INVESTIGATIONS ON CERTAIN BIOCHEMICAL CHANGES AND PHYLLOSPHERE MICROFLORA OF HEVEA BRASILIENSIS AS INFLUENCED BY NITROGENOUS FERTILIZER APPLICATION AND CORYNESPORA CASSIICOLA INOCULATION

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to my Parents

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Certificate

This is to certify that the thesis entitled **Investigations on Certain Biochemical Changes and Phyllosphere Microflora of *Hevea brasiliensis* as Influenced by Nitrogenous Fertilizer Application and *Corynespora cassiicola* Inoculation** is an authentic record of the research work carried out by **Mrs. Annakutty Joseph** under my scientific supervision and guidance at the Rubber Research Institute of India, Kottayam, in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** of the Mahatma Gandhi University, under the Faculty of Science and no part thereof has been presented for the award of any other degree, diploma or associateship in any University.

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Declaration

I hereby declare that this thesis entitled **Investigations on Certain Biochemical Changes and Phyllosphere Microflora of *Hevea brasiliensis* as Influenced by Nitrogenous Fertilizer Application and *Corynespora cassiicola* Inoculation** has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar title for recognition.

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CONTENTS

Chapter 1

INTRODUCTION

Natural rubber (*Hevea brasiliensis* Muell. Arg.) is an important plantation crop in India which occupies 5.33 lakh ha with an annual production of 5.49 lakh tonnes. India is the fifth largest rubber growing country and ranks fourth in natural rubber production. Over 90 per cent of the natural rubber is produced in Kerala State and it plays a major role in the economy of this state. One of the major constraints to rubber cultivation is the diseases causing considerable damage to trees and yield loss. Almost all parts of the rubber tree, succumb to the attack of various fungal pathogens. The leaf spot disease caused by *Corynespora cassiicola* (Berk and Curt) Wei is an important nursery disease. Even though this disease was considered as minor and non-significant in the rubber plantations in India, it is now assuming importance by attacking mature plants as well. Recent epidemics of the disease forced countries like Sri Lanka and Indonesia to destroy some susceptible high yielding clones. The disease becomes more serious over years and some clones which are classified as resistant or tolerant become highly susceptible to *C. cassiicola*. Now this disease is a potential threat to the rubber plantations in the world.

In nurseries, the disease is observed during the period of February-May. Both young and mature leaves are affected, the former being more susceptible. Severe incidence leads to heavy leaf fall which ultimately

affect the growth of the plants and thereby reduce the percentage of buddable plants in seedling nurseries.

Disease incidence is determined by genetic factors of both host and pathogen. Disease resistance is the rule and susceptibility is the exception. Plants have their own built-in defence mechanism against almost all microorganisms, but in a few cases, the so-called pathogens overcome the defence barrier with their offensive chemicals and cause disease. It is now becoming increasingly clear that many types of resistance involve biochemical interaction between host tissues and invading pathogen. Environment and other cultural practices are also important for susceptibility of the host and pathogenicity of the organism. Mineral nutrition of the plants has pronounced influence in determining the infection and disease development.

Indiscriminate application of fertilizers especially application of heavy doses of nitrogenous fertilizer predisposes the plants to diseases. Rajalakshmy *et al.* (1979) reported a heavy incidence of *Corynespora* leaf spot disease in rubber seedlings with higher nitrogen level and low incidence in its absence. A higher susceptibility of *Hevea* seedlings to *Helminthosporium* attack is reported owing to the higher level of nitrogenous fertilizer application (Jones and Hilton, 1958). The chemical constituents of the plants are altered by the application of fertilizers especially nitrogen. Biochemical resistance/susceptibility against any disease depends mainly on pre-existing, pre-formed or induced toxic chemicals of the host or pathogen. Several physiological processes in the host are stimulated due to the activation of host enzymes, during host pathogen interaction. Studies on biochemical basis of resistance is gaining importance during recent years in view of the need for minimising the pollution of biosphere and preservation of ecological balance

endangered by indiscriminate use of chemicals. Biochemical nature of disease resistance involved in rubber to various other diseases was attempted by several workers (Figari, 1965; Hashim *et al.*, 1980; Giesemann *et al.*, 1986; Sanier *et al.*, 1992; Garcia *et al.*, 1995a, b). However no attempt is made so far to find out biochemical changes in rubber seedlings due to nitrogenous fertilizer application and *Corynespora* infection.

The conditions prevailing at the leaf surface of the host may also play a major role in predisposing the plants to diseases. The surface of the plants harbours a number of microorganisms and the various activities of these microorganisms may affect the ability of pathogens to begin or continue the growth, if the plants are to be infected. These interactions partly depend on the metabolites available on the leaf surface. These metabolites, containing nutrients and inhibitory substances may directly affect the growth of microorganisms or encourage certain groups to dominate the environment and function as antagonist of the pathogen. The nutrient composition of these metabolites is influenced by nutrient composition of the leaf tissues which is controlled by the fertilizer applied.

The resistance or susceptibility of a plant to leaf disease is the sum of biochemical reactions of the host and the interaction of leaf surface microflora. Hence a study on biochemical changes and phyllosphere microflora of rubber seedlings under graded levels of nitrogen and C. *cassiicola* inoculation was carried out. Such studies may help to unravel the mysteries of disease resistance/susceptibility and enable the pathologists to engineer plants to win the battle even when the pathogens have an array of offensive chemicals.

Chapter 2

REVIEW OF LITERATURE

Natural rubber (*Hevea brasiliensis*) is one of the most important plantation crops in India. Like many other crops, *Hevea* is also subjected to the attack of number of fungal pathogens. *Corynespora* leaf disease caused by *Corynespora cassiicola* is one of the important leaf disease of rubber. A recent survey on the intensity and the importance of diseases of *Hevea* in natural rubber producing countries ranked *Corynespora* leaf disease as the fourth most serious disease (Allen and Cronin, 1994). This disease was reported for the first time in India during 1958 in a seedling rubber nursery (Ramakrishnan and Pillay, 1961). George and Edathil (1980) reported the occurrence of *Corynespora* leaf disease incidence in mature trees. Recently *Corynespora* infection has been found to be much damaging on mature trees in South Canara district of Karnataka State. Young leaves emerged after defoliation in February-March are severely affected causing heavy defoliation, die-back of the twigs and yield loss.

In nurseries, infection is observed on young leaves during the sunny months of November to May. The common symptoms developed on leaves are circular or irregular brown spots of about 1-10 mm in diameter, usually surrounded by an yellow halo. The number of spots may vary from a few to many and even a single lesion on the mid rib of a leaflet can cause defoliation of the leaves (Liyanagae *et al.*, 1986). Repeated defoliation and refoliation

results in growth retardation and reduction in the number of buddable plants in seedling nurseries.

Some of the factors favouring the development of pathogen are high humidity, temperature ranging from 28–30°C (Situmorang *et al.*, 1996) light intensity agromanagement practices as well as clones in the case of mature plants. Ananth and Menon (1965) reported partial shading for the control of this leaf spot disease. Rajalakshmy *et al.*, (1978) observed an increased *Corynespora* disease incidence in the presence of higher dose of nitrogen and low incidence in its absence. A higher susceptibility of *Hevea* seedlings to *Helminthosporium* sp. attack is also reported owing to the higher level of nitrogenous fertilizer application (Jone and Hilton, 1958). Higher disease susceptibility with excess dose of nitrogen has been reported in a number of crops. Kang *et al.* (1993) reported a higher *Corynespora* leaf spot development in cucumber in the presence of higher nitrogen. Plants treated with increased concentration of nitrogen showed increased susceptibility to late blight of potato (Phukan, 1993), *Septoria* epidemics in winter wheat (Leitch and Jenkins, 1995). High nitrogen levels in rice plants is usually related to decreased disease resistance, especially when potassium is deficient (Sridhar, 1969; Prasad and Regunathan, 1972; Kothandaraman, 1984). The susceptibility of plants to various diseases due to excessive nitrogenous fertilizer application is attributed to the changes in biochemical constituents coupled with composition of leaf leachates and phyllosphere microbial population.

Integrated disease management aims at reduced input of synthetic fertilizers, pesticides and fungicides. Identification of adverse effect of

excessive fertilizer application based biochemical changes as well as the reaction of plants to the invasion of pathogens are essential to develop effective disease management practices.

2.1 Biochemical changes

2.1.1 Changes in phenolic compounds

Phenolic compounds are widely distributed in plant kingdom. These includes simple phenols, coumarins, flavonoids and complex phenols such as tannins, lignins, lignans, phenyl glycosides, anthocyanins leucoanthocyanins, anthoxanthins etc. Presence of high concentration of phenolic compounds is considered to be one of the major factor for an incompatible host pathogen interaction (Farkas and Kiraly, 1962; Vidhyasekaran, 1988, 1997). Infection of plants by pathogens induces marked changes in plants (Mahadevan, 1991; Anwar *et al.*, 1995; Vidhyasekaran, 1997). The phenolics and their oxidation products such as quinones which accumulate near the wounded and infected tissues are highly fungitoxic and their possible role in disease resistance has been extensively studied by many investigators (Sequeira, 1983; Vidhyasekaran, 1988). Phenols accumulation is usually higher in resistant genotype than in susceptible ones (Arora and Wagle, 1985; Luthra *et al.*, 1988, Saini *et al.*, 1988; Velazhahan and Vidhyasekaran, 1994).

Considerable quantitative and qualitative changes in phenolics are found to take place upon infection by plant pathogens (Kuc, 1963; Arora and Wagle, 1985; Luthra *et al.*, 1988). Anwar *et al.* (1995) observed no qualitative changes in phenolic compounds, however their concentration increased due to infection more particularly cinnamic and chlorogenic acids in sorghum and maize leaves infected by *Perenosclerospora sorghi*. Sindhan and Parashar (1996)

reported an increase in total phenols due to infection by *Puccinia* in ground nut leaves. Tan and Low (1975) observed an active defence reaction for the first time in *Hevea* leaves. They found a fluorescent blue phytoalexin in the resistance reaction to *Colletotrichum gloeosporioides*. The phytoalexin was later identified as scopoletin (Giesemann *et al.*, 1986) and it accumulated in leaves with total resistance and with high level of partial resistance to *Microcyclus ulei* (Garcia *et al.*, 1995a, b). Hashim *et al.* (1980) found quiercetin to be higher in leaves from resistant clones to *M. ulei*.

One of the functions of the phenolics in plants is the detoxification of toxins elicited by pathogens (Vidhyasekaran, 1997). Individual phenolics differ widely in their capacity to inhibit the pathogens and detoxify the toxins produced by them. Vidhyasekaran (1973) had compared the toxicity of different phenols to the growth of *Gloeosporium ampelophagum* the grape wine anthracnose fungus and found that O.D phenols are highly fungitoxic.

Reduction of phenols in plants due to enhanced application of nitrogenous fertilizers is an established fact in physiological plant pathology. Wakimoto and Yoshii (1958), Prasad and Regunathan (1972) and Muralidhar (1982) reported that higher dose of nitrogen application decreased the polyphenol content of rice plants. Kiraly (1964) observed application of nitrogen fertilizers in large amounts tends to increase susceptibility of wheat to rust fungus and at the same time a decrease in the total phenol level in tissues. Phenol content and the activity of phenol oxidising enzymes decreased in tomato plants after high nitrogen fertilization (Sarhan *et al.*, 1982).

2.1.2 Changes in carbohydrates

Sugars form the major source of energy for the development of plant pathogen both inside and on the surface of the host plants. It is one of the indices of the type of metabolism inherent to the plant and determining both its capacity for active resistance to their parasite and the degree of predisposition to those diseases caused by facultative parasites (Rubin and Artsikhovskaya, 1963).

Many workers investigated the carbohydrate metabolism in response to pathogenic invasion and the results are contradictory. Otani (1955) and Tokunaga (1959) in their studies in rice blast disease, found direct correlation between sugar content and susceptibility of plants to diseases. Appa Rao (1956) investigated the total carbohydrate level in blast resistant CO·4 and susceptible ADT·10 rice varieties and found that leaves contained almost the same amount of carbohydrate throughout the growth period except at the 'ear head' stage where the resistant CO·4 registered a higher carbohydrate content. Sindhan and Parashar (1996) observed low sugar content in resistant groundnut leaves in comparison to susceptible leaves. However, the amount of total sugars and reducing sugars was found to be higher in smut resistant wheat plants than susceptible varieties.

Application of nitrogenous fertilizers tends to decrease total sugars in plants. In a study with rice, Ramakrishnan (1966) noted that healthy leaves of blast susceptible CO·13 rice cultivar contained large quantities of sugars than the blast resistant CO·29 variety. Prasad and Regunathan (1972) reported that the resistant CO·4 contained more sugars than a susceptible ADT·10. They also recorded augmented levels of soluble sugars and starch in both the

varieties upon the addition of nitrogenous fertilizers. Though enormous literatures are available on the effect of nitrogen on disease incidence as well as changes in metabolism of plants, no such report is available with regard to rubber plants.

Invasion of plant pathogens in plants also altered the carbohydrate level. Ramakrishnan (1966) observed that blast diseased tissues of both susceptible CO·13 and resistant CO·29 rice varieties in general showed a reduction in reducing sugar content as compared to that of healthy ones. The decrease was particularly evident in first and second day after inoculation. A progressive decrease in the amount of reducing, non-reducing and total sugars was recorded in brinjal following inoculation with *Phomopsis vexans* (Sharma *et al.*, 1993). Irrespective of susceptibility/resistance, a decrease in total sugars and reducing sugars was observed by Sindhan and Parashar (1996) in wheat infected with *Urocystis agropyri.* But Gupta *et al.* (1992) reported an increase in the total sugar in groundnut leaf due to infection by leaf spot pathogens.

Starch, one of the insoluble carbohydrate distributed in different parts of plants is also reported to be altered by application of nitrogenous fertilizers as well as invasion by plant pathogens. In groundnut, the carbohydrate content decreased after infection in all the cultivars (Sindhan and Parashar, 1996). Blast susceptible ADT·10 rice variety contained more starch than the resistant CO·4 and its level reduced due to nitrogen application and increased due to *P. oryzae* inoculation in both the varieties (Prasad and Regunathan, 1972). Kothandaraman (1984) reported a reduction in starch content due to

increased level of nitrogen application in rice plants while *P. oryzae* inoculation increased the level of starch in tissues.

2.1.3 Changes in nitrogenous compounds

2.1.3.1 Changes in amino nitrogen

The plant pathogens metabolize various nitrogenous compounds in addition to carbohydrates and such compounds are considered to be important in disease resistance. Changes in carbohydrate metabolism occurring as a result of the disease also have a marked effect on nitrogen metabolism of diseased plants. Nitrogen metabolism of host plants in response to pathogenic invasion has been investigated by several workers. In general, accumulation of soluble nitrogenous compounds in diseased tissues has been reported (Subrahmanyan *et al.*, 1976; Reddy *et al.*, 1977; Ahmad *et al.*, 1982). However, decrease in nitrogenous compounds following infection has been reported by Patel and Walker (1963 a, b), Mishra and Siradhana (1980) and Waltare and Ayers (1980). Bhargava and Khare (1988) reported higher amount of total amino acids in resistant cultivars of chick pea as compared to susceptible ones. On the other hand, Alam *et al.* (1993) reported a high amino nitrogen content in susceptible variety of banana. Singh and Chohan (1977) reported higher amount of amino acids in the chlorotic region of cucurbit fruits. A progressive decrease in the amount of amino acids was recorded in brinjal leaves and fruits after inoculation with *Diaporthe vexans* (Sharma *et al.*, 1993). Sindhan and Parashar (1996) also reported a decrease in the content of amino acids due to infection in tolerant and susceptible groundnut varieties.

Nitrogenous fertilizer application tends to increase amino nitrogen content in plant tissues. Robinson and Hodges (1981) reported increased

amino acid content in presence of higher levels of nitrogen in *Poa pratensis* and pathogenesis by *Drechslera sorokiniana.*

The importance of individual amino acids for the growth of pathogen has been proved by several workers. Leach (1919) proposed that certain parasites and physiological races require specific nutrients furnished only under the susceptible condition of the host. Tinline (1963) found that lysineless methionineless strains of *Cochliobolus sativus* could not cause severe infection on wheat but lysine and methionine was supplied, severe disease incidence occurred. Some of the amino acids like glutamic acid and aspartic acid are found more in susceptible plants (Shishiyama *et al.*, 1969) and in plants under higher nitrogenous fertilizer level resulting in susceptibility to disease (Tanaka, 1963). While there are many reports to show that some amino acids induce resistance. Kuc *et al.* (1959) and Kannaiyan *et al.* (1973) observed phenylalanine, tryptophan, methionine, cysteine and lysine are the amino acids responsible for disease resistance.

2.1.3.2 Changes in total nitrogen

Plant pathogens alter the content of total nitrogen of plants upon infection and it has been well studied (Shaw, 1963; Anthoni Raj, 1977). Vidhyasekaran (1988) reported enhanced total nitrogen upon increased nitrogenous fertilizer application and suggested that the total nitrogen in tissues are responsible for growth and production of toxin and enzyme by the pathogen. He also found that some proteins in the host cell wall may inhibit the enzymes and induce resistance.

Tolerant varieties of mustard and groundnut, were reported to contain less amounts of total nitrogen than the susceptible plants (Gupta *et al.*, 1995). Following infection nitrogen concentration decreased in all the groundnut cultivars but the decrease was quite substantial in susceptible variety. Luthra *et al.* (1988) reported higher level of nitrogen in varieties resistant to downy mildew in comparison to susceptible ones. Nema (1991) observed no appreciable changes in nitrogen content between tolerant and susceptible varieties of betel wine. But infection led to a small depletion but not appreciable is observed among different varieties. Blast susceptible rice plants are reported to contain greater amounts of total nitrogen than the resistant plants (Sridhar, 1969; Prasad and Regunathan, 1972). Application of nitrogenous fertilizer enhanced the total nitrogen content of the rice plants (Prasad and Regunathan, 1972; Kothandaraman, 1984). Rajalakshmy *et al.* (1979) reported an increase in nitrogen content in rubber seedlings applied with higher dose of nitrogen. Rice plants infected by *P. oryzae* registered a higher level of total nitrogen (Tokunaga *et al.*, 1959; Zeoldos, 1962). Similar results were recorded in the blast CO·13 rice plants in response to *P. oryzae* infection (Ramakrishnan, 1966; Sridhar, 1969; Prasad and Regunathan, 1972). On the other hand, in CO·29 tissues the total nitrogen decreased post-infectionally (Ramakrishnan, 1996). However, Sridhar (1969), Prasad and Regunathan (1972) observed an enhanced level of total nitrogen in the infected CO·29 rice plants.

2.1.3.3 Changes in protein

Host parasite interaction exert a pronounced effect on protein synthesis and enzyme activity of host plants and the speed or extent of such

reaction may be important among the factors which determine the resistance or susceptibility of the host (Stahmann, 1967). Changes in the protein content of tissues have been reported by several workers (Shaw, 1963; Andebrhan *et al.*, 1980). The crude protein was considerably high in healthy rice plants of susceptible varieties, as compared to resistant varieties. But after infection there was a significant increase of protein in resistant plants but decreased significantly in susceptible plants. This decrease was attributed to the pathogen rather than the host (Goodman *et al.*, 1967). Enhanced protein synthesis appears to be a universal phenomenon in incompatible host pathogen interaction. *De novo* synthesis of new proteins has also been reported. Enhanced protein synthesis in the primary leaves of oats in relation to resistance expression at the early stages of infection with incompatible race was reported by Yamamoto and Tani (1982). When incompatible bacteria, *Pseudomonas syringae* were injected into the leaves of tobacco, new host proteins were found to be synthesized and this induced resistance to the secondary infection (Ahl *et al.*, 1981).

Nitrogenous fertilizer application is found to enhance the protein nitrogen level of resistant and susceptible cultivars to blast disease (Prasad and Regunathan, 1972). Kundu and Sircar (1969) also reported the increased protein level in rice plants fertilized with nitrogen than in unfertilized control plants. Infection led to increase in levels of protein in rice plants than healthy ones (Zeoldos, 1962). Ramakrishnan (1966) observed an initial decrease of protein in susceptible CO·13 plants. But it registered a higher level at later stages. In the infected CO·29 rice tissues, general decrease in protein nitrogen was observed (Ramakrishnan, 1966). Sridhar and Mahadevan (1968) observed that CO·13 leaves synthesised protein within 15 minutes of

inoculation with *P. oryzae* and suggested that the host cell reacted readily to the trigger given by the pathogen. Prasad and Regunathan (1972) observed a general increase of crude protein in resistant CO·4 and susceptible CO·13 plants. However, the susceptible plants registered a lower level of protein after 120 h at higher levels of nitrogen application.

