in intercellular spaces of resistant capsicum plants wherein they could not find invasion of cells by pathogen. Higher activity of peroxidase in conjunction with polyphenol oxidase and OD phenol was attributed to the resistance in chilli (Markose, 1996). Paul (1998) also reported higher activity of peroxidase enzyme in brinjal under diseased condition.

5.2.3.4.4 Polyphenol oxidase

Polyphenol oxidase is the key enzyme in disease resistance and its level depend upon shifting of sugars for its synthesis. In fact its polymerization capacity depends on levels of dihydroxy phenols and peroxidase.

After inoculation, enzyme activity decreased substantially in all the plant parts and genotypes studied (Table 21). Decrease in enzyme activity was maximum in *P. nigrum* compared to *P. colubrinum*. Maximum decrease was observed in Kalluvally followed by Panniyur 1 and *P. colubrinum* (Fig.12). Both in leaves and stems of *P. colubrinum*, activity increased linearly whereas in Panniyur 1 increase was slow. However, in Kalluvally, leaves and stems enzyme activity decreased after third minute.

Steady increase in activity implies that there was continuous production of toxic compounds like quinone and promotion of plant growth by auxins. Whereas steady or decrease in enzyme activity can be attributed to inhibition of production of toxic compounds in course of plant growth. Comparative analysis of leaves and stems of susceptible, tolerant and immune genotype and their reaction with polyphenol oxidase revealed that linear increase in enzyme activity imparts greater resistance.

Accelerated activity in immune species indicates that *P. colubrinum* is endowed with greater potentiality to activate polyphenol aggressively, effecting more production of lignin, quinone and tannins thus the advance of assilating pathogen could be effectively checked.

The specific activity of polyphenol oxidase remain higher while that of peroxidase remained lower in tolerant *Brassica carinata* and *B. napus* as compared to *B. juncea* and *B. campestris* but after infection with alternaria leaf blight pathogen activity of both enzymes increased comparatively at much faster rate in the susceptible species. Activity of polyphenol oxidase was considerably higher at initial stages of plant growth in all species which markedly dropped at later stages. (Gupta *et al.*, 1995).

Thus from the study it is evident that there exists negative correlation between peroxidase and polyphenol oxidase enzyme activities. Rich and Horsfall (1954) concluded from their experiments that the susceptibility of fungi to the phenolics seemed to be related to the property of polyphenol oxidase contained in the material.

So it can be concluded from the present study that anatomical and biochemical differences observed in *Piper* spp. can effectively be used for screening the genotypes against tolerance / resistance to *Phytophthora capsici*. The selection of tolerant / immune genotype via screening will be helpful to evolve a resistant / tolerant plant type and also in the selection of parents for further breeding programmes. Keeping view of the changes in biochemical constituents on inoculation with pathogens, possibility of evolving a management strategy for the control of the disease cannot be ruled out.

Summary

SUMMARY

Anatomical and biochemical investigations on *Phytophthora* foot rot disease reaction in *Piper* spp. were conducted in the Department of Plantation Crops and Spices and Biochemistry Laboratory of the College of Horticulture, Vellanikkara during 1996-1998. The salient findings of the study are summarised in this chapter.

The two species viz., *Piper colubrinum* L. and *P. nigrum* L. and two genotypes of *P. nigrum* viz., Panniyur-1 and Kalluvally differed significantly in the various anatomical and biochemical parameters tested. The various characters studied are compared among the three genotypes.

Anatomical features of *Piper* spp.

Sl. No.	Anatomical parameters	*Piper nigrum* L.		*P. colubrinum* L.
		Panniyur-1	Kalluvally	
1	2	3	4	5
I. Anatomical features of leaf				
1.	Upper cuticle thickness (µm)	Thickest 0.44-0.88	Thick 0.15-0.30	Thicker 0.10-0.50
2.	Upper epiderm cells size (µm²)	Small 19.92-183.00	Smaller 14.94-133.20	Smallest 9.96-20.75
3.	Inter cellular spaces (µm)	Small 0.83-8.30	Smaller 1.66-4.98	Smallest 0.16-1.66
4.	Spongy parenchyma cells size (µm²)	Small 956.00-4364.00	Smaller 876.00-1912.00	Smallest 3.28-790.00
5.	Palisade tissue thickness (µm)	Thick 8.30-11.62	Thicker 6.64-13.28	Thickest 9.96-16.60

Contd.