2.1.4 Changes in ascorbic acid

Ascorbic acid plays an important role in the metabolism of plants. It may occur as such or in its oxidised form, the dehydro ascorbic acid, nearly in all plant tissues. Ascorbic acid accumulates in resistant plants and suppresses symptom development in many host pathogen interactions. Ascorbic acid acts as a respiratory carrier (Alberg, 1961) and it is a good reducing agent reducing toxic quinones to less toxic phenols (Uritani and Lechika, 1953) and this reduction has a significant role in altering the defence mechanisms (Rubin *et al.*, 1939; Sridhar and Mahadevan, 1979).

Prasad and Regunathan (1972) reported a higher level of ascorbic acid in resistant CO·4 rice cultivar than in the susceptible ADT·10. An increase in total sugar content in rotting tomato fruits infected by *Scleroticum rolfsii* was observed by Prasad *et al.* (1989). Decrease in ascorbic acid content was also reported in lemon fruits (Babu and Reddy, 1990) and in banana (Singh, 1993) following infection by pathogens. Malhotra (1993) reported a decrease in ascorbic acid content to a greater extent in susceptible varieties of tomato seedlings than resistant varieties.

Nitrogen fertilization increased the ascorbic acid content of both resistant and susceptible rice varieties (Sridhar, 1969; Prasad and Regunathan,

1972). Sridhar (1969) reported a reduction in the ascorbic acid level upon *P. oryzae* inoculation in both resistant and susceptible rice varieties. However Prasad and Regunathan (1972) observed a reduction in susceptible cultivar upon infection with *P. oryzae* and the same was observed only in initial stages in the case of resistant plants. Farkas and Kiraly (1958) showed that ascorbic acid may induce susceptibility in a few sustainer and may inhibit polyphenol oxidase, which is involved directly or indirectly in resistance of host plants to pathogenic microorganisms. Kiraly and Farkas (1962) showed that ascorbic acid accumulated in rust infected susceptible wheat varieties while it declined in infected resistant tissues.

2.1.5 Changes in oxidative enzymes

2.1.5.1 Ascorbic acid oxidase

This enzyme, one of the terminal oxidases, is widely distributed in plants. Szent-Gyorgyi (1931) first demonstrated that this plant enzyme could easily oxidise ascorbic acid. Toyoda and Suzuki (1960) reported increased ascorbic acid oxidase activity in *P. oryzae* inoculated rice plants. They stated that the major part of ascorbic acid might be oxidised to dehydro ascorbic acid which failed to reduce toxic quinones thus protecting the tissues against *P. oryzae* infection. Kiraly and Farkas (1957) observed an increase in ascorbic acid oxidase activity which paralleled with the increase in respiration in resistant cultivars of wheat following infection. However Vidhyasekaran *et al.* (1972) observed that both ascorbic acid content and ascorbic acid oxidase were higher in susceptible young leaves of grape wine. Tamari *et al.* (1963 and 1967) observed that piricularin-binding protein, a copper containing enzyme secreted by *P. oryzae* oxidised the ascorbic acid.

Nitrogenous fertilizer application reduces this enzyme level in both blast-resistant and blast-susceptible rice varieties (Sridhar, 1969). Prasad and Regunathan (1972) recorded an increased level of ascorbic acid oxidase with the increase in nitrogen application in blast susceptible ADT·10 rice variety. However, the activity of ascorbic acid oxidase fluctuated with different levels of nitrogenous fertilizer in the blast resistant CO·4 rice variety. In general, a reduction in ascorbic acid oxidase in susceptible and resistant plants due to *P. oryzae* inoculation was reported by them. Sridhar and Ou (1974) noted an enhanced ascorbic acid activity in rice plants grown under high nitrogen level upon infection by *P. oryzae*.

2.1.5.2 Peroxidase

Peroxidase, an iron containing enzyme found in plants mainly catalyse the oxidation of phenolic compounds through PO-H_2O_2 system (Bonner, 1950; Srivastava, 1987). Alteration in this oxidative enzyme in plants in response to pathogenic infection have been well documented (Hammerschmidt *et al.*, 1982; Shimoni *et al.*, 1991). Many workers (Uritani, 1976; Arora and Wagle, 1985) have concluded that the peroxidase and polyphenol oxidase activities are related to resistance while some others (Vidhyasekaran *et al.*, 1973; Nadolny and Sequeira, 1980) have reached opposite conclusion. Interestingly peroxidase itself was found to inhibit the development of mycelium of wheat rust pathogen (*Puccinia graminis* f. sp. *triciti*) in *in vitro* culture (Macko *et al.*, 1968). Fehrmann and Dimond (1967) found a strict positive correlation between peroxidase activity in different organs of potato plant and resistance to *Phytophthora infestanes*. High peroxidase activity was reported in non-infected groundnut variety resistant to *Puccinia arachidi* sp. (Velazhahan

and Vidhyasekaran, 1994). Nema (1991) observed more peroxidase and polyphenol oxidase in resistant and moderately susceptible varieties of betelvine. However Gupta *et al.* (1990, 1992) observed a lower specific activity of peroxidase in tolerant varieties of mustard and groundnut varieties. Increase in peroxidase have been correlated with resistance in many species like cucurbits cotton, tobacco, wheat and rice (Young *et al.*, 1995; Dalisay and Kuc, 1995). Significant peroxidase activity was observed in *Hevea* resistant to *Corynespora cassiicola* compared to susceptible clones (Breton *et al.*, 1996). *Hevea* clones resistant to SALB possessed significantly lower peroxidase and susceptible varieties had higher activity and this enzyme increased with infection, leading to earlier detection in resistant leaves (Hashim *et al.*, 1980). On the other hand, Gupta *et al.* (1990 and 1995) reported a sharp increase in the activity of this enzyme in *Brassica* species resistant and susceptible to *Alternaria* sp. after infection and the increase was considerably higher in susceptible cultivars.

Matsuyama and Kazaka (1981) reported that nitrogen application enhances the peroxidase activity of healthy rice leaves and it increased upon infection within 24 hours in resistant variety while in susceptible variety there was a slight increase in 3-5 days after infection.

2.1.5.3 Polyphenol oxidase

Phenolics are oxidised to quinones by polyphenol oxidase (PPO) and peroxidase (PO). PPO, one of the copper containing enzymes, may be involved in the terminal oxidase in the diseased tissues of some plants (Kosuge, 1969). Like PO, PPO activities are related to resistance (Fehrmann and Dimond, 1967; Uritani, 1976; Nema, 1991). No difference in the

PPO activity in the unaffected leaves of rubber and in apple with varying intensities of resistance was observed in South American leaf blight of *Hevea* (Hashim *et al.*, 1980) and apple scab (Hanusova, 1969). On the other hand, a significant difference in the activity of this enzyme was observed in the infected plants of apple and it seemed to be suppressed after inoculation in the resistant *Hevea* leaves. Melouk and Horner (1972) also detected lower PPO activities in diseased peppermint infected with *Phoma strasseri* Moesz. They attributed the lowering PPO activities to continuous contact between the enzyme and the oxidised substrate which inhibited enzyme activity. Gupta *et al.* (1990, 1992) reported a high activity of PPO in resistant varieties of mustard and groundnut. In response to infection, the activity of the enzyme increased sharply in both the varieties. However, the increase was more associated with susceptibility. On the other hand, an increase in PPO activity was reported in resistant varieties of groundnut leaves and decrease in susceptible variety due to infection by *Puccinia arachidis* sps. (Velazhahan and Vidhyasekaran, 1994). A rapid initial increase in PPO activity in the resistant tomato plants, followed by a very high activity during 12–24 h after inoculation with *Fusarium oxysporum* f. sp. *lycopersici*. On the other hand, no increase in PPO activity after infection was found in susceptible tomato plants (Vidhyasekaran, 1997).

2.1.6 Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL)

Phenylalanine ammonia-lyase and tyrosine ammonia-lyase are the key enzymes in the phenyl propanoid pathway and may be involved in phenolic synthesis (Burrell and Rees, 1974). Aromatic compounds arise from phenylalanine and tyrosine by the action of PAL and TAL (Neish, 1961).

Koukol and Conn (1961) and Young *et al.* (1966) reported that these enzymes are widely distributed in plant kingdom. In disease resistance, these enzymes are increasingly recognised to play an important role in the conversion of phenylalanine and tyrosine to coumaric acid.

Sadasivan (1968) suggested the role of phenylalanine and tyrosine in the disease resistance mechanism of rice varieties against *Drechslera oryzae* and *P. oryzae*. Koti Reddy (1972) reported that *P. oryzae* inoculation increased the activity of PAL and TAL and the activity of the former is more. Increased activity of these two enzymes in bacterial blight of rice, especially in resistant reaction was reported by Purushothaman (1974), Mohanty *et al.* (1982). Lamb *et al.* (1987) reported that the induction of PAL in incompatible interaction was much rapid than that of compatible reaction. PAL activity was found to be activated approximately two fold in the incompatible interaction when potato leaves were infected with *Phytophthora infestans* (Fritzemeier *et al.*, 1987). PAL is the key enzyme for the synthesis of phenolics, phytoalexins and lignins, the three factors responsible for disease resistance (Vidhyasekaran, 1988b). Induction of PAL activity has been reported in cow pea inoculated with *Phytophthora vignora* (Ralton *et al.*, 1988) in Eucalyptus inoculated with *Verticillium albo-atrum* (Lee *et al.*, 1992), in beans infected with *Colletotrichum lindemuthianum* (Lamb *et al.*, 1992) in rice infected with *Helminthosporium oryzae* (Vidhyasekaran *et al.*, 1992) and in barley leaves infected with *Erysiphe graminis* f. sp. *hordei* (Shiraishi *et al.*, 1995) as resistant reaction.

Nitrogen fertilization reduced both PAL and TAL in rice plants (Matsuyama and Dimond, 1973; Kothandaraman, 1984). *P. oryzae* inoculation also reduced the activity of lyase enzymes (Kothandaraman, 1984).

2.2 *In vitro* studies on the production of hydrolytic enzymes

Hydrolytic enzymes are extremely important in pathogenesis because they provide the pathogens chemical means of entrance into the host and a process where by nutrients can be digested. These enzymes are secreted by the infecting pathogens and/or activated in the host tissues during infection and this determine the ability of pathogen to cause disease (Albersheim *et al.*, 1969). Pectolylic, cellulolytic and proteolytic enzymes secreted by the pathogen have been reported to be involved in pathogenesis (Sathiayanathan and Vidhyasekaran, 1978; Ramaraj and Vidhyasekaran, 1986; Kolattukudy, 1985; Dori *et al.*, 1995).

2.2.1 Cellulolytic enzymes

Cellulose is the major cell wall polysaccharide and is composed of glucose units in the chain configuration connected by β-1,4 glycoside bonds. Cellulolytic enzyme degrade cellulose and its derivatives. Tanaka (1963) reported that *P. oryzae* produces cellulases in culture. Isolates of *Colletotrichum gloeosporioides* produced C_1 and C_x enzymes in culture (Senaratna *et al.*, 1991). Several other pathogens are also known to produce different cellulases *in vitro* and they have been detected in infected tissues also (Vidhyasekaran, 1974; Suzuki *et al.*, 1983; Dori *et al.*, 1995). Komarajah and Reddy (1988) reported the production of cellulases by *C. cassiicola*, the seed borne fungus of methi. Singh and Kunene (1980) observed an enhanced

activity of cellulase in *P. oryzae* when nitrogen level in the medium was increased. β-glucosidase releases reducing sugars from glycosides or oligosaccharides. This enzyme is particularly important in disease resistance mechanism where phenols are released from phenolic glycosides. In the infected tissues break-down of glycosides may take place as a result of the action of glycosidases secreted by the pathogen (Dimond, 1955). Several investigators reported the presence of β glucosidase system in plant pathogens (Senaratna *et al.*, 1991).

2.2.2 Pectinolytic enzymes

The capacity to produce pectic enzymes is widespread in pathogenesis. Plant pathogens produce multiple forms of different types of pectic enzymes (Collmer and Keen, 1986). The pectic substances are classified into two major group, the pectin (pectinic acid) and pectic acid (polygalacturonic acid) (Vidhyasekaran, 1993) and the enzymes are pectin esterases and polygalacturonases. Important reviews on the significance of pectic enzyme in plant disease are Bateman (1966), Wood (1960), Bateman and Basham (1976). *Fusarium solani* f. sp. *pisi*, the pea pathogen produces all the pectin degrading enzymes (Crawford and Kolattukudy, 1987). *Magnaporthe grisea* (*P. oryzae*), the rice blast pathogen produced pectin methyl esterase (PME) pectinlyase and polygalacturonase (PG) (Bucheli *et al.*, 1990). Two polygalacturonases were detected in the culture filtrate of *Aschochyta pisi* the leaf and pod rot pathogen of pea (Hoffman and Turner, 1984). *Helminthosporium nodulosum*, the finger millet pathogen and *H. oryzae*, the rice pathogen produce PME, endo-PG, exo-PG, polygalact *trans*-eliminase (PGTE) and pectin lyase (Vidhyasekaran, 1972, 1974 a, b, c, 1978). Multiple pectic

enzymes production by several other pathogens have been reported (Ramaraj and Vidhyasekaran, 1986; Valsangiacomo *et al.*, 1992; Khare *et al.*, 1994).

2.2.3 Protease

Cell walls contain a protein rich in hydroxyproline, in addition to the polymeric carbohydrates (Ginzburg, 1961; Lamport, 1973). Proteases are involved in degradation of plant cell wall proteins (Hislop *et al.*, 1982; Movahedi and Heale, 1990 a, b). One of the well documented studies on the secretion of proteolytic enzymes was on *P. oryzae* by Otani (1959). Mahadevan (1970) reported the production of proteolytic enzymes and the existence of m-RNA in *P. oryzae*. He also reported the production of proteolytic enzymes by the cotton wilt pathogen (*F. oxysporum f. vasinfectum*) and *H. oryzae*, responsible for the brown leaf spot of paddy. Degradation of RNA by *Phytophthora infestans* the late blight pathogen, was demonstrated by Page (1959). Hancock and Miller (1965) detected protease activity in alfalfa plant tissues infected with *Stremphylium botryosum*. Saxena *et al.* (1995) reported that metabolism of tomato cultivars is greatly affected by *in vivo* activity of proteolytic enzyme of *Fusarium solani*.

2.3 Toxins

The success of a pathogen in becoming established on a host may depend largely on the action of antimetabolites or toxins secreted by the pathogen (Goodman *et al.*, 1967). There is an enormous amount of literature on toxic metabolites of pathogens in culture fluids and the host tissues and their role in plant disease (Tamari, 1968; Sato, 1970). Onesirosan *et al.* (1975) reported that isolates of *Corynespora cassiicola* highly pathogenic to tomato

produce toxin in synthetic medium which induced symptom in susceptible but not in resistant plants. Toxin production in synthetic medium by *C. cassiicola* causing severe leaf spot and leaf fall in *Hevea* was reported by Liyanagae *et al.* (1986). They observed that the crude toxin could cause characteristic spots in rubber leaves similar to that incited by the pathogen. Breton *et al.* (1996) in their preliminary study showed that the isolated toxin is probably a low molecular weight glycoprotein and susceptibility of *Hevea* clones is highly linked to the efficiency of their defence mechanism against the *Corynespora* toxin. Breton (1997) purified the toxin, *Cassiicoline* from the extract and confirmed the main role of *Cassiicoline* in the aggressiveness of *C. cassiicola.*

Tamari *et al.* (1963) observed that piricularin produced by *P. oryzae* inhibited the activities of peroxidases, catalases and cytochrome oxidase. Although at higher concentrations, piricularin inhibited the respiratory rate of rice seedlings at an extremely low concentration of 1/1 ^{xxxx} it stimulated respiration (Tamari *et al.*, 1963 and 1967). Vidhyasekaran *et al.* (1992) reported a decrease in phenolic content and peroxidase and phenylalanine ammonia-lyase (PAL) activities in rice leaves at advanced stage of infection and they attributed it to the action of toxin produced by the fungus *H. oryzae.* They suggested that the major role of toxin in pathogenesis is the suppression of defence mechanism of plants.

2.4 Changes in the composition of leaf leachates

Aerial parts of plants as well as roots leak and absorb materials (Baker and Cook, 1974) and the exudation from leaves may take place through ectodermata, stomata and hydathodes (Preece and Dickinson, 1971; Baker

and Cook, 1974). Substances leached from plants include a great diversity of materials such as inorganic nutrients alkaloids, phenols, vitamins and other organic substances (Brillova, 1971; Blakeman, 1972; Chet *et al.*, 1975; Good, 1974). The qualitative and quantitative nature of the leachates is influenced by type and age of the plants, soil fertility, climate and health of plants (Lepp and Fairfax, 1976). Since leachates contain compounds which can be utilised as nutrients by microorganisms and may also contain substances which inhibit their germination and growth, they are of considerable importance to the microbial population of plant surfaces. All these aspects of leaf leachates and their influence on phyllosphere microorganisms were reviewed by Tukey (1971), Godfrey (1976) and Battacharyya and Purkayastha (1983). Hence the present review is confined to the effect of plant nutrition and disease on leachate composition. Last and Deighton (1965) reported that nutrient leaching is often greater from plants deficient in nutrients. However Purushothaman *et al.* (1976) observed an enhanced level of sugars and amino acids in the leaf washings of rice plants, applied with higher doses of nitrogen in combination with phosphate and potassium. But the leaf NPK levels did not influence the presence of nutrients on the leaf surface. Huber and Watson (1974) reported that nitrogen forms affect the composition of leaf and root exudates and bring out an effect similar to that of host constituents.

It is well known that infection of plants by any pathogen causes an imbalance in the metabolism of plants. The stress caused by the pathogen reflects in all the metabolic activities of the host plant including the leaf exudation pattern. Leaves from healthy and vigorous plants are much less susceptible to leaching than leaves which are injured; whether injury be induced by pests or nutritional and physiological disorders (Tukey, 1971).

Oblisami *et al.* (1973) observed higher levels of reducing sugars and amino acids in virus infected leaves of green gram. They also reported the presence of more number of amino acids in diseased plants than in the healthy plants. However, Beute (1973) reported lesser carbohydrate level in virus infected *Gladiolus* plants. Blast disease development in rice is reported to be closely related to the leaf exudate and its composition (Subha Rao and Suryanarayana, 1957). Suryanarayana (1958) reported that the exudate of blast susceptible CO·13 rice plant contain large amounts of glutamine which stimulate the germination of *P. oryzae*. On the other hand, blast resistant CO·29 exuded little glutamine and the same increased in both the varieties when heavy nitrogenous fertilizer was applied. Analysing the leaf exudates of blast resistant and blast susceptible rice varieties in controlled environmental conditions, Saxena (1978) concluded that the presence of alanine, serine, threonine, aspartic and glutamic acid was common for both the varieties and the susceptible varieties in addition, have asparagine, tyrosine and proline. Mohanty and Gangopadhyay (1981) reported the presence of increased amounts of total sugars along with amino acids especially DL glutamine and proline which markedly influenced spore germination of *P. oryzae* in susceptible plants, while the presence of L-histidine was found to be non-conducive to spore germination in leaf exudates of resistant variety.

2.5 Changes in phyllosphere microorganisms

Leaves of higher plants harbour a number of microorganisms which includes casuals and residents. Ruinen (1956) coined the term phyllosphere for the presence of microorganisms on the surface of leaves. Phyllosphere

microorganisms include a variety of bacteria, filamentous fungi, yeasts and actinomycetes (Ruinen, 1956; Leben, 1961). A number of reviews are available in this subject (Ruinen, 1961; Preece and Dickinson 1971; Battacharyya and Purkayastha, 1983).

Type of plant species, age and health of the plant, season, climatic factors, location, agricultural operations like fertilizer applications, pest and disease control, all these factors cause a wide variation in quantitative and qualitative nature of phyllosphere microflora (Karling, 1965; Dickinson *et al.*, 1975; Khara and Singh, 1981; Kothandaraman, 1984).

Saprophytes and plant pathogens on the leaf surface have both associative and antagonistic activities are wisely utilized for the control of plant diseases (Cruickshank and Perrin, 1963; Baily, 1971). Some of the phyllosphere microorganisms are also found to favour the development of the plant diseases (Clark and Lorbeer, 1977) while plant pathogens favour the survival of saprophytes (Choudhary and Verma, 1980).

Last (1955) isolated more sporobolomyces from leaves of wheat grown in plots manured for many years by nitrogen phosphorous and potassium than from plants grown without manuring. Purushothaman *et al.* (1976) observed more number of bacteria, fungi and actinomycets at tillering stage of rice. Nitrogen at 100 and 150 kg/ha along with phosphorous and potassium increased the number of microorganisms on the leaf surface of rice.