1	2	3	4	5
6.	Vascular bundle size (µm^2)	Big 1513.00-1640.00	Biger 912.00-2390.00	Biggest 2390.00-3984.00
7.	Mucilage canal size (µm^2)	Big 10.40-14.94	Bigger 6.64-80.60	Biggest 12.14-85.71
8.	Collenchyma tissue thickness (µm)	Thick 8.30-11.62	Thicker 9.96-14.94	Thickest 8.30-15.30
9.	Lower epiderm cell size (µm^2)	Smallest 13.9-16.6	Small 41.5-49.80	Smaller 16.7-49.80
10.	Lower cuticle thickness (µm)	Thicker 0.70-1.20	Thickest 0.17-2.49	Thick 0.17-1.66
11.	Stomata per unit area	Most 73.00-130.00	More 52.00-90.00	Many 41.00-71.00
II. Anatomical features of stem				
1.	Hypoderm thickness (µm)	Thickest 24.94-41.50	Thicker 23.24-28.32	Thick 8.3-14.94
2.	Chlorenchyma thickness (µm)	Thick 9.60-17.00	Thicker 11.6-23.00	Thickest 11.17-37.81
3.	Cortex thickness (µm)	Thick 20.00-69.00	Thicker 60.00-76.00	Thickest 66.00-99.60
4.	Inter cellular spaces (µm)	Smallest 0.16-0.33	Smaller 0.16-0.90	Small 0.90-1.50
5.	Peripheral bundle number	Few 12.00-30.00	Few 18.00-32.00	Many 24.00-44.00
6.	Peripheral bundle size (µm^2)	Large 79.86-876.00	Larger 278.00-1192.00	Largest 717.00-1992.00
7.	Pith size (µm^2)	Largest 1340.00-1634.00	Large 613.00-841.00	Larger 695.00-1003.00
III. Anatomical features of root				
1.	Epiderm thickness (µm)	Thick 12.40-14.11	Thicker 16.16-19.64	Thickest 16.16-24.97

Contd.

1	2	3	4	5
2.	Cortical cell size (μm^2)	Smallest 19.92-157.70	Smaller 119.50-182.60	Small 39.81-332.00
3.	Inter cellular spaces (μm)	Larger 1.33-1.67	Largest 1.50-2.10	Large 1.50-1.66
4.	Pericycle thickness (μm)	Thick 6.64-9.13	Thicker 6.64-11.62	Thickest 8.30-13.28
5.	Pericycle cell size (μm^2)	Large 29.90-50.80	Larger 39.90-59.80	Largest 39.90-79.70
6.	Stele size (μm^2)	Largest 10372.00-11872.00	Larger 5845.00-7139.00	Large 2790.00-4012.00
7.	Vascular bundle size (μm^2)	Larger 438.30-511.30	Largest 730.40-2390.00	Large 132.80-310.00

Biochemical characterisation of *Piper* spp.

Biochemical parameters	Panniyur 1	Kalluvally	*P. colubrinum* L.
1	2	3	4
Total phenols (mg/g)	Low 2.123-2.987	Medium 3.245-4.516	High 3.437-5.539
Ortho dihydroxy phenols (mg/g)	Low 3.527-5.093	High 5.210-7.627	Medium 4.863-7.667
Reducing sugar (mg/g)	Low 1.615-2.474	Medium 2.374-3.444	High 3.304-4.170
Non reducing sugar (mg/g)	High 1.652-2.464	Medium 1.444-1.976	Low 1.350-1.792
Total free amino acid (%)	Low 0.017-0.030	Medium 0.023-0.035	High 0.028-0.047
Glucanase enzyme activity (mg/g)	High 0.967-1.749	Medium 0.857-1.307	Low 0.646-1.098

Contd.

1	2	3	4
IAA oxidase enzyme acitivy (mg/g)	High 0.794-2.075	Low 0.476-1.943	Medium 0.253-1.940
Peroxidase enzyme activity (units/litre)	Low 127.982-200.000	Medium 168.016-422.414	High 196.783-430.175
Polyphenol oxidase enzyme total activity	Low 0.061-0.268	Medium 0.049-0.260	High 0.098-0.287

Comparison of biochemical parameters in *Phytophthora capsici* inoculated and uninoculated plants of *Piper* spp.

	Panniyur-1		Kalluvally		*P. colubrinum* L.	
	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
1	2	3	4	5	6	7
Total phenol (mg/g)	Low 2.123-2.239	Medium 5.628-6.413 Increased (170%)	Medium 3.245-3.645	High 6.126-7.328 Increased (107%)	High 3.437-5.539	Medium 4.973-7.504 Increased (39%)
Ortho dihydric phenol (mg/g)	Low 4.537-5.093	Low 2.223-4.973 Decreased (34%)	High 7.060-7.627	Low 1.157-3.306 Decreased (229%)	Medium 5.997-7.667	Low 1.303-2.433 Decreased (266%)
Reducing sugar (mg/g)	Low 2.375-2.474	High 1.753-3654 Increased (12%)	Medium 2.374-3.425	Low 1.690-1.963 Decreased (56%)	High 3.304-3.711	Medium 1.516-2.224 Decreased (88%)
Non reducing sugar (mg/g)	High 1.652-2.464	High 1.595-1.787 Decreased (22%)	Medium 1.488-1.976	Medium 0.330-1.440 Decreased (95%)	Low 1.440-1.792	Low 0.074-0.232 Decreased (958%)
Total free amino acid (%)	Low 0.017-0.030	Low 0.029-0.031 Increased (25%)	Medium 0.023-0.035	Medium 0.016-0.049 Decreased (6.3%)	High 0.039-0.047	High 0.037-0.099 Increased (58%)

Contd.