Changes in the metabolites take place in diseased plants which may be reflected in the composition of nutrients in leaf exudates with consequent

changes in microbial population in the phyllosphere region. Sadasivan and Prasad (1973) reported more number of bacteria and fungi and less actinomycetes in tapioca plants infected by *Cercospora henningaii*. Oblisami *et al.* (1973) reported the increased population of fungi and actinomycetes in healthy and increased population of bacteria including *Azotobacter* in virus diseased green gram plants. Apple leaves infected by *Venturia inaequalis* and *V. pirine* harboured more bacteria and fungi. The actinomycete population was not affected by the disease (Stadelmann and Schwinn, 1979). Disease resistant pearl millet plants have higher populations of fungi and bacteria compared to susceptible cultivar under healthy and diseased condition (Chandrakumar and Balasubramanian, 1981). Wadje and Deshpande (1979) observed more fungal population in cotton leaves infected by *Xanthomonas malvacearum*.

2.6 Appressoria formation in the presence of phyllosphere microorganisms

Most of the fungi penetrate through the host cuticle or stomata by forming a cushion like adhesive structure, the appressorium on the host surface. This develops rapidly in many fungi when spores are grown in plant or non-plant surfaces, while in other cases the development is in response to specific host surface. Physical and chemical structures of the host surface and environmental condition are reported to stimulate or suppress the formation of appressoria (Grover, 1971; Emmet and Parbery, 1975).

Brodie (1975) observed the development of appressoria in *Colletotrichum dematium* f. sp. *spinacea* immediately after the germination of spores when *Pseudomonas* sp. was added in large numbers with the inoculum on beet leaves. In the absence of bacteria also, the spores germinated with

large germ tube. Lenne and Parbery (1976) have observed clusters of bacteria surrounding lysed conidia and germ tube of C. *gloeosporioides* on leaves. Because bacteria normally failed to lyse appressoria it was suggested that these might survive under conditions where germtubes and conidia were destroyed. It was shown *in vitro* that a higher proportion of germinated conidia developed appressoria in the presence of bacteria than when these were absent.

Stimulation of appressoria formation by bacteria occurred both in the presence and absence of added nutrients. Blakeman and Parberg (1977) reported that two isolates of bacteria, *Pseudomonas* sp. isolate 14 and UV·3 known to compete strongly for amino acids on leaf surface stimulated formation of appressoria by C. *acutatum* on both glass and sugar beet leaves. Appressoria reached maturity more frequently on leaves than on glass. However, in the presence of added nutrients *Pseudomonas* isolate 14 failed to increase the number of appressoria. It was suggested that deprivation of either of the two major nutrients sources on plant leaves, namely simple sugars or aminoacid might stimulate formation of appressoria of C. *acutatum*. Blakeman and Brodie (1977) observed the inhibition of *Phoma betae* and *Cladosporium herbarum* conidia germination and reduced germ tube length with increased number of appressoria formation by *Pseudomonas* sp. They concluded that C. *lindemuthianum* and probably C. *dematium* as well respond to low nutrient condition.

Chapter 3

MATERIALS AND METHODS

3.1 Establishment of plants and inoculation with *C. cassiicola*

3.1.1 Raising of rubber seedlings

Germinated *Hevea* seeds were planted in porcelain pots filled with top soil collected from the field. Four seedlings were planted in each pots and watering was carried out regularly. After 40 days of planting, first dose of fertilizer was applied to the plants. Fertilizer dosage was calculated in such a way that each pot gets the treatments comparable to that recommended for nursery plants.

N - 500 kg ha^{-1}

P - 250 kg ha^{-1}

K - 100 kg ha^{-1}

Half dose of the nitrogen and full dose of the phosphorous and potassium in the form of ammonium sulphate, rock phosphate and muriate of potash respectively were applied to the pots. Nitrogen was applied in four different concentrations i.e., 25, 50, 100 and 200 per cent of the recommended dose, and phosphorous and potash in the recommended dosage as below.

	N	P	K	
1.	125	250	100	kg ha^{-1}
2.	250	250	100	kg ha^{-1}
3.	500	250	100	kg ha^{-1}
4.	1000	250	100	kg ha^{-1}

After the fertilizer application, the top soil was gently forked for uniform distribution of fertilizers. Fifteen days after first application the plants were applied with remaining half dose of nitrogenous fertilizer.

3.1.2 Inoculation

The pathogen, *C. cassiicola* was grown in Potato Dextrose Agar (PDA) medium for abundant sporulation, and spore suspension was prepared with sterile distilled water so that each ml. contains 7×10^4 spores and sprayed on plants uniformly. Inoculated plants were covered with transparent polythene bags for 24 h to maintain high humidity. Equal number of plants were kept as control and sprayed with the same volume of distilled water and covered for 24 h.

3.1.3 Disease assessment

Leaves in the topmost whorl were assessed visually for the severity of the disease on the eighth day of inoculation based on the intensity of spots developed and deformation of the leaves using a 0–5 scale as given below.

	No. of spots	Grade	Disease intensity
1.	No spots	0	Not diseased
2.	1–10	1	Very mild disease
3.	11–25	2	Mild disease
4.	26–50	3	Severe disease
5.	51–75	4	Very severe disease with deformation of leaves
6.	76–100	5	Very severe disease with deformation of leaves and leaf fall

The per cent disease index was calculated using the formula

$$\frac{\text{Sum of all the disease ratings}}{\text{Total number of ratings x maximum disease grade}} \times 100$$

3.2 Biochemical changes

3.2.1 Sampling of leaves

Leaf samples were collected for the biochemical analysis and enzyme studies at random from every treatment at 0, 24, 48, 72, 96 and 168 h of inoculation. Samples collected at 168 h of inoculation only were used for phyllosphere and leaf leachates studies.

3.2.2 Determination of dry weight

The leaf bits were gently pressed in folds of filter paper to remove surface moisture. The tissues were transferred to a previously weighed moisture bottle and the fresh weight was determined. The bottles were kept in a hot air oven at 105°C for 25 h removed, cooled in a desiccator and reweighed. The processes of oven drying, cooling and weighing were repeated until constant weight was obtained.

3.2.3 Estimation of phenols, sugars and amino nitrogen

3.2.3.1 Ethanol extraction of plant materials

Leaves were separated, chopped and used for ethanol extraction after removing excess moisture by blotting them between folds of filter paper. Exactly 1 g of the chopped material was plunged into 15 ml of boiling 80 per cent ethanol, extracted for 5 min in a hot water bath and cooled in running tap water. The material was homogenised by grinding in a porcelain mortar

and pestle and squeezed through two layers of cheese cloth. The residue was transferred back to 5 ml of boiling 80 per cent ethanol and re-extracted for 5 min, cooled and filtered through Whatman No. 41 filter paper. A jet of ethanol was used to wash the filter paper. The final volume was adjusted to 20 ml with 80 per cent ethyl alcohol. The residue was used for starch estimation.

3.2.3.2 Quantitative estimation of total phenols

Total phenols were estimated by employing Folin-Ciocalteu reagent (Bray and Thorpe, 1954).

Estimation

Folin-Ciocalteu reagent was diluted to 1 N with equal volume of distilled water and 1 ml of the same was added to 1 ml of the alcohol extract in a 25 ml marked boiling tube followed by 2 ml of 20 per cent sodium carbonate and the mixture was heated in a boiling water bath for exactly one min. The blue colour was diluted to 25 ml with glass distilled water. The percentage of light transmittance was determined in a Spectronic-20 colorimeter at 725 nm. Total phenol was calculated from a standard curve plotted from concentration of catechol.

3.2.3.3 Quantitative estimation of *ortho*-dihydroxy phenols

Ortho-dihydroxy phenols were estimated by the method described by Johnson and Sohaal (1952) employing Arnow's reagent which is specific to *ortho* groups. To one ml of the alcoholic extract in a 25 ml marked boiling tube, 1 ml of 0.5 N hydrochloric acid, 1 ml of Arnow's reagent prepared by

dissolving 10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of glass distilled water and 2 ml of 1N sodium hydroxide were added. The volume was raised to 25 ml with distilled water and the light pink colour was read in the 'Spectronic-20' colorimeter at 522 nm. Reagent blank contained 1 ml of distilled water in the place of extract. *Ortho*-dihydroxy phenols in the unknown were calculated from a standard curve plotted using catechol.

3.2.3.4 Determination of reducing sugars

Reducing sugars in the alcohol extract were determined by the Nelson's method (1944) (Composition of reagent, see Annexure 2).

Estimation

To 1 ml of alcohol extract in a 25 ml marked boiling tube, 1 ml of mixture of reagent 'A' and 'B' prepared by mixing 25 parts of reagent 'A' with 1 part of reagent 'B' was added. The mixture was heated for 20 min in a boiling water bath cooled in tap water and 1 ml of the arsenomolybdate reagent was added. The solution was thoroughly mixed and diluted to 25 ml with glass distilled water. Reagent blank contained 1 ml of distilled water in the place of extract. The resulting blue colour was read in a 'Spectronic-20' colorimeter at 497 nm. Reducing sugars were calculated from glucose standards.

3.2.3.5 Determination of non-reducing sugars

3.2.3.5.1 Hydrolysis of the extract

Non-reducing sugars present in the alcohol extract were first hydrolysed to reducing sugars (Inman, 1962) and then estimated. Exactly 1

ml of the alcohol extract was taken in a boiling tube and evaporated to dryness in a water bath. One ml of the glass distilled water and 1 ml of 1 N sulphuric acid were added to the residue. The mixture was hydrolysed by heating at 49°C for 30 min over a thermostat water bath. The solution was neutralised with 1 N sodium hydroxide using methyl red indicator.

3.2.3.5.2 Estimation

Total sugars of the hydrolysed sample were estimated by the Nelson's method as mentioned earlier. Non-reducing sugars were calculated by subtracting the reducing sugar value from that of total sugars and were expressed in glucose equivalents.

3.2.3.6 Determination of amino nitrogen

Amino nitrogen was determined by the ninhydrin method of Moore and Stein (1948). (Composition of reagent, see Annexure 2)

Estimation

To 1 ml of the alcohol extract in a boiling tube, 1 drop of methyl red indicator was added and the extract was neutralised with 0.1 N sodium hydroxide, if necessary. To this solution, 1 ml of ninhydrin reagent was added, mixed thoroughly by shaking and aluminium caps were placed on the tubes. The mixture was heated for 20 mts in a water bath. The tubes were removed, cooled in running tap water, 5 ml of diluent solution added and the contents thoroughly mixed. The purple colour of the solution was read in a 'Spectronic-20' colorimeter at 475 nm. Blanks consisted of 1 ml of distilled

water in the place of alcohol extract. Amino nitrogen was calculated from the standard graph prepared from glutamic acid.

3.2.4 Quantitative estimation of starch

Starch in the samples were estimated by the method of Sumner and Somers (1949).

Two hundred mg of finely powdered, 80 per cent alcohol insoluble residue, dried in an oven at 60°C for two consecutive days were placed in a glass stoppered 100 ml Erlenmeyer flask. Three ml of 6 N hydrochloric acid were added to the flask and steamed in an autoclave at 110°C for 1 h. The flasks were cooled and the solution was neutralised by using concentrated sodium hydroxide. The volume was raised to 25 ml with distilled water. An aliquot of 1 ml was withdrawn and glucose was estimated by Nelson's (1944) method. The amount of starch was determined by multiplying the amount of estimated glucose by the factor 0.9.

3.2.5 Determination of total nitrogen in rubber seedlings

Total nitrogen in the sample was determined by microkjeldahl method (Jackson, 1962).

The samples were dried at 70°C for 48 h and powdered. Fifty mg of the powdered sample dried at 105°C for 6 h was transferred in to a digestion flask and digested with 2 g of potassium sulphate, 40 mg of mercuric oxide and 25 ml of concentrated sulphuric acid. Gently heated the flask until frothing ceased and heating continued more strongly until the solution was cleared. After cooling, 10 ml of distilled water was added and warmed to dissolve the solute material. Blanks were prepared using the reagents alone.

Estimation

The digested sample was transferred in to the distillation flask. A quantity of 2 ml sodium hydroxide-sodium thiosulphate in 100 ml of water was added and steam distilled. The liberated ammonia was collected in to 5 ml of 4 per cent boric acid solution (in water), containing 2-3 drops of methyl red-bromocresol green indicator (prepared by mixing five parts of 0.2 per cent alcoholic bromocresol green solution with one part of 0.2 per cent alcoholic methyl red solution). The distillate was titrated against 0.02 N hydrochloric acid. The end point was chosen as the appearance of green colour. The blank digest was also run in the same way. Nitrogen in the sample was calculated by employing the factor, 1 ml of 1 N acid is equivalent to 14 mg of nitrogen.

3.2.6 Determination of crude protein

The protein content of the tissue was derived by multiplying the total nitrogen content of plant tissues by the factor 6.25 (A.O.A.C. 1960).

3.2.7 Separation and quantitative determination of simple phenolic compounds

Phenols were extracted from plant tissues and were separated by thin layer chromatographic technique. Some of them were identified and the quantities estimated by the procedure scaled down by Seikel (1964).

3.2.7.1 Extraction of phenols from plants

Ten grams of the fresh tissues were chopped into small bits, plunged into 50 ml of 80 per cent boiling ethanol and extracted for 10 min in a hot water bath. After cooling, the extract was decanted. The tissues were

homogenized by grinding in a porcelain pestle and mortar and strained through a cheese cloth. The residue was transferred back to 25 ml of boiling 80 per cent ethanol and re-extracted for 5 min cooled and filtered. The extracts were pooled and filtered through Whatman No. 40 filter paper. At the end of filtration, the filter paper was again washed with about 5 ml of 80 per cent ethanol. The filtrates were concentrated to 20 ml at 40°C under reduced pressure. The concentrated ethanol extract was then extracted thrice by shaking with 20 ml portions of petroleum ether in a separating funnel in about ten hour period. The upper petroleum ether layer was removed and discarded. The residual solution was acidified to pH 3 with 1 N hydrochloric acid and extracted with four 20 ml volumes of redistilled ethyl ether in a 12 h period. The upper etherial fractions were removed, combined and evaporated to dryness at 40°C. The residue was redissolved in 1 to 2 ml of 70 per cent ethanol and used for chromatography.

3.2.7.2 Chromatographic separation

Fifty µl of the extract were spotted on thin layer chromatographic plates (TLC) of 28 x 23 cm size. The chromatogram was developed at room temperature (28 ± 1°C) bidimensionally (ascending). For the first direction, the upper phase of mixture (6:7:3 v/v) of benzene-acetic acid-water (Seikel, 1964) was used and the sheets developed for 3½ h without equilibrating the sheet in the solvent. The developed sheet was removed and dried until the odours of benzene and acetic acid faded away. For the second direction, at right angle to the first a 3 per cent solution of sodium chloride in 0.1 N hydrochloric acid (Seikel, 1964) was run for 1½ h. The chromatogram was removed from the solvent air dried for 12 h.

Since caffeic and protocatechuic acids stayed at the base of the chromatogram along with flavonols in the above solvents, they were eluted from the chromatogram in 1 per cent acetic acid (Feenstra *et al.*, 1963), rechromatographed and developed ascendingly in n-butanol-acetic acid water (4:1:1 v/v) for 6 to 7 h and again developed in 2 per cent acetic acid for 1 h. The sheet was removed from the solvent system and air dried.

3.2.7.3 Identification

The resolved constituents in the sheets were examined in ultraviolet light with and without ammonia fumes (sp. gr. 0.88). The chromatograms were also sprayed with the following chromatographic reagents (Annexure 2).

a) diazotized sulphanilic acid
b) tetraazotized benzidine
c) ferric chloride–potassium ferricyanide
d) alkaline silver nitrate
e) ferric chloride
f) sodium molybdate and
g) 2% copper sulphate

The characteristic colours developed were compared by co-chromatography with the known samples and some of the phenols were identified referring to the Rf values and colours under the stated conditions.

3.2.7.4 Quantitative estimation of identified phenols

The chromatograms were developed in quadriplicates for each sample. Two sheets were used for spraying with diazotized sulphanilic acid and ferric chloride reagents. The other two sheets were examined under the

ultraviolet lamp and the phenolic acid spots were outlined lightly with a pencil. The spots from the sheet were scraped and eluted with 3 ml of absolute methanol for 12 hr. The eluant was then transferred to another test tube. One ml of Folin-Ciocalteu reagent and 2 ml of 20 per cent sodium carbonate were added and heated in a boiling water bath for exactly 1 min. The blue coloured solution was cooled, centrifuged at 2,100 g for 5 min and the volume of the supernatant was raised to 6 ml with distilled water. A portion scraped from the chromatogram, from the position along side the phenolic spots was treated in the same way and used as blank. The absorbance was determined in a 'Spectronic 20' colorimeter at 725 nm (Seikel, 1964). The quantity of individual phenolic acids in the samples were calculated from the standard graphs prepared with known phenolic acids.

3.2.8 Quantitative estimation of individual amino acids

3.2.8.1 Preparation of samples

Alcohol extract similar to that of the extract for analysis of phenols, sugars and amino acids was used in this study (Mahadevan and Sridhar, 1982).

Twenty ml of the alcohol extract was taken in a separating funnel and the chlorophyll was removed by partitioning twice with equal volumes of petroleum ether. The petroleum ether fractions were discarded. The pigment free alcohol extract was evaporated to near dryness at 40°C *in vacuo*. The residue was suspended in 5 ml of distilled water. The sample was slowly passed through an anion exchange column (Dowex-1, cl^- form) to remove metallic salts. The flow of the liquid was so adjusted with the stopper that only 3 to 4 drops of the effluent were collected per min. Care was taken to

collect up to the last drop of the effluent. Washing the column finally with 5 ml of glass distilled water helped collection of the effluent without any loss. The clear solution was passed through a Dowex-50 x H^+ form (20–50 mesh) cation exchange resin column. Before loading the column, it was washed thoroughly with water until the pH of the effluent was close to neutrality. The flow rate was adjusted to approximately 20 drops per min. When all the solution was passed through the column, it was washed with distilled water and then eluted the amino acids with 50 ml of 2M ammonium hydroxide. The elute was evaporated to dryness until no ammonia odour was detected. The residue was redissolved in 5 ml of distilled water and the pH was adjusted to 2.5 with 4M formic acid to release any remaining ammonia. The solution was evaporated to dryness and the residue dissolved in 5 ml of distilled water.

3.2.8.2 Chromatographic separation of amino acids (Block *et al.*, 1958)

Fifty µl of the samples were spotted on thin layer chromatographic sheet (28 x 23 cm) and developed ascendingly for 10 hr. The solvent system for the first direction consisted on *n*-butanol-acetic acid-water (4:1:1 v/v) and for the second direction phenol-water (3:1 v/v). Distilled phenol was used. The air dried chromatograms were sprayed with 0.1 g of ninhydrin in 95 ml of 90 per cent ethyl alcohol and 5 ml of distilled water and immediately dried at 100°C for 15 min in an oven. The coloured spots developed were identified with co-chromatogram using known amino acids.

3.2.8.3 Quantitative estimation of amino acids (Demetriades, 1956)

The spots were scraped from the chromatogram and transferred to test tubes containing 4.0 ml of the eluant (3.6 ml of 75 per cent ethyl alcohol and 0.4 ml of 0.1 per cent copper sulphate). Elution was done till there had been no trace of pink colour. The colour intensity of the eluates was read in a 'Spectronic-20' colorimeter at 475 nm using appropriate blanks. The quantity of the amino acids present was calculated from standard graphs prepared with authentic samples of the amino acids.

3.2.9 Estimation of ascorbic acid

Ascorbic acid content of the plant was estimated by the visual titration method based on the reduction of 2,6-dichlorophenol-indophenol dye (Roe, 1954) (Composition of reagent, see Annexure 2).

3.2.9.1 Extraction of plant materials

Two g of the leaf material, cut into small bits, were thoroughly crushed in a porcelain mortar and pestle with 5 ml of 0.5 per cent oxalic acid and a pinch of acid washed sand for 3 min and filtered through two layers of cheese cloth. To ensure complete extraction, the residue was re-extracted and filtered through the cheese cloth with minimum quantity of oxalic acid. The extract was pooled and centrifuged at 2,100 g for 20 min. and the final volume was raised to 10 ml with 0.4 per cent oxalic acid so that 5 ml of the extract represented one g of tissues. Ascorbic acid content of this extract was estimated.

3.2.9.2 Estimation

Five ml of the oxalic acid extract were placed in a white porcelain dish and titrated against the standardized indophenol reagent until the pink endpoint persisted for 15 seconds. Ascorbic acid content was calculated by employing the formula,

$$I \times S \times D/A \times 100/W = \text{mg of ascorbic acid/100 g of tissue}$$

where

I = ml of indophenol reagent used in titration

S = mg of ascorbic acid reacting with 1 ml of reagent

D = volume of the extract in ml

A = aliquot titrated in ml

W = weight of the sample in mg

The result was expressed in oven dry tissue basis.

3.3 Oxidative Enzymes

3.3.1 Assay of polyphenol oxidase, peroxidase and ascorbic acid oxidase

(Composition of reagent, see Annexure 2).

3.3.1.1 Enzyme extraction from plants

One g of the plant material cut into small bits, was crushed in 4 ml of chilled 0.1 M sodium phosphate buffer at pH 7.1 with a pinch of chilled acid washed sand in a previously chilled porcelain pestle and mortar. The extract was strained through two layers of cheese cloth, and the volume was made up to 5 ml with the buffer, centrifuged at 2100 g for 30 min and the supernatant was used as the enzyme source (Sridhar *et al.*, 1969). The activity of polyphenol oxidase (PPO) and peroxidase (PO) was determined in

a 'Spectronic-20' colorimeter while that of ascorbic acid oxidase was followed up in a UV spectrophotometer. Cuvettes containing the same concentrations of the reaction mixture as that of the treatments, except the enzyme source added to them, and heated at 100°C for 30 min on a water bath were maintained as controls.

3.3.1.2 Estimation

3.3.1.2.1 Polyphenol oxidase

The reaction mixture contained 0.5 ml of 0.2 M phosphate buffer at pH 7.0, 0.5 ml of 0.1 M catechol, 1.5 ml of glass distilled water and 0.5 ml of the enzyme extract. Changes in absorbance at 495 nm (Matta and Dimond, 1963) were recorded at 30 seconds interval for 3 min.

3.3.1.2.2 Peroxidase

The assay system consisted of 0.1 ml of the tissue extract as enzyme source, 1.0 ml of 0.001 M pyrogallol in 0.05 M phosphate buffer at pH 6.5, 0.1 ml of 2 per cent (0.588 M) hydrogen peroxide and 1.8 ml of glass distilled water. Changes in absorbance at 470 nm were recorded at 30 seconds interval for a period of 3 min (Hampton, 1963).

3.3.1.2.3 Ascorbic acid oxidase activity

To 0.1 ml of the plant extract, 1 ml of 0.2 M phosphate buffer at pH 6.2, 0.1 ml of 0.001 N ascorbic acid and 1.8 ml of glass distilled water were added and changes in absorbance at 265 nm were recorded at 30 seconds interval up to 3 min (Oberbacher and Vines, 1963).

3.3.1.3 Expression of enzyme activity

Enzyme activity was expressed in terms of units (U). One unit is that amount which will catalyse the transformation of one micromole of the substrate per minute at 28±1°C (Anonymous, 1965).

3.4 Assay of phenylalanine ammonia–lyase and tyrosine ammonia-lyase

(Composition of reagent, see Annexure 2)

3.4.1 Extraction of enzymes (Biehn *et al.*, 1968)

Plant tissues weighing 3 g were cut into small bits and disintegrated in cold redistilled acetone (1 g of tissue/12 ml) for two min in a chilled pestle and mortar. The homogenate was filtered in a Buchner funnel with Whatman No. 1 filter paper and the residue was washed twice with cold acetone (10 ml acetone per g of tissue). After drying for 15 min in a fume hood, the acetone powder was dried further under reduced pressure at room temperature for at least 1 h and then stored at 0°C.

For the assay of enzymes, the acetone powder was suspended in 0.1 N borate buffer at pH 8.8 (0.1 g of powder/6 ml). The mixture was occasionally stirred, filtered, through a double cheese cloth and the filtrate was centrifuged at 2,100 g at 4°C for 15 min.

3.4.2 Estimation

3.4.2.1 Assay of phenylalanine ammonia-lyase activity

The reaction mixture contained 1.5 ml borate buffer extract containing the enzyme, 1 ml of 0.05 M phenylalanine and 2.5 ml of 0.1 M borate buffer at

pH 8.8. The mixture was incubated for 1 h in a test tube at 40°C and the reaction was stopped by the addition of 0.1 ml of 5 M hydrochloric acid.

A zero time control prepared as described above was assayed at the same time. The acidified reaction mixture was extracted with 7.5 ml of ether. Two and a half ml of the other phase were removed and evaporated to dryness under a stream of air. The residue that remained was dissolved in 5 ml of 0.05 M sodium hydroxide and the absorbance at 268 nm was determined in a spectrophotometer.

3.4.2.2 Assay of tyrosine ammonia-lyase activity

All the methods used to assay PAL were followed to measure TAL except that the substrate was replaced with tyrosine. *p.* coumaric acid from the reaction mixture was partitioned with ether and the content measured in a spectrophotometer at 333 nm.

3.5 Effect of nitrogen on the *in vitro* production of cellulase (C_1 and C_x) β-glucosidase, pectinolytic enzymes and protease of *C. cassiicola*

(Composition of reagent, see Annexure 2)

The effect of nitrogen on the production of cellulases (C_1 and C_x) β-glucosidase pectinolytic enzyme and protease was determined. Modified Czapek's medium devoid of sodium nitrate (Annexure 1) was used to obtain the enzyme source.

To the modified Czapek's medium, cellulose, salicin and pectin were added separately as sole carbon source to obtain the enzyme source for cellulases, β-glucosidase and pectinolytic enzymes, respectively. Salicin was incorporated at 0.25 per cent level while cellulose and pectin were added at

3 per cent level. However, to obtain protease source, casein was added at 3 per cent level substituting carbon and nitrogen sources in the medium. The media were dispensed in 50 ml quantities into 250 ml Erlenmeyer flasks. Sodium nitrate was incorporated into these aliquot in such a way so as to give 250, 500, 750, 1000, 1250, 1500, 1750 and 2000 ppm nitrogen. Medium devoid of nitrogen served as the control. The flasks were sterilized at 1.4 kg/cm^2 for 20 min, inoculated with 5 mm discs of actively growing culture of *C. cassiicola* and incubated for 15 days. At the end of the incubation period, the biomass was filtered and its dry weight was determined. The culture filtrate obtained from different treatments were centrifuged at 2,100 g for 20 min and the cell free culture filtrate served as the enzyme source.

3.5.1 Assay of cellulolytic enzymes

3.5.1.1 Assay of cellulase (C_1) (Norkrans, 1950)

The reaction mixture consisted of 1 ml of cellulose solution (the concentration of which was adjusted to approximately 0.85 absorbance at 610 nm), 4 ml of 0.2 M sodium acetate acetic acid buffer at pH 5.6 and 5 ml of the enzyme extract. Absorbance was determined immediately at 610 nm in a Spectronic-20 colorimeter and incubated at room temperature. At the end of 24 h the absorbance was again measured and the enzyme activity was expressed as units (1 unit = 0.01 absorbance at 610 nm), calculated as difference in absorbance.

3.5.1.2 Assay of cellulase (C_x) (Husain and Dimond, 1960)

The cellulase (C_x) activity was determined by loss in the viscosity of carboxy methyl cellulose (CMC).

Carboxy methyl cellulose of 0.5% concentration was prepared in 0.2 M sodium acetate acetic acid buffer at pH 5.6 and to 4 ml of the same, 2 ml of the buffer and 2 ml of the culture filtrate were added. The final pH of the reaction mixture was adjusted to 5.6, transferred to Ostwald Fensk viscometer (size 150), placed in a water bath at 32+1°C and the viscosity losses were determined at 0, 5, 15, 30 and 60 intervals and the reading at 60 min was used for comparison. The percentage loss in the viscosity was calculated by employing the formula

$$\text{Percentage loss in viscosity} = \frac{T_0 - T_1}{T_0 - T_w} \times 100$$

where

T_0 = flow time at 0 time (sec)

T_1 = flow time at each intervals (sec)

T_w = flow time of double distilled water (sec)

3.5.1.3 Assay of β-glucosidase

The reaction mixture contained 4 ml of 0.25 per cent salicin in sodium acetate-acetic acid buffer at pH 5.6, 1 ml of the same buffer, 1 ml of 4 per cent sodium fluoride (to prevent contamination) and 2 ml of the culture filtrate. The release of reducing sugar from salicin was determined at 0, 1, 2 and 4 h following the method of Nelson (1944).

3.5.2 Assay of pectinolytic enzymes

3.5.2.1 Protopectinase (Mahadevan and Chandramohan, 1967)

The activity of protopectinase was determined by the action of culture filtrates on potato medullary discs.. To 15 ml of the culture filtrate adjusted to pH 6.5 taken in 10 cm sterile petridishes, potato discs of 1 mm thickness and

8 mm diameter, cut by means of a sharp razor blade were placed. One ml of 4 per cent sodium fluoride was added to prevent bacterial contamination. The plates were incubated at room temperature and the coherence of the potato discs was tested by touching them with a glass rod at 4, 8 and 12 h intervals. The loss of coherence of potato discs was expressed in units of 5 (Mahadevan and Chandramohan, 1967). Heated inactivated culture filtrate and distilled water served as controls.

3.5.2.2 Polygalacturonase (PG)

The change in viscosity of sodium polypectate was employed to determine the PG activity.

Sodium polypectate, 0.75 per cent was dissolved in distilled water and the pH was adjusted to 5.2. To 4 ml of sodium polypectate, 2 ml of 0.2 M sodium acetate-acetic acid buffer pH (5.2) and 2 ml of culture filtrate was added. The pH of the reaction mixture was adjusted to 5.2 and immediately transferred to Ostwald Fensk viscometer in water bath at 30±1°C, viscosity losses were measured and the per cent loss in viscosity was calculated as detailed earlier.

3.5.2.3 Pectin transeliminase (PTE) and polygalacturonase trans-eliminase (PGTE)

Pectin *trans*-eliminase and polygalacturonase *trans*-eliminase were assayed by loss in viscosity of pectin and sodium polypectate respectively (Ayers *et al.*, 1966). Production of thiobarbituric acid reacting substances (Neukom, 1960) and the releases of compounds maximally absorbing at 232 nm (Bateman, 1966) were determined.

Loss in viscosity

The viscosity loss of pectin was determined with Ostwald Fensk viscometer, size 150 while that of sodium polypectate was determined employing the viscometer size 300. Both the assays were made at 30°C in a water bath. The final reaction mixture contained 4 ml of 1 per cent citrus pectin or 1.2 per cent sodium pectate at pH 8.6 in 0.025 M borate buffer, 2 ml of the same buffer and 2 ml of the culture filtrate.

The reaction mixture was quickly transferred into the respective viscometers and the relative viscosity of the mixture at various intervals up to 2 h was determined (Wallace *et al.*, 1962).

3.5.2.4 Production of thiobarbituric acid (TBA) reacting substances

Production of thiobarbituric acid reacting substances from pectin and sodium polypectate were determined. Three ml of the enzyme substrate mixture under test were withdrawn and centrifuged at 2100 g for 2 min. To the clear supernatant, 10 ml of 0.1 M thiobarbituric acid and 5 ml of 0.5 N hydrochloric acid were added and the mixture was boiled in a water bath for 1 h. The test tubes were cooled, the volume was made up and the absorbance of the mixture was determined in a 'Spectronic-20' colorimeter between 450-500 nm employing blank withdrawn at 0 time to adjust the optical density to 0.

3.5.2.5 Release of compounds absorbing maximally at 232 nm

Transeliminative split of pectin and sodium polypectate by the enzyme was confirmed further by the following experiment (Bateman, 1966; Kathirvelu and Mahadevan, 1967).

The reaction mixture was clarified by adding 3 ml of 5 per cent trichloro acetic acid (TCA) and the precipitated enzyme proteins were removed by centrifuging at 2,100 g for 20 min. The absorption maximum of the clear supernatant was recorded in a spectrophotometer at a wave length of 210-310 nm with appropriate controls.

3.5.3 Assay of protease (Davis and Smith, 1955)

The reaction mixture contained 10 ml of 1 per cent casein dissolved in 0.1 M sodium phosphate buffer at pH 7 in a test tube to which 5 ml of the same buffer and 5 ml of the enzyme source were added and incubated at 30°C in a water bath. From this mixture, aliquot of 5 ml were withdrawn at hourly intervals and pipetted out in a test tube containing 5 ml of 5 per cent trichloro acetic acid. The mixture was allowed to stand at room temperature for 1 h and the precipitated proteins were removed by centrifuging at 2,100 g for 20 min.

One ml of the supernatant was transferred to a test tube containing 1 ml of Folin-ciocalteu reagent. To this 2 ml of 20 per cent sodium carbonate was added and boiled exactly for 1 min in a water bath. After cooling, the volume was made up to 10 ml with distilled water, centrifuged and clear supernatant was read in a 'Spectronic-20' colorimeter at 725 nm. Standards prepared from tryptophan were used to calculate the unknown. Suitable

controls were maintained. The enzyme activity was expressed as amino acids released in tryptophan equivalents.

3.6 Toxin production in *Corynespora cassiicola*

3.6.1 Toxin production in modified Czapek's broth medium

Fifty ml aliquot of modified Czapek's broth devoid of sodium nitrate were dispensed separately in 250 ml Erlenmeyer flasks. Sodium nitrate was added to give 250, 500, 750, 1000, 1250, 1500, 1750 and 2000 ppm. nitrogen levels into the flasks. Three flasks were maintained for each treatment. The pH was adjusted to 6.5, sterilized at 1.4 kg/cm^2 for 20 min and inoculated with 5 mm disc of *C. cassiicola* obtained from 7 days old culture in modified Czapek's agar medium. The flasks were incubated at room temperature (28±1°C) for 15 days. At the end of incubation period, the mycelium was filtered through Whatman No. 41 filter paper and the pH was measured with a pH meter. Then the pH was adjusted to 3 with 2 N acetic acid and centrifuged at 2,100 g for 20 min. The supernatant was microscopically examined for the presence of cells and only cell free extracts were used. Ten ml of the filtrate was extracted with equal volume of peroxide-free ether consecutively for three times over a period of 8 h. The ether was evaporated off in a hot water bath at 40°C, the residue was redissolved in 1 ml of distilled methanol and used for assay of toxin(s) employing *Bacillus subtilis*.

3.6.2 Toxin production in rubber leaf extracts

Leaves of rubber seedlings raised with different levels of nitrogen as stated earlier were used for getting leaf extract. Leaves were collected 5 days after the application of second dose of nitrogenous fertilizer. Fifty g of the chopped leaves were plunged into 200 ml of distilled water, boiled for

15 min, filtered, pH adjusted to 6.5, dispensed in 50 ml aliquot into 250 ml. Erlenmeyer flasks and sterilized at 1.4 kg/cm^2 for 20 min. The flasks were inoculated with C. *cassiicola*, incubated for 15 days and toxin extracted from the filtrate as described earlier. Uninoculated leaf extracts treated in the same way served as control.

3.6.3 Assay of toxin

The toxin was assayed for its ability to inhibit the growth of *B. subtilis* (Mahadevan, 1966 b). 100 µl portions of the extracts were applied to sterile filter paper disc of 1 cm diameter under aseptic conditions and dried by means of a hair dryer. The discs were placed on *B. subtilis* seeded nutrient glucose agar (Annexure 1) plates with appropriate control, incubated at room temperature (28±1°C) for 8 h and the inhibition was calculated by employing the formula of Smale and Kiel (1966).

$$\text{Area of inhibition } A = \pi(R_1-R_2)(R_1+R_2)$$

where

R_1 = width of zone of inhibition + the radius of the assay disc in mm

R_2 = radius of the assay disc in mm

3.6.4 Detoxification of toxins of *C. cassiicola* by plant phenolics (Sridhar, 1969)

One ml of the toxin extract from modified Czapek's broth medium was separately mixed with 1 ml of solution containing *p*. hydroxybenzoic acid, *p*. coumaric acid, caffeic acid, protocatechuic acid, ferulic acid, vanillic acid and chlorogenic acid individually so that the final concentration of phenolic acids in the reaction mixture will be 0.002, 0.004 and 0.008 M. Inhibitory

activity of 100 µl of the mixture against *B. subtilis* was tested as described earlier.

3.7 Determination of nutrients on the leaf leachates

3.7.1 Collection of leaf leachates (Schneider and Sinclair, 1975)

About 10 g of leaf samples were collected and plunged into 500 ml Erlenmeyer flasks containing 100 ml distilled water. The flasks were shaken for 30 min in a rotory shaker. The leaf washings thus collected were centrifuged at 2,100 g for 10 min and the clear supernatant was condensed at 40°C to 5 ml.

3.7.2 Estimation of phenols, amino nitrogen and reducing sugars

The condensed leaf washings were used for the estimation of total phenols following the procedure of Bray and Thorpe (1954) and total sugars by the method of Nelson (1944) and Inman (1962). The amino nitrogen content of the leachate was analysed following the procedure of Moore and Stein (1957). The area of the leaf in each flasks was also measured in a leaf area meter and the quantity of total phenols total sugars or amino acids leached in 30 min per square cm area of the leaf was calculated.

3.8 Enumeration of phyllosphere microflora of *Hevea* (Dickinson, 1965)

3.8.1 Collection of leaf sample

The leaf samples were collected from the plants treated with different levels of fertilizers as mentioned earlier on the seventh day of inoculation with *C. cassiicola* at random leaving copper brown leaves. Nearly 10 g of the leaf samples were collected with the help of sterile forceps and placed inside a sterile 250 ml Erlenmeyer flask containing 100 ml of distilled water. The flasks were properly labelled and the samples were used for the microbial assay within 1 h after collection.

3.8.2 Estimation of microbial population

The leaf samples in the sterile water blanks were shaken for 15 minutes on a rotary shaker and ten fold serial dilutions were prepared. One ml aliquot was plated with respective medium for bacteria, filamentous fungi and yeasts. (The composition of the media was given in the Annexure 1) (Leben, 1972). The incubation time for both bacteria and filamentous fungi was 4 to 5 days at 28±1°C and for yeasts 7 days at 20°C (Leben, 1972). After the incubation period, the colonies of bacteria, filamentous fungi and yeast were counted. Colonies of yeast were not categorized as yeast unless typical budding cells were observed. The data on microbial population were expressed as number per square area of leaf by measuring the area of leaf from each flask as mentioned earlier. The colonies were transferred to nutrient agar slants for bacteria and potato dextrose agar slants for filamentous fungi and yeasts.

3.9 Effect of phyllosphere microorganisms on the spore germination and appressoria formation in *C. cassiicola*

3.9.1 Preparation of bacterial and yeast cultures

Twenty five isolates each of bacteria and yeast were selected in each fertilizer treatment at random from the plates used for the enumeration of phyllosphere microorganism for this study. The isolates of bacteria and yeast were cultured in ¼ strength nutrient glucose broth for 3 days. The cultures were centrifuged at 2,100 g for 30 min at 20°C. Cells thus removed were washed twice with 10 ml portion of the sterile distilled water and again centrifuged. The bacterial and yeasts cell counts were adjusted to 4×10^6/ml by the addition of sterile distilled water.

3.9.2 Preparation of *C. cassiicola* spore suspension

Spores of *C. cassiicola* were collected from 9 days old culture in potato dextrose medium. The conidial suspension was passed through double layered sterile cheese cloth and the spore suspension was centrifuged. It was then washed twice with 10 ml portions of sterile distilled water followed by centrifuging. Finally the counts of conidia were adjusted to 20×10^4 per ml.

The rubber leaf leachates, collected as per the procedure mentioned earlier and sterilized by filtration was used in this study.

3.9.9 Spore germination studies

Equal quantities of *C. cassiicola* spore suspension and suspensions of bacteria or yeasts were mixed. The mixture was added to equal quantity of rubber leaf leachates so that the mixture contained 7×10^4 conidia of *C. cassiicola* and 1×10^6 bacteria or yeast cells per ml. Droplets of 50 µl of the above mixture were placed in sterile cavity slides, incubated for 16 h at 28±1°C and the spore germination and the appressorial formation were observed. Slides with only spores of *C. cassiicola* in the leaf leachates served as control. The percentage of the isolates of the bacteria and yeast in each fertilizer treatment which induced the spore germination and appressorial formation in *C. cassiicola* was calculated.

3.10 Statistical analysis of the experimental data

The experimental data was subjected to statistical analysis using CRD and two factor RBD.