Continued

1	2	3	4	5	6	7
Glucanase enzyme activity (mg/g)	High 1.445-1.749	High 1.376-3.670 Increased (58%)	Medium 1.190-1.307	Medium 0.575-1.803 Decreased (5%)	Low 0.646-1.098	Low 1.075-1.135 Increased (27%)
IAA oxidase activity (mg/g)	High 0.794-0.936	Low 0.139-0.513 Decreased (165%)	Low 0.476-0.953	Medium 0.446-1.327 Increased (24%)	Medium 0.253-1.251	High 0.713-1.411 Increased (41%)
Peroxidase enzyme activity (units/litre)	Low 147.181-200.00	Low 344.699-399.415 Increased (114%)	Medium 280.081-422.414	Medium 351.153-735.608 Increased (55%)	Medium 368.548-430.175	High 1111.827-1269.438 Increased (198%)
Polyphenol oxidase enzyme total activity (OD value)	Low 0.071-0.268	Medium 0.069-0.242 Decreased (9%)	Medium 0.098-0.260	Low 0.047-0.080 Decreased (180%)	High 0.260-0.287	High 0.240-0.278 Decreased (6%)

(Figures in parentheses indicate percent increase or decrease in inoculated plants over the uninoculated plants)

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* Originals not seen

APPENDIX-I

Chemical composition of media used in the study

Quantity (mg / l)

(1) Composition of Potato Dextrose Agar Medium

Potato	200 g
Agar	20 g
Distilled water	1 l
Dextrose	20 g

(2) Composition of Carrot Agar Medium

Carrot	200 g
Agar	18 g
Distilled water	1 l

ANATOMICAL AND BIOCHEMICAL INVESTIGATIONS ON *PHYTOPHTHORA* FOOT ROT DISEASE REACTION IN *Piper* spp.

By

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ABSTRACT OF A THESIS

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ABSTRACT

Anatomical and biochemical investigations on *Phytophthora* foot rot disease reaction in *Piper* spp. were carried out in the Department of Plantation Crops and Spices and Biochemistry Laboratory of the College of Horticulture, Vellanikkara during 1996 to 1998.

The study revealed that the two species viz., *Piper colubrinum* L. and *P. nigrum* L. and two genotypes of *P. nigrum* viz., Panniyur 1 and Kalluvally differed significantly in the various anatomical and biochemical parameters studied.

The immune genotype was characterised by compact arrangement of cells, small epidermal, mesophyll and spongy parenchyma cells, thick palisade and collenchyma tissues, large vascular bundles and mucilage canals, thick lower epiderm with large cells and less number of stomata per unit area in the leaves. The stem was characterised by thin cuticle, epiderm and hypoderm, small sized epidermal cells, thick cortex, large cortical cells with more inter- cellular spaces, thick chlorenchyma and sclerenchyma, more number of large peripheral and medullary vascular bundles, large mucilage canals and small indistinct pith. It had thick root epiderm,less number of root hairs, small cortical cells, thick endoderm, big stele, pericycle cells and vascular bundles.

In contrast, the susceptible genotype was characterised by thin palisade and collenchyma tissues, small vascular bundles and mucilage canals, thin lower epiderm with small cells and more number of stomata per unit area. The stems of the susceptible genotype exhibited thicker cuticle with large epidermal cells, more epidermal appendages, thick hypoderm with small cells, thin cortex with small cortical cells arranged with less intercellular spaces and thin sclerenchymatic tissues. It had thin root epiderm, more number of epidermal appendages, small cortical cells with large stele, thin pericycle with small cells and distantly placed small vascular bundles.

The tolerant genotype Kalluvally exhibited somewhat intermediate values to *P. colubrinum* and Panniyur 1 in all the anatomical characters tested.

In the biochemical parameters studied, *P. colubrinum* registered high content of total phenol, reducing sugar, total free amino acid and higher activities of peroxidase and polyphenol oxidase enzymes. The susceptible genotype Panniyur 1 was marked by low of total and OD phenols, reducing sugars, total free amino acids, peroxidase and polyphenol oxidase activities and high glucanase and IAA oxidase activities. The tolerant genotype Kalluvally registered medium values for total phenols, reducing and non reducing sugars, total free amino acids and enzymes like peroxidase, glucanase and polyphenol oxidase. However, the content of OD phenol was high and IAA oxidase activity was low in Kalluvally.

On inoculation with *Phytophthora capsici*, the content of total phenol, reducing sugar, total free amino acid and enzyme activities like glucanase and peroxidase increased in Panniyur 1 whereas the contents of OD phenol, non reducing sugar and activities of IAA oxidase and polyphenol oxidase decreased. In *P. colubrinum* the contents of total free amino acids and peroxidase enzyme activity increased at a higher rate than in the other genotypes while the content of OD phenol, reducing and non reducing sugars decreased drastically on inoculation. Kalluvally exhibited similar trend as that of *P. colubrinum* and registered somewhat intermediate values between *P. colubrinum* and Panniyur 1 in almost all the biochemical parameters tested. However, decrease in glucanase activity and a greater reduction in polyphenol oxidase activity were observed in Kalluvally on inoculation.

The anatomical and biochemical differences observed in *Piper* spp. can effectively be used for screening the genotypes for tolerance / resistance to *Phytophthora capsici* and for the management of the disease.

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