Chapter 4

RESULTS

4.1 Disease incidence

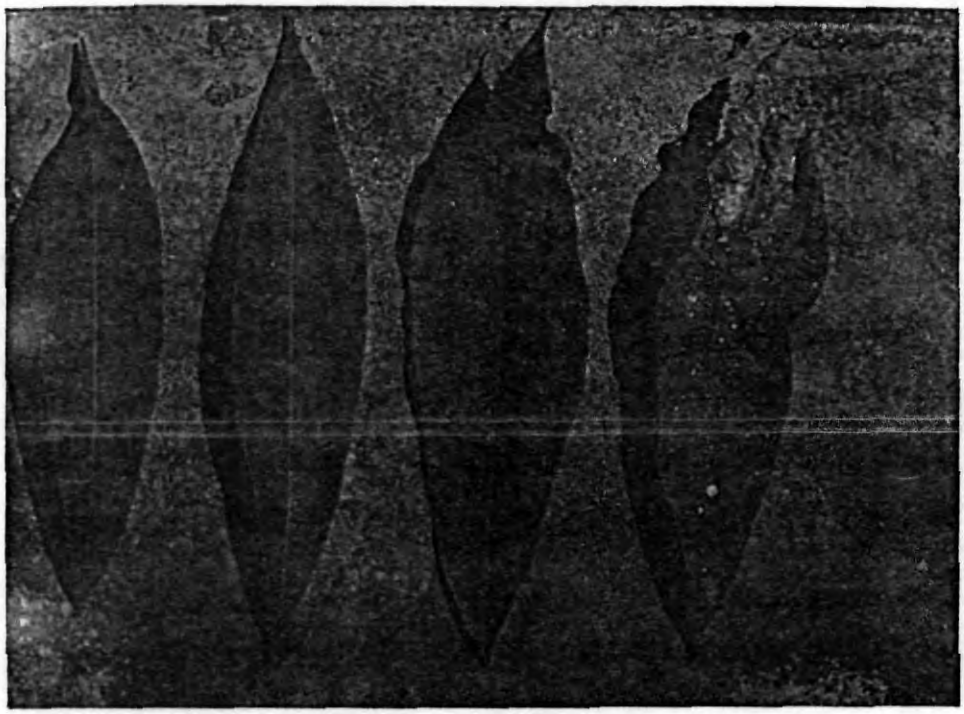
Leaves of *Hevea* seedlings applied with different levels of nitrogenous fertilizer were observed on the eighth day of inoculation for disease intensity. The disease index per cent increased significantly with an increase in the level of nitrogen with the maximum disease score in the 200 per cent of the recommended dose of nitrogen (Table 1). Variation in the symptom expression was also observed with different levels of nitrogen. Typical leaf spots of *C. cassiicola* developed in plants receiving 100 and 200 per cent nitrogenous fertilizer level. Deformity in the leaves was observed in plants receiving 200 per cent nitrogen. At 25 and 50 per cent nitrogen level, the spots were comparatively small and most of them were of pin head size (Plate 1).

Table 1 **Effect of different levels of nitrogen on *Corynespora* leaf spot disease intensity**

Nitrogen level (Per cent of recommended dose)	Per cent disease index
25	24.80
50	38.55
100	45.91
200	62.48

CD (P=0.05) - 3.64

Plate 1 **Disease development in rubber seedlings applied with different levels of nitrogen (% of recommended dose)**



25 50 100 200

4.2 Biochemical constituents

4.2.1 Changes in total phenols (Table 2)

Nitrogenous fertilizer application to rubber seedlings considerably altered the content of total phenols. Increasing the level of nitrogen significantly reduced the total phenols. Total phenols content increased with increase in the sampling period. Upon infection by C. *cassiicola,* the content of total phenols increased significantly at 25 and 50 per cent recommended dose of nitrogenous fertilizer. However, at 100 and 200 per cent nitrogen level total phenols got reduced upon inoculation and the reduction was much pronounced at 200 per cent nitrogen. The increase in total phenols due to infection by *C. cassiicola* is more after 48 h of inoculation in the 25 per cent nitrogen treatment and at 24 h in the case of 50 per cent nitrogen treatment.

Table 2 **Changes in total phenols* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	632	665	720	740	792	760	718.66	635	726	840	800	810	792	767.76
50	600	646	690	715	782	770	700.50	605	805	790	795	825	832	775.33
100	515	560	572	612	710	750	619.83	512	480	495	530	605	680	550.33
200	446	482	496	510	605	620	526.50	450	380	350	392	440	540	425.00

CD (P=0.05) Interaction - 11.70
CD (P=0.05) Mean - 4.77
CD (P=0.05) Interaction - 10.59
CD (P=0.05) Mean - 4.32

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 4.64
50% N - 5.30
100% N - 4.94
200% N - 4.75

* mg g^{-100}

4.2.2 Changes in *ortho*-dihydroxy phenols (Table 3)

The content of O.D. phenols in rubber seedlings was significantly altered by the application of different doses of nitrogen as in the case of total phenols. At 25 and 50 per cent nitrogen, the reduction was not much. On the other hand, at 100 and 200 per cent there was significant reduction in O.D. phenols. The per cent decrease in O.D. phenols was more at 200 per cent nitrogen treatment. The level of O.D. phenols increased with the frequency in sampling period.

As in the case of total phenols, the O.D. phenols also increased upon infection by *C. cassiicola*. The increase in O.D. phenols upon infection starts from the time of inoculation and reaches a maximum at 48 to 96 h. At higher levels of N, *viz.*, 100 and 200 per cent, *C. cassiicola* inoculation resulted in a drastic reduction in O. D. phenols and the reduction was much pronounced on 48 and 72 h in the case of 100 per cent and 24 to 72 h in the case of 200 per cent nitrogen.

4.2.3 Changes in phenolic acids

The phenolic acids in the extracts of rubber seedlings at different treatments were separated by thin layer chromatography. Eight phenolic acids *viz.*, *p.* hydroxybenzoic acid, ferulic acid, *p.* coumaric acid, vanillic acid, caffeic acid, chlorogenic acid, protocatechuic acid and cinnamic acid were identified by co-chromatography with authentic samples. Six more unidentified spots were noted in the extracts of healthy plants under all levels of nitrogen treatment. In the infected plant one more spot was observed which was also not identified.

Table 3 **Changes in *ortho*-dihydroxy phenols* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	146	155	180	165	159	173	163.08	145	163	217	191	205	185.5	184.41
50	138	152	173	166	156	170	159.17	139	161	199	182	173	180	172.33
100	114	138	163	153	160	173	150.17	115	125	120	109.5	129.5	147	124.33
200	74	95	124	108.8	97	114	102.00	75	57	68	73	81	93	74.5

CD (P=0.05) Interaction - 9.5 CD (P=0.05) Interaction - 3.409

CD (P=0.05) Mean - 1.56 CD (P=0.05) Mean - 1.39

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 3.18

50% N - 4.26

100% N - 4.12

200% N - 3.69

* mg g^{-100}

Among the eight identified phenolic acids, *p.* hydroxybenzoic acid registered a high level followed by ferulic acid, *p.* coumaric acid, vanillic acid, caffeic acid, chlorogenic acid, protocatechuic acid and cinnamic acid. The content of different phenolic acids was significantly altered by application of graded levels of nitrogenous fertilizer, period of sampling and *C. cassiicola* inoculation.

4.2.3.1 *p.* hydroxybenzoic acid (Figure 1)

The level of *p.* hydroxybenzoic acid in rubber seedlings in general did not show much variation at 25 and 50 per cent nitrogen dosage and further increase in the nitrogen level caused a considerable reduction in *p.* hydroxy benzoic acid. The reduction was much pronounced at 200 per cent nitrogen. *C. cassiicola* inoculation increased the *p.* hydroxybenzoic acid at 25 and 50 per cent nitrogen level however at 100 and 200 per cent nitrogen it got reduced.

4.2.3.2 Ferulic acid (Figure 2)

Ferulic acid content was not significantly altered by an increase in the level of nitrogen application but at 200 per cent nitrogen application there was a reduction in the level of ferulic acid. Period of sampling also did not show any uniform alteration. At 25 and 50 per cent nitrogen level, *C. cassiicola* inoculation though augmented the ferulic acid content up to 48 h, it showed a reduction thereafter which was not significant. At higher nitrogen level *viz.*, 100 and 200 per cent, ferulic acid content was reduced due to infection. At 100 per cent nitrogen level, the reduction due to infection was significant from 48 h while at 200 per cent the reduction was significant from 96 h only.

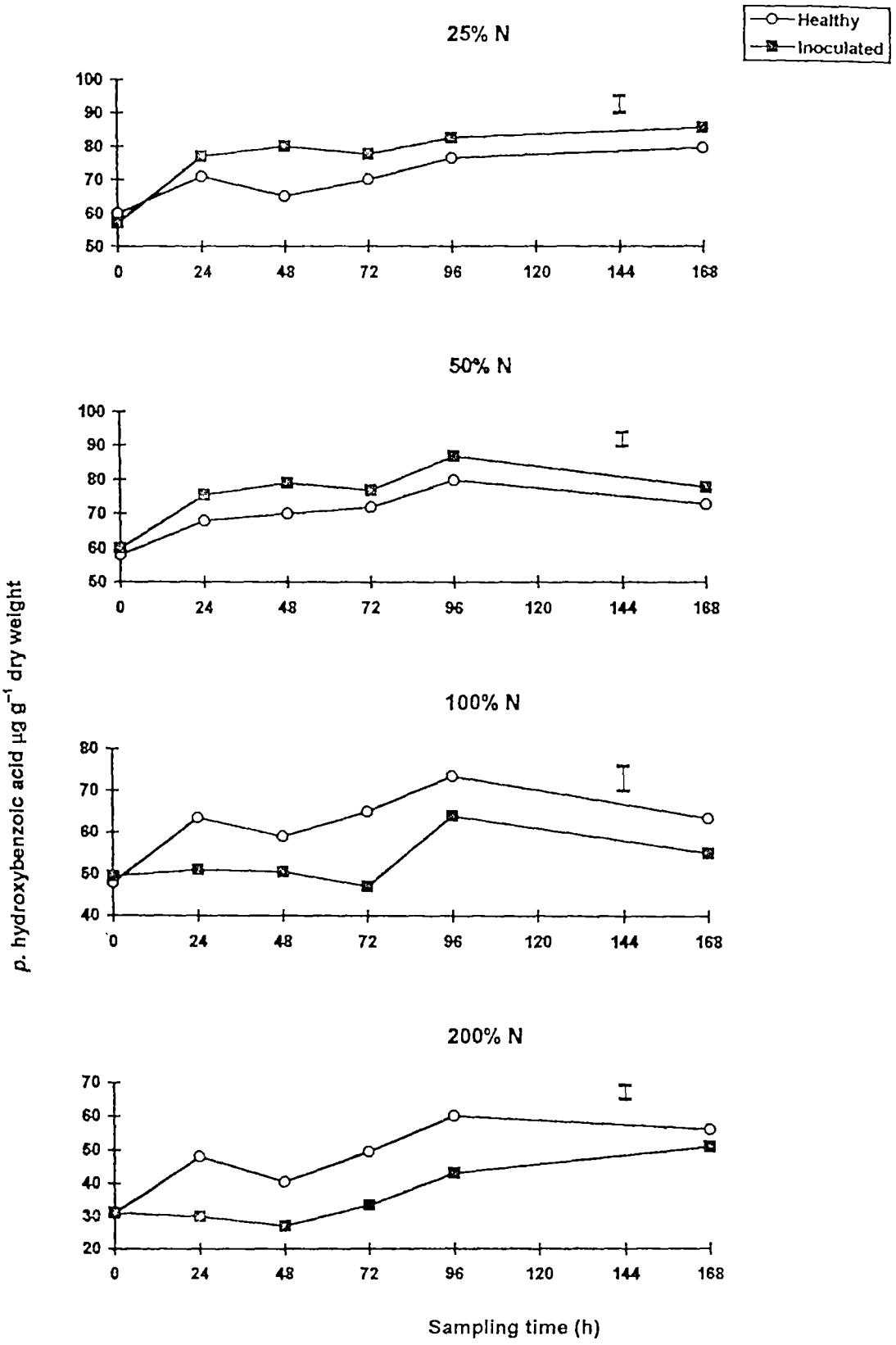


Figure 1 Changes in *p.* hydroxybenzoic acid in the healthy and C. *cassiicola* inoculated rubber seedlings at different levels of nitrogen

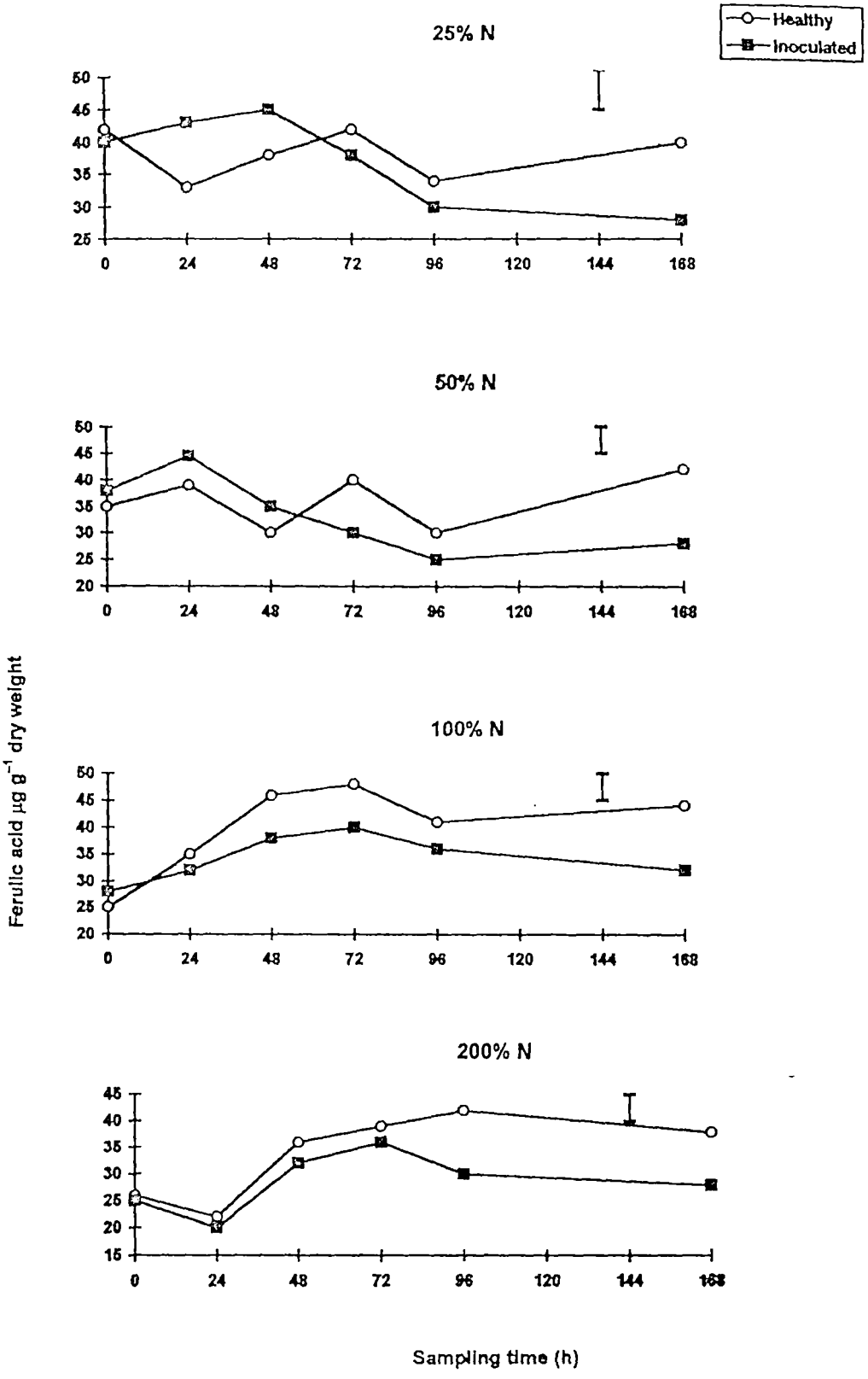


Figure 2 Changes in ferulic acid in the healthy and C. *cassiicola* inoculated rubber seedlings at different levels of nitrogen

4.2.3.3 *p.* coumaric acid (Figure 3)

p. coumaric acid decreased with an increase in nitrogenous fertilizer up to 100 per cent and further increase in nitrogen did not have much impact. At 25 and 50 per cent nitrogen level, *C. cassiicola* inoculation increased the *p.* coumaric acid upto 48 h and thereafter followed a significant reduction. At 100 and 200 per cent nitrogen level no specific trend was observed. However, in the 48 h of sampling *p.* coumaric acid content showed an increase in the infected plants.

4.2.3.4 Vanillic acid (Figure 4)

The level of vanillic acid in general was reduced due to an increase in nitrogenous fertilizer application. The content of vanillic acid increased with the increase in sampling time however it was not much pronounced *C. cassiicola* inoculation increased the vanillic acid at 25 and 50 per cent nitrogen level. At 100 and 200 per cent nitrogen, *C. cassiicola* inoculation caused a reduction in vanillic acid content.

4.2.3.5 Caffeic acid (Figure 5)

Caffeic acid reduced significantly with an increase in the level of nitrogen. The changes in caffeic acid due to *C. cassiicola* inoculation varied with different levels of nitrogenous fertilizer application. At 25 and 50 per cent nitrogen, there was an increase due to inoculation whereas at 100 and 200 per cent of nitrogenous fertilizer treatment, *C. cassiicola* inoculation reduced the caffeic acid content throughout the experiment except in the sampling of 168 h of inoculation.

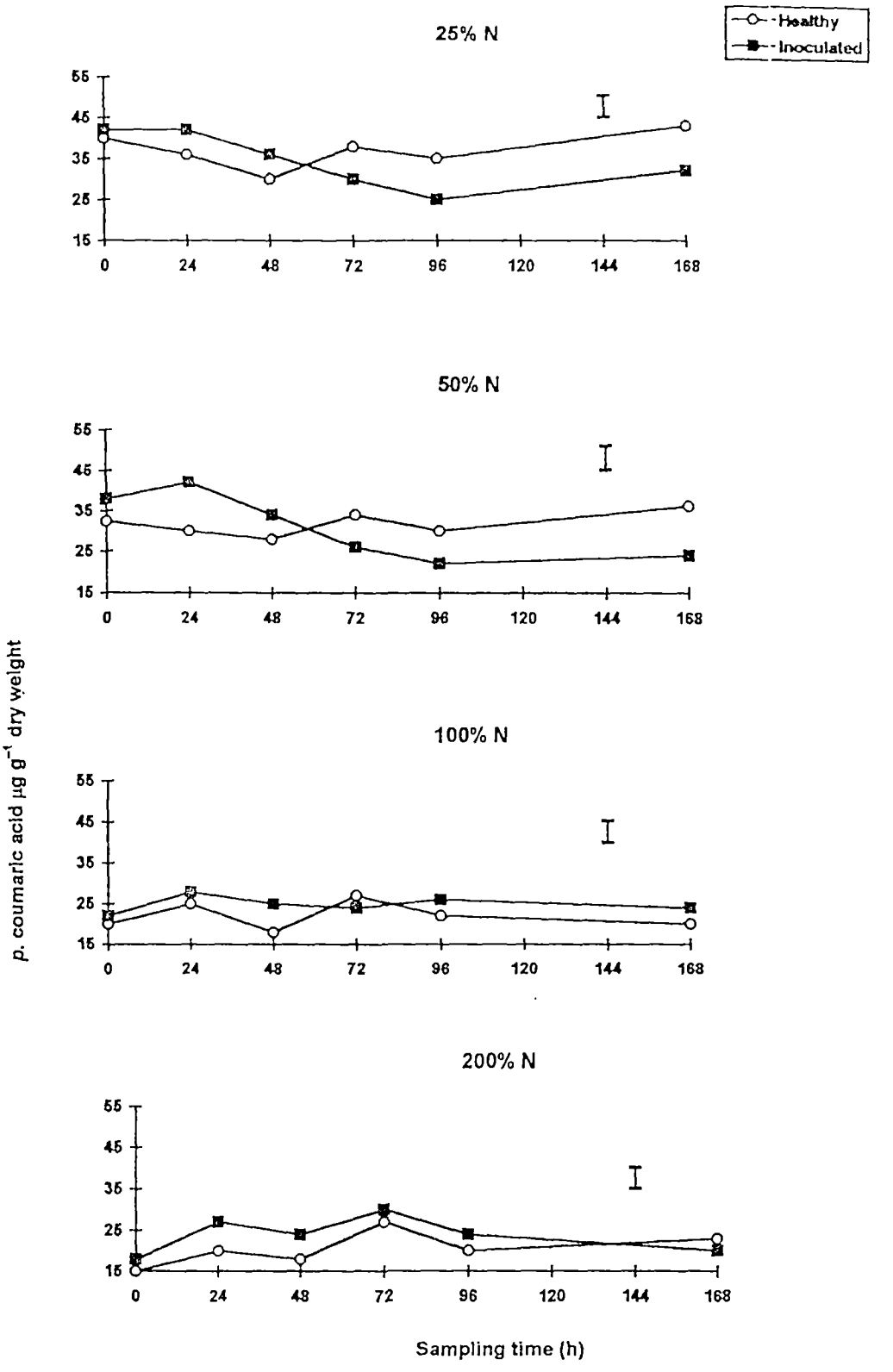


Figure 3 Changes in *p.* coumaric acid in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

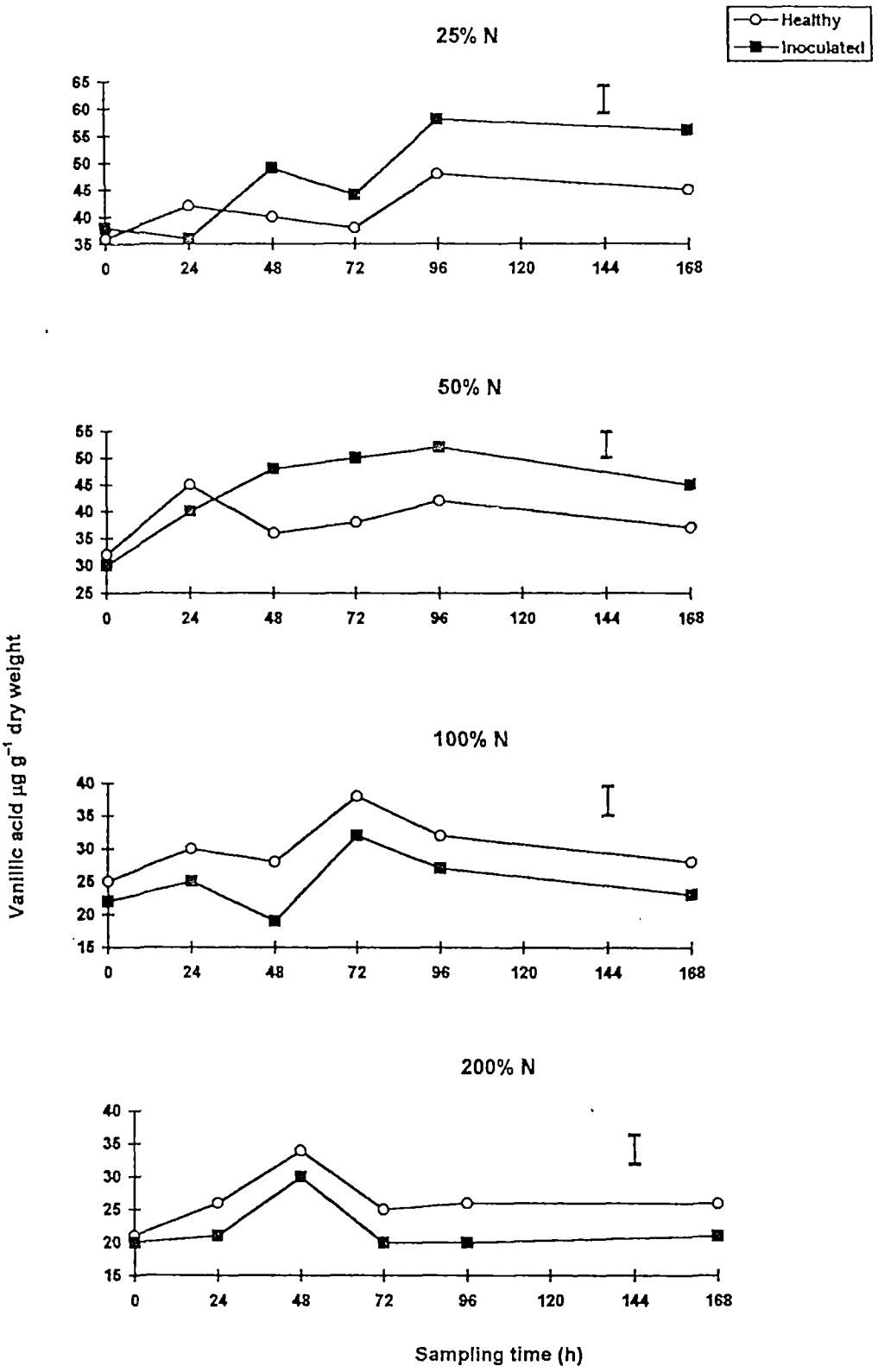


Figure 4 Changes in vanillic acid in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

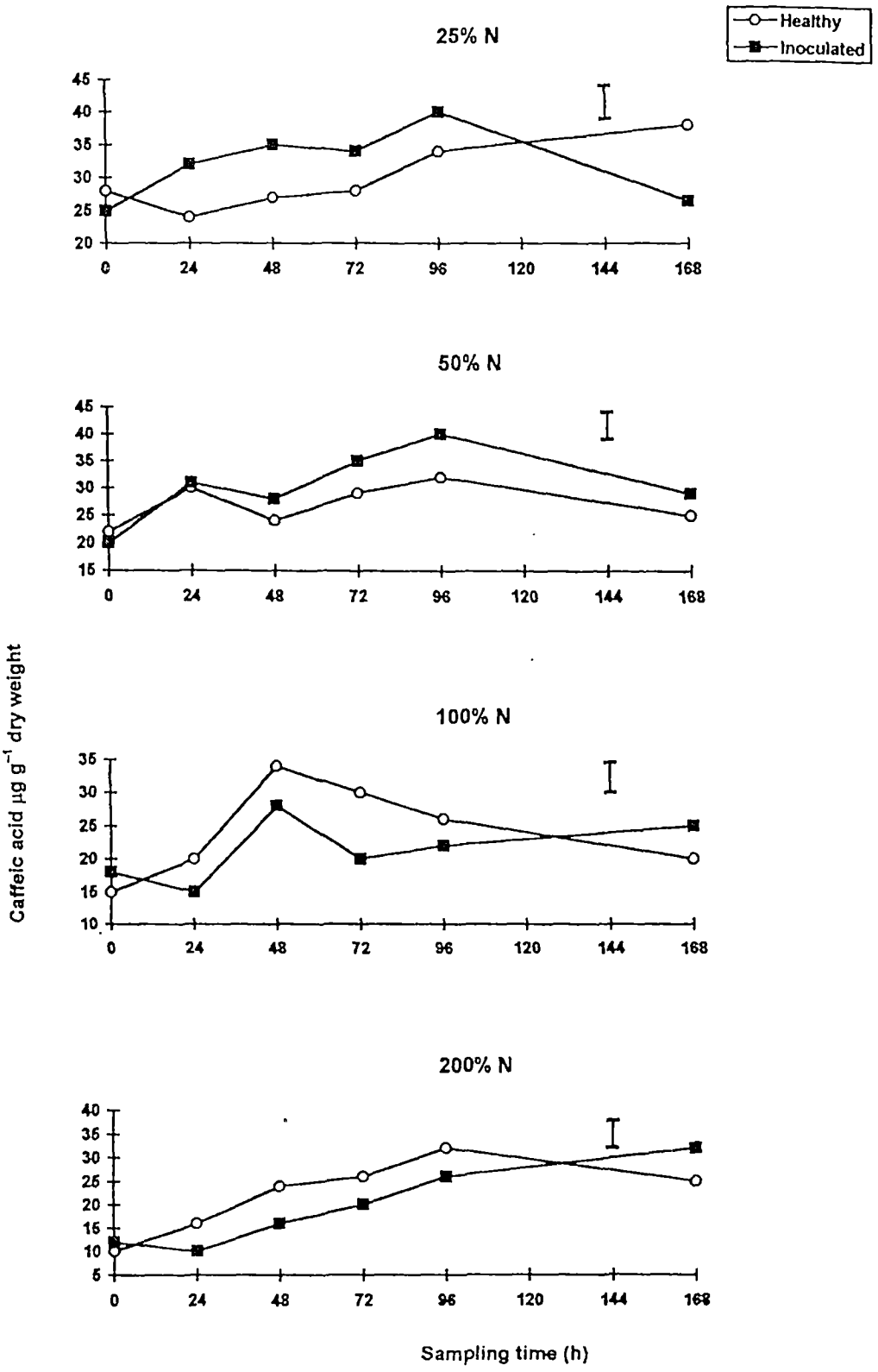


Figure 5 Changes in caffeic acid in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

4.2.3.6 Chlorogenic acid (Figure 6)

Graded levels of nitrogenous fertilizer application resulted in the reduction of chlorogenic acid. In general, an increase in the level of chlorogenic acid was noticed with the increase in the period of sampling. *C. cassiicola* infection augmented the level of chlorogenic acid up to 72 h in the case of 25 and 50 per cent nitrogen. In the case of 100 and 200 per cent nitrogen, *C. cassiicola* inoculation did not significantly alter the content of chlorogenic acid.

4.2.3.7 Protocatechuic acid (Figure 7)

Protocatechuic acid content in rubber seedlings applied with 25 and 50 per cent nitrogenous fertilizer was same and further increase in the level of nitrogen resulted in a reduction of protocatechuic acid which is more apparent in the case of 200 per cent nitrogen application. With the increase in period of sampling, there was an increase in the level of protocatechuic acid. At 25 and 50 per cent nitrogenous fertilizer application, *C. cassiicola* inoculation resulted in an augmented level of protocatechuic acid till the end of the experiment. But at 100 and 200 per cent nitrogen level, *C. cassiicola* infection raised the level of protocatechuic acid upto 48 h of inoculation and thereafter it got reduced.

4.2.3.8 Cinnamic acid (Figure 8)

Increase in nitrogenous fertilizer application up to 100 per cent level reduced the cinnamic acid significantly and the reduction thereafter was not significant. *C. cassiicola* inoculation increased the level of cinnamic acid initially and at later stages, there was a reduction of this phenolic acid in all the fertilizer treatments though not significant.

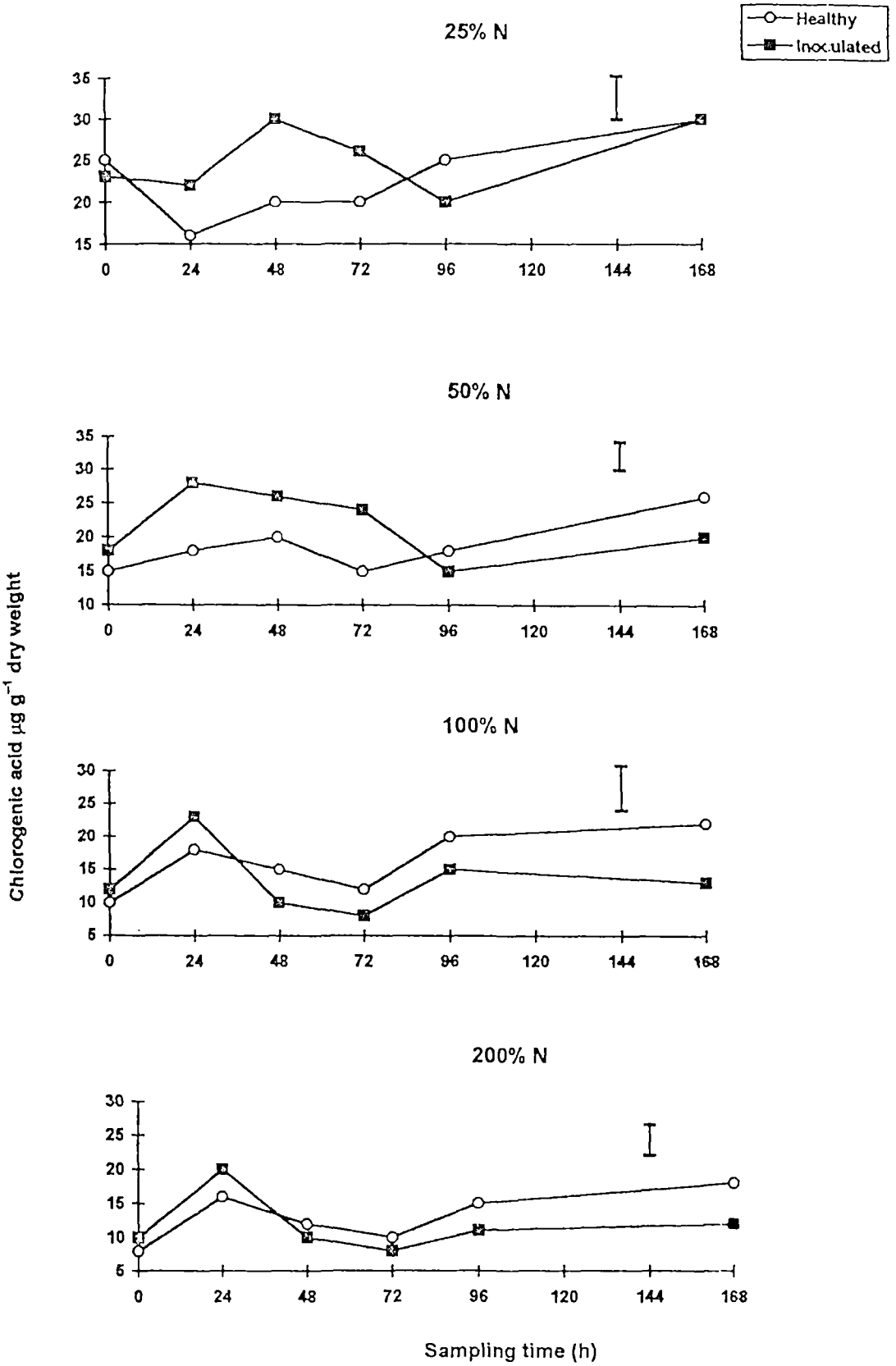


Figure 6 Changes in chlorogenic acid in the healthy and C. *cassiicola* inoculated rubber seedlings at different levels of nitrogen

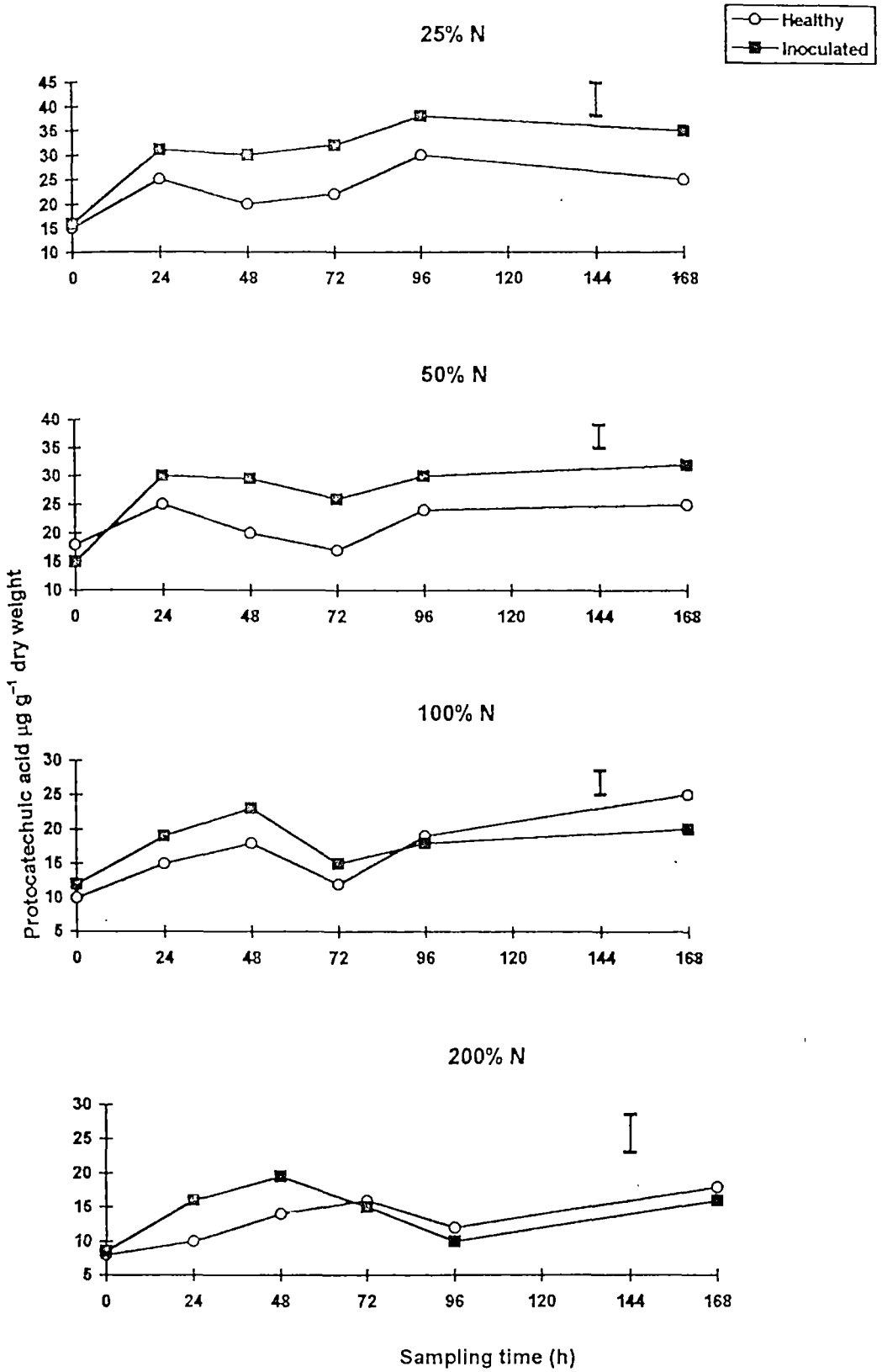


Figure 7 Changes in protocatechuic acid in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

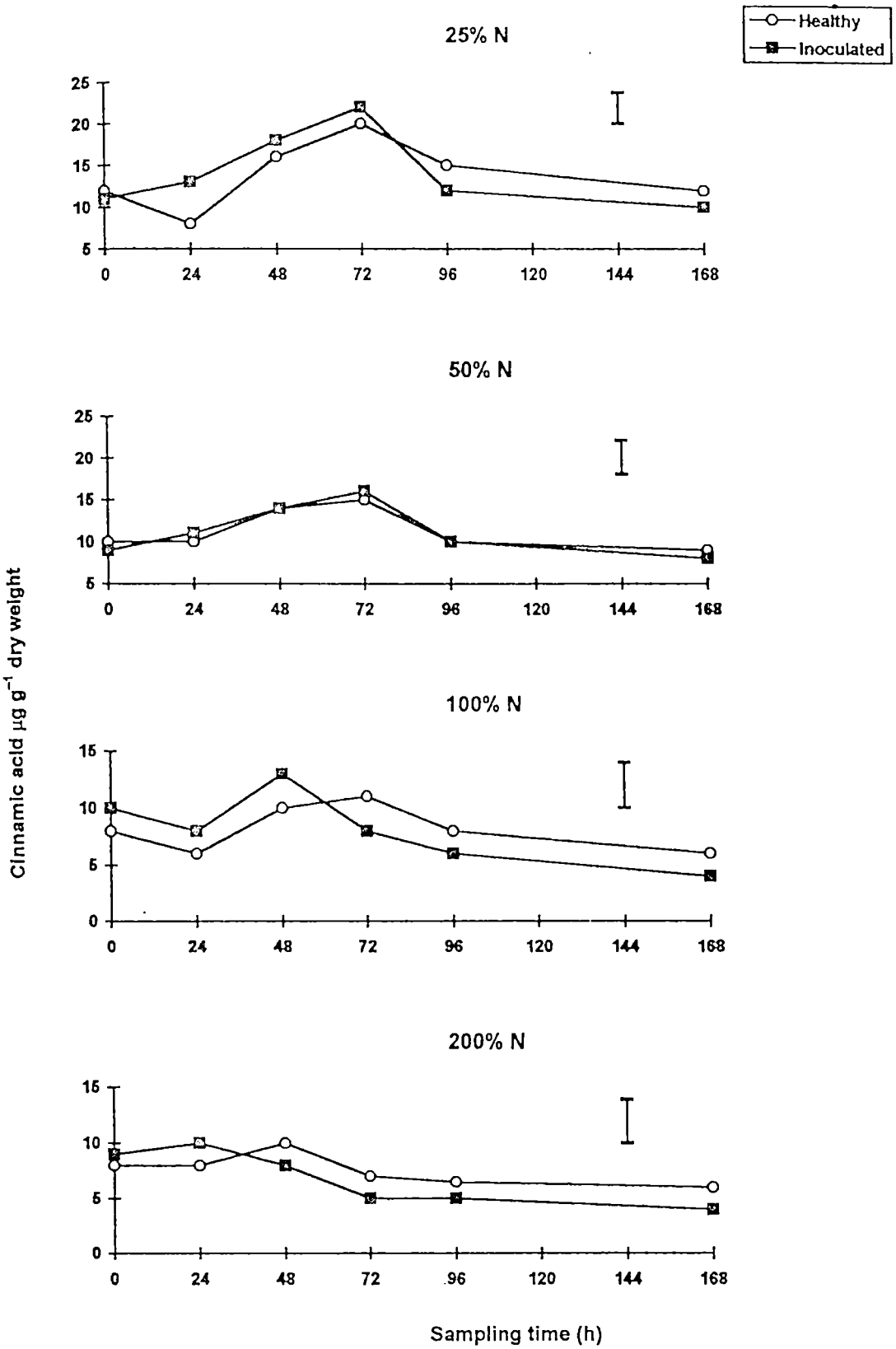


Figure 8 Changes in cinnamic acid in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

4.2.4 Changes in reducing sugars (Table 4)

In general, an increase in levels of nitrogen application markedly increased the level of reducing sugars. The content of reducing sugars increased significantly with increase in sampling period in the case of seedlings applied with 25, 50 and 100 per cent nitrogenous fertilizer while a reduction in reducing sugar was recorded in 200 per cent nitrogen treatment. Irrespective of the level of nitrogenous fertilizer application, *C. cassiicola* inoculation reduced the reducing sugar considerably. The reduction was not much at the end of the period in the case of 25, 50 and 100 per cent nitrogen application. At 200 per cent nitrogen level, the percentage reduction of reducing sugar due to *C. cassiicola* inoculation was very high.

4.2.5 Changes in non-reducing sugar (Table 5)

Increased levels of nitrogenous fertilizer application gradually reduced the content of non-reducing sugars. The level of non-reducing sugars at different period of sampling did not show a definite pattern. However, on an average there was a reduction of non-reducing sugars upon increase in sampling period. *C. cassiicola* inoculation reduced the content of non-reducing sugars in all the treatments. The reduction was noticed from the beginning of sampling at 25 and 50 per cent nitrogen whereas the reduction was noticed only after 48 h of inoculation in the case of 100 and 200 per cent nitrogen application.

Table 4 **Changes in reducing sugars* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	610	580	630	660	705	745	655.0	606	560	600	622	693	739	636.66
50	640	595	665	720	695	675	665.5	634	580	612	696	680	659	643.5
100	720	678	716	765	736	710	720.83	715	656	688	720	712	690	696.83
200	810	792	786	810	759	740	782.83	806	750	679	680	597	572	680.66

CD (P=0.05) Interaction - 6.139 CD (P=0.05) Interaction - 6.917

CD (P=0.05) Mean - 2.51 CD (P=0.05) Mean - 2.415

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 5.63

50% N - 7.17

100% N - 8.34

200% N - 4.62

* mg g^{-100}

Table 5 **Changes in non-reducing sugars* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	1085	1104	1076	1056	1068	1075	1077.83	1081	1087	1058	1042	1032	1060	1060.00
50	1045	1015	1030	1050	1072	1088	1046.33	1039	1006	986	1020	1056	1052	1026.50
100	990	1034	982	920	860	828	947.66	1000	1052	969	895	826	816	926.33
200	946	974	945	955	927	892	936.83	950	1005	920	898	878	870	920.17

CD (P=0.05) Interaction - 6.92
CD (P=0.05) Mean - 2.82
CD (P=0.05) Interaction - 6.32
CD (P=0.05) Mean - 2.503

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 6.95
50% N - 7.65
100% N - 7.17
200% N - 5.69

* mg g^{-100}

4.2.6 Changes in starch (Table 6)

Starch content of rubber seedlings varied at different levels of nitrogenous fertilizer. An increase in the dose of nitrogenous fertilizer reduced the level of starch in tissues. The starch content also decreased with increase in sampling time in 25 and 50 per cent nitrogen treatment. Irrespective of nitrogen level, *C. cassiicola* inoculation resulted in an increase in starch content.

4.2.7 Changes in amino nitrogen (Table 7)

The content of amino nitrogen increased significantly with increase in nitrogen level. In healthy seedlings of all the nitrogen treatments, there was a reduction in amino nitrogen with increase in sampling period. *C. cassiicola* inoculation led to a reduction in the level of amino nitrogen upto 24 h and thereafter it increased considerably in 25, 50 and 100 per cent nitrogen treatments. However at 200 per cent nitrogen fertilizer application *C. cassiicola* inoculation recorded a lower level of amino nitrogen.

4.2.8 Quantitative analysis of amino acids

Rubber seedlings applied with different levels of nitrogen contained 14 amino acids in detectable quantities. Asparagine content was maximum followed by phenylalanine, glutamine, methionine, tyrosin, aspartic acid, alanine, leucine, cysteine, glutamic acid, arginine, histidine and glycine. Apart from this 14 amino acids, tryptophan was also present but its quantity was so less for estimation. Application of nitrogen at different levels and *C. cassiicola* inoculation altered the content of the above amino acids during different periods of sampling. The extent of alteration was not uniform for all amino acids. Therefore the changes with respect to each amino acid are given below.

Table 6 **Changes in starch* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	142	138	135	130	125	128	133.00	140	144	148	142	135	133	140.30
50	135	132	130	128	126	125	129.33	136	142	137	136	132	136	136.50
100	125	120	122	118	120	121	121.00	124	130	132	125	130	128	128.16
200	112	110	105	102	100	102	105.16	110	116	115	108	106	110	110.83

CD (P=0.05) Interaction - 3.86 CD (P=0.05) Interaction - 4.54

CD (P=0.05) Mean - 1.575 CD (P=0.05) Mean - 1.851

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 4.53

50% N - 4.62

100% N - 4.45

200% N - 4.78

* mg g^{-1}

Table 7 **Changes in amino nitrogen* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	460	505	480	468	440	424	462.83	456	490	528	504	469	451	483.00
50	730	760	743	728	692	630	718.83	725	748	760	745	726	658	727.00
100	850	890	835	804	791	770	823.33	848	862	870	820	813	808	836.83
200	1050	1100	1076	1120	1080	1041	1077.83	1047	1060	1020	1072	1039	1007	1073.50

CD (P=0.05) Interaction - 5.192
CD (P=0.05) Mean - 2.12
CD (P=0.05) Interaction - 5.49
CD (P=0.05) Mean - 2.241

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 5.03
50% N - 5.97
100% N - 5.83
200% N - 5.38

* mg g^{-100}

4.2.8.1 Asparagine (Figure 9)

Asparagine increased with increase in nitrogen levels from 25 to 200 per cent of recommended dose. The level of asparagine in all treatments increased upto 48 h and thereafter reduced in both healthy and inoculated plants. *C. cassiicola* inoculation significantly increased asparagine in all the nitrogenous treatment throughout the sampling period.

4.2.8.2 Phenylalanine (Figure 10)

An increase in the level of nitrogenous fertilizer application to rubber seedlings from 25 to 200 per cent increased the phenylalanine content in general. With an increase in sampling time, there was an increase in phenylalanine in 25, 50 and 100 per cent treatment while a reduction was noticed in the case of 200 per cent. Inoculation with *C. cassiicola* caused a reduction in phenylanine in plants treated with 25 and 50 per cent nitrogen while in 100 and 200 per cent treatment there was an increase in phenylalanine content.

4.2.8.3 Glutamine (Figure 11)

Increased application of nitrogen augmented the level of glutamine in rubber seedlings. Glutamine content decreased with an increase in sampling period. *C. cassiicola* inoculation in general increased the content of glutamine. The increase was significant in plants fertilized with 25 per cent nitrogen . In all other treatments, the increase was not significant.

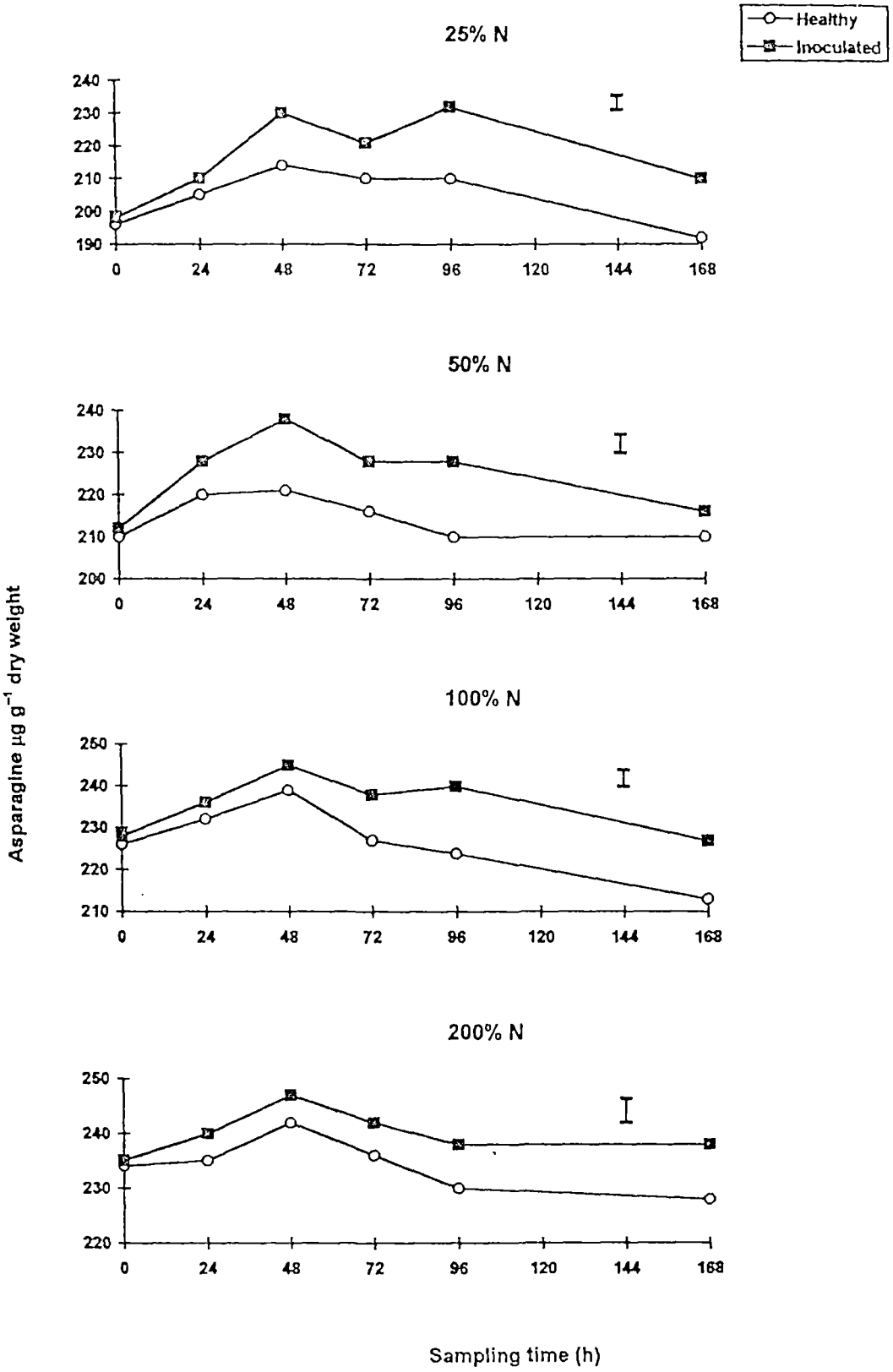


Figure 9 Changes in asparagine in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

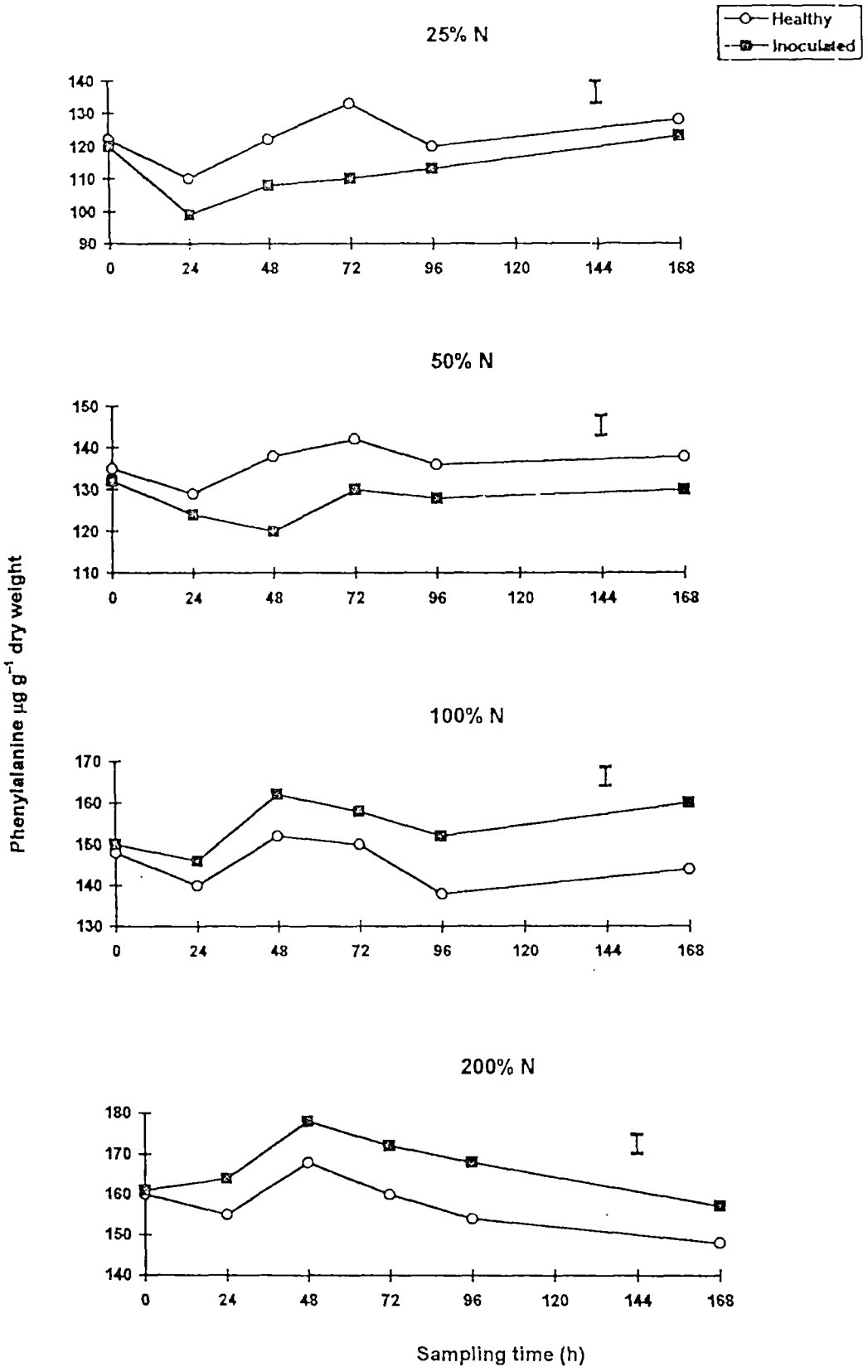


Figure 10 Changes in phenylalanine in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

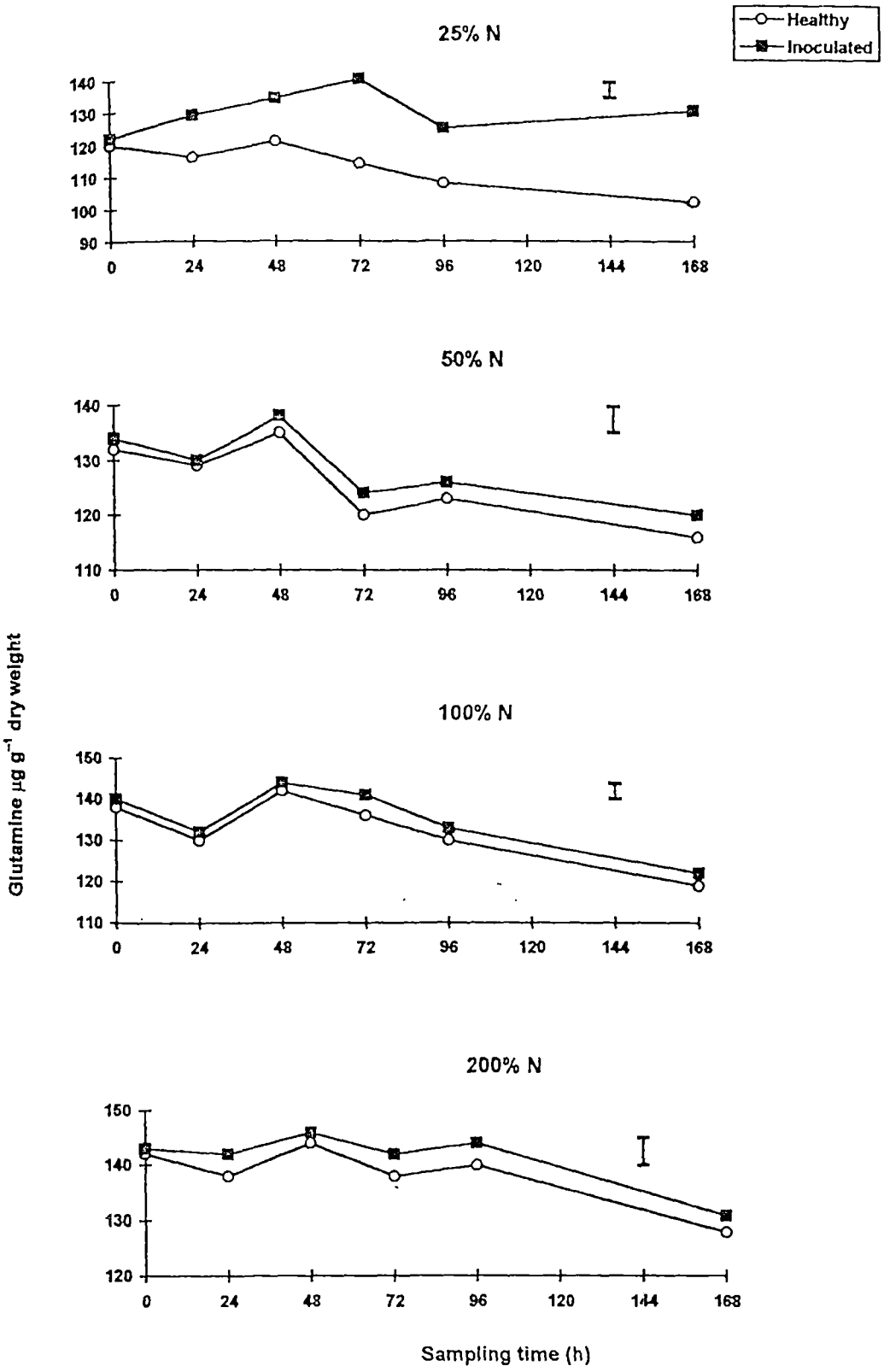


Figure 11 Changes in glutamine in the healthy and C. *cassiicola* inoculated rubber seedlings at different levels of nitrogen

4.2.8.4 **Methionine** (Figure 12)

Levels of methionine in rubber seedlings increased due to an increase in application of nitrogen. The content of methionine was decreased with increase in sampling period. In general *C. cassiicola* inoculation caused a significant reduction in the content of methionine in all the treatments.

4.2.8.5 **Tyrosine** (Figure 13)

The content of tyrosine in rubber seedlings increased with an increase in the levels of nitrogen. With the increase in sampling periods from 0 to 48 h there was an increase in tyrosine content and thereafter a declining trend up to 168 h was observed. Development of leaf spot disease caused a significant reduction in the level of tyrosine in 25 and 50 per cent nitrogen treatment up to 72 h and thereafter the reduction was not significant. At 100 and 200 per cent nitrogen level significant increase in the levels of tyrosine was registered.

4.2.8.6 **Aspartic acid** (Figure 14)

Increased application of nitrogenous fertilizer augmented the aspartic acid content in tissues of rubber seedlings. In general, the level of aspartic acid decreased with an increase in sampling period in both healthy and infected tissues except in 25 per cent nitrogen treatment. *C. cassiicola* inoculation reduced the aspartic acid level by 24 h of inoculation and thereafter it increased in plants receiving 25, 50 and 100 per cent nitrogen. However at 200 per cent nitrogen level, the reduction was maintained upto 96 h.

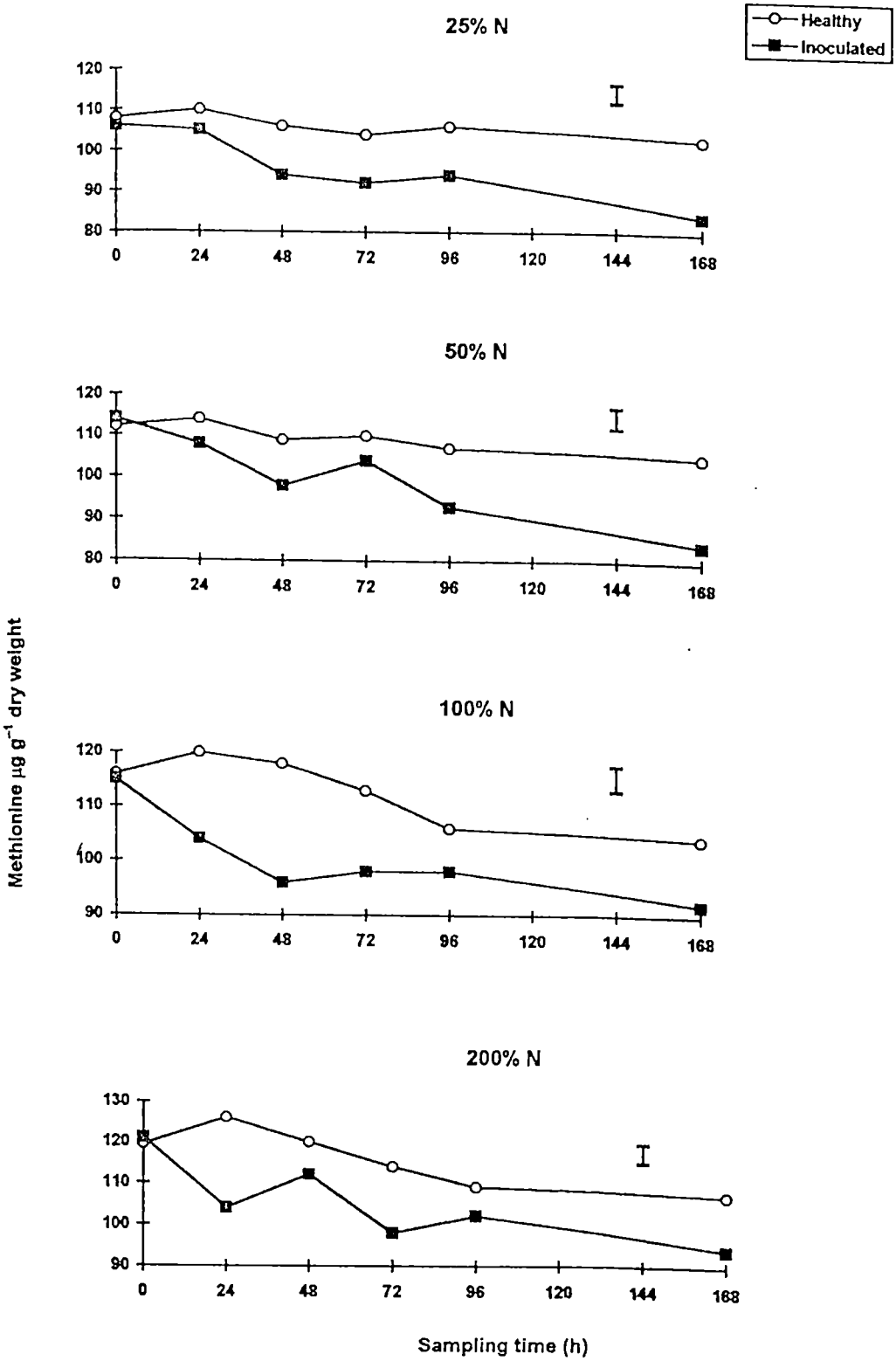


Figure 12 Changes in methionine in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

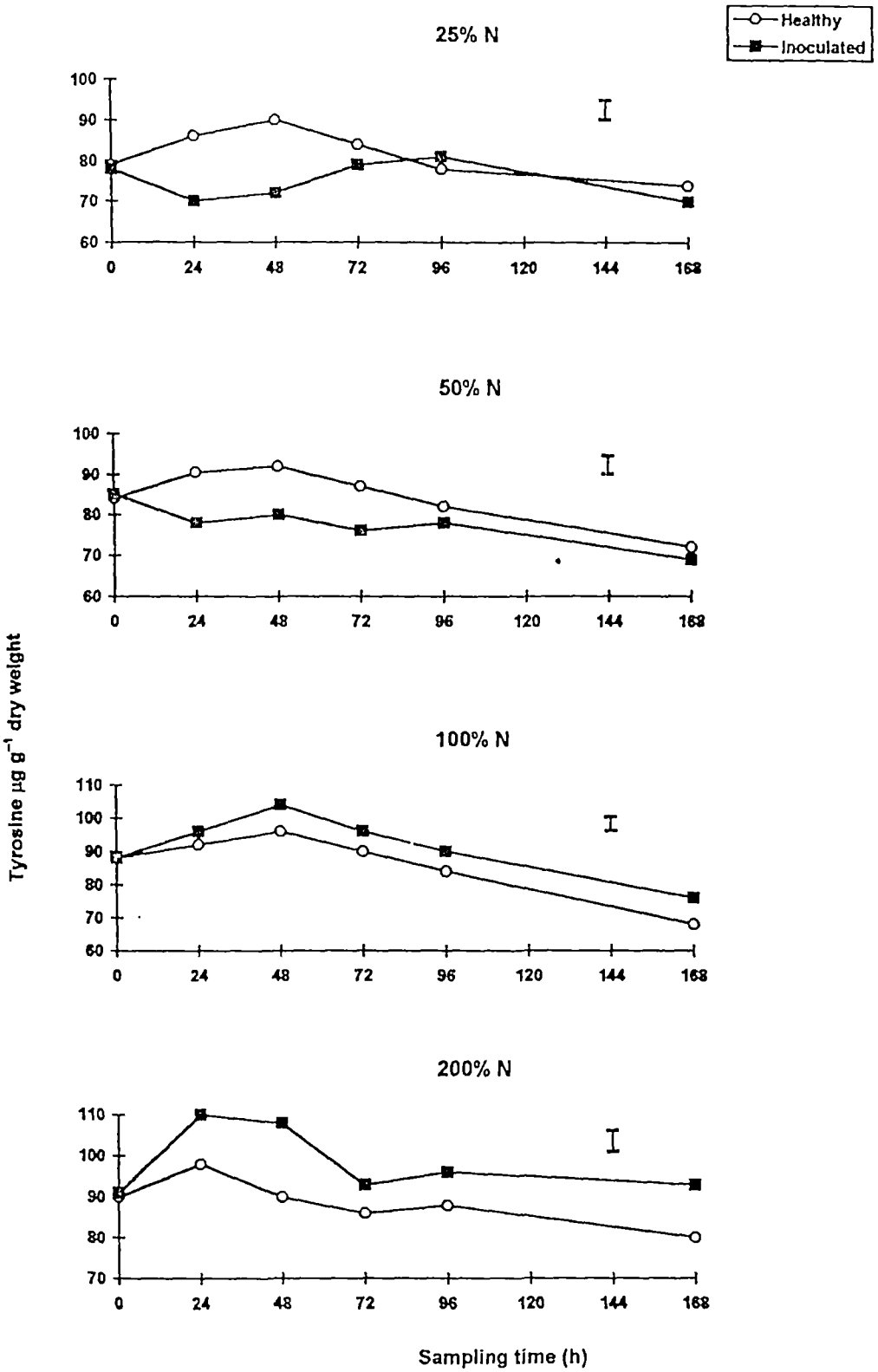


Figure 13 Changes in tyrosine in the healthy and C. *cassiicola* inoculated rubber seedlings at different levels of nitrogen

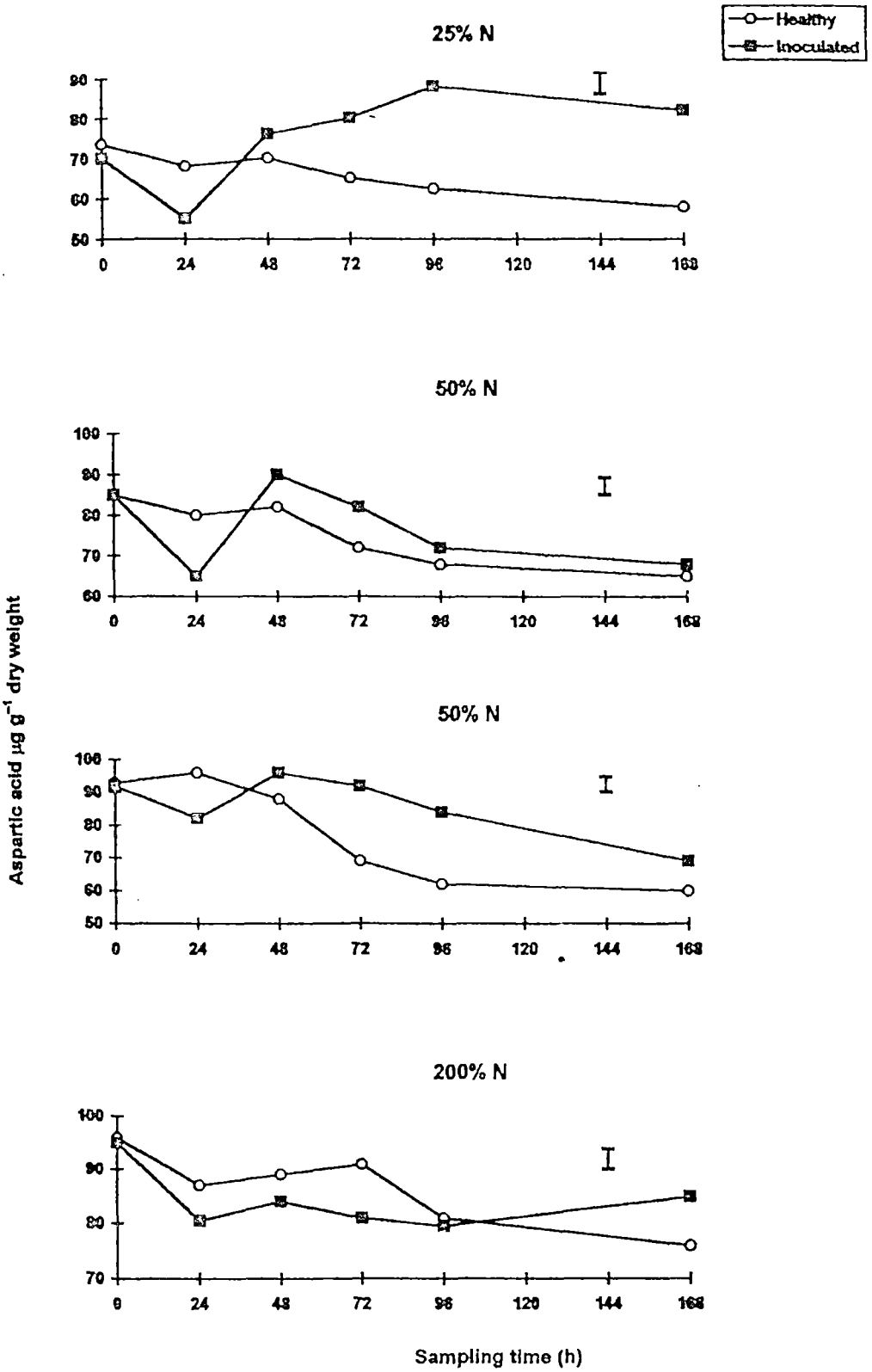


Figure 14 Changes in aspartic acid in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

4.2.8.7 **Glycine** (Figure 15)

Application of graded levels of nitrogenous fertilizer did not significnatly alter the level of glycine in rubber seedlings up to 100 per cent level. The content of glycine increased generally during the initial hours of sampling but was not significant. *C. cassiicola* inoculation increased the content of glycine in treatments receiving 25 and 50 per cent nitrogen but the increase was significant only at 72 and 96 h in 50 per cent nitrogen treated plants. At 100 and 200 per cent level glycine content decreased and this reduction was much significant when compared to the increase in the former treatments.

4.2.8.8 **Alanine** (Figure 16)

The content of alanine increased with increase in the level of nitrogenous fertilizer. In general, an increase in alanine content was observed with increase in sampling periods at all levels of nitrogen. Inoculation of *C. cassiicola* caused a decrease in the level of alanine up to 48 h and thereafter either an increase or no appreciable change was recorded.

4.2.8.9 **Leucine** (Figure 17)

Level of leucine increased significantly with increase in the level of nitrogenous fertilizer application. With increase in sampling time the level of leucine increased up to 48 h and thereafter it decreased in all the levels of nitrogen but was not significant. Inoculation decreased the content of leucine in plants received with graded levels of nitrogen up to 49 h and followed an increase. The level of decrease was more in 25 and 50 per cent of nitrogen.

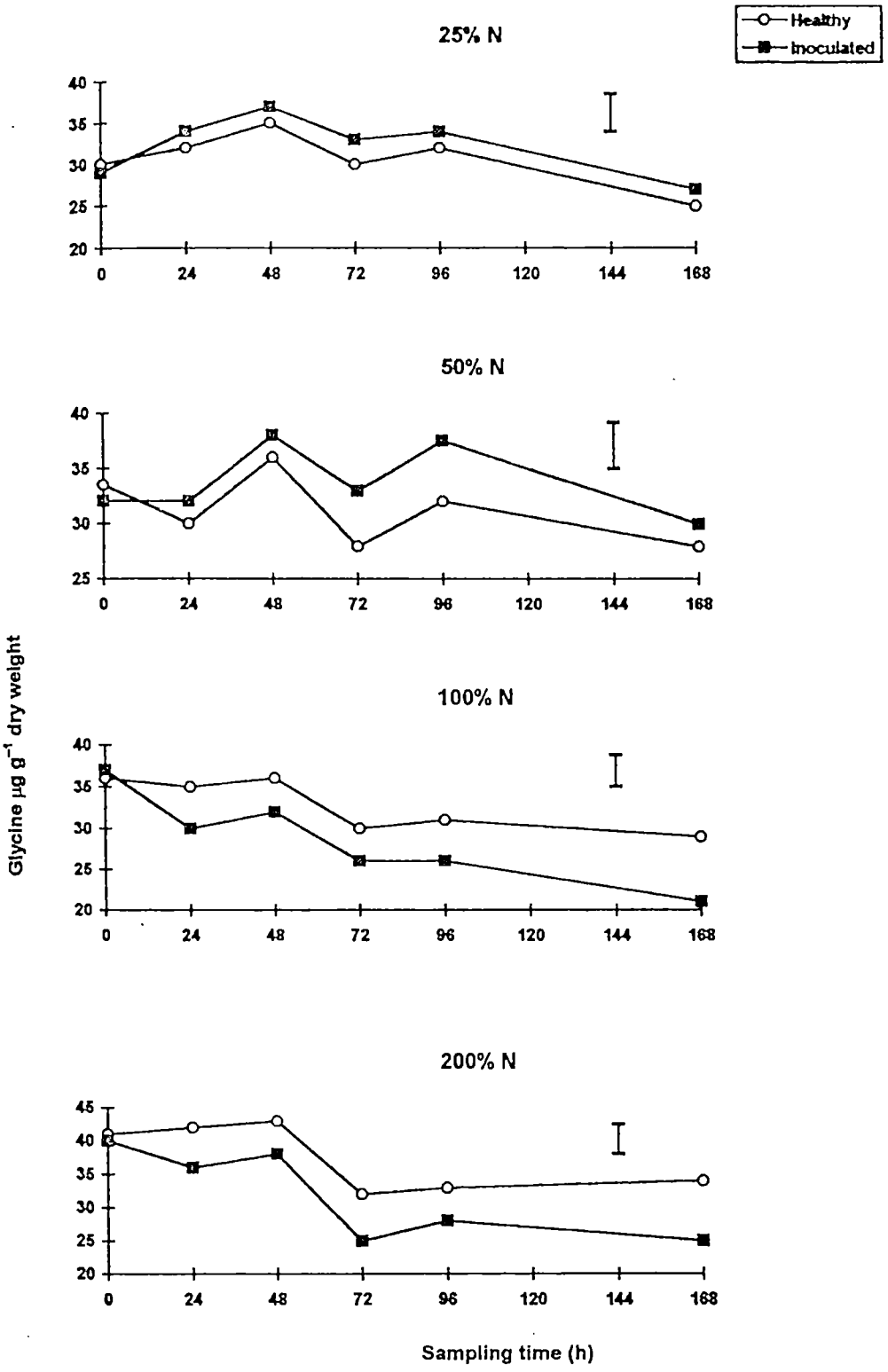


Figure 15 Changes in glycine in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

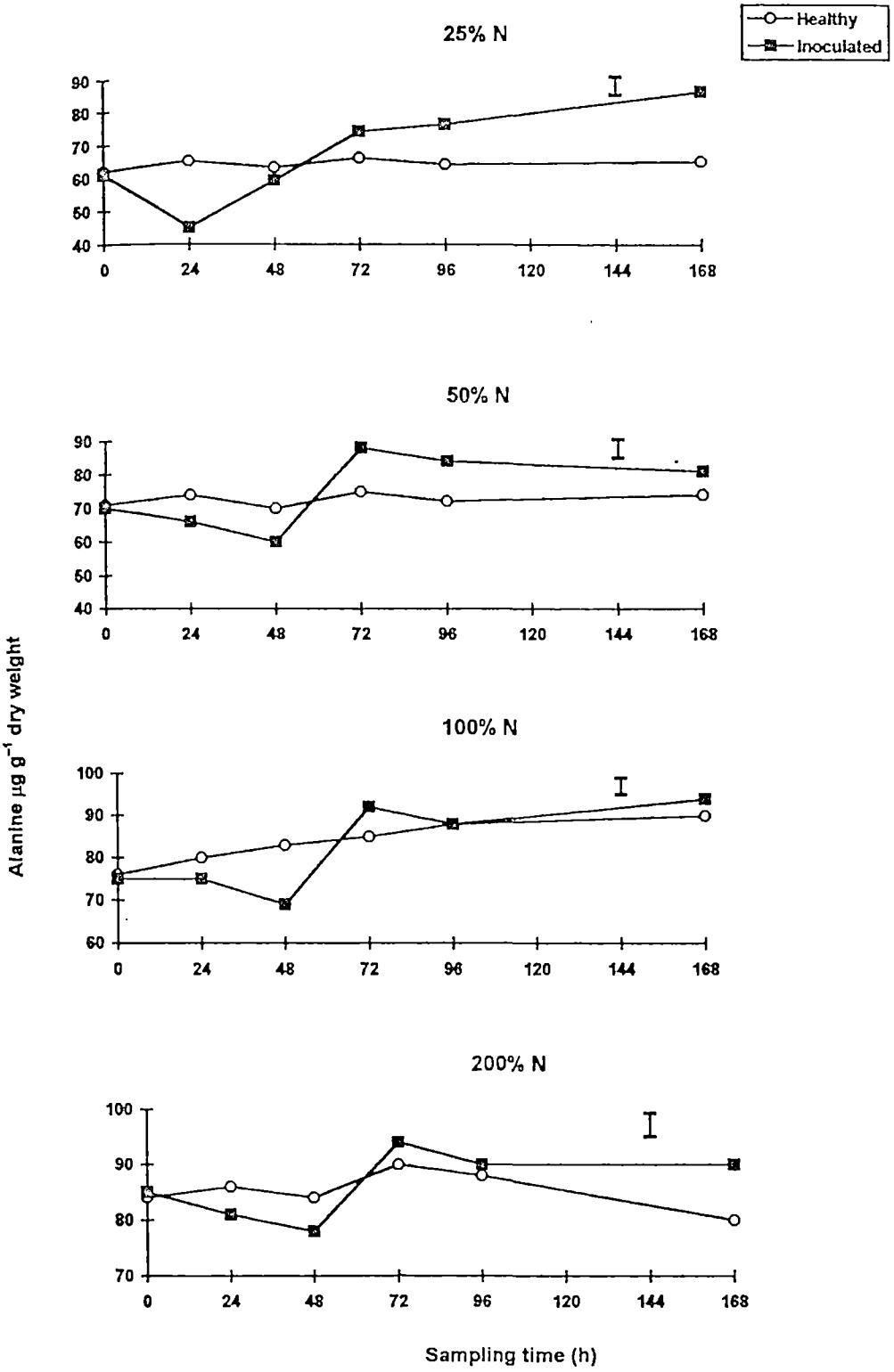


Figure 16 Changes in alanine in the healthy and C. *cassiicola* inoculated rubber seedlings at different levels of nitrogen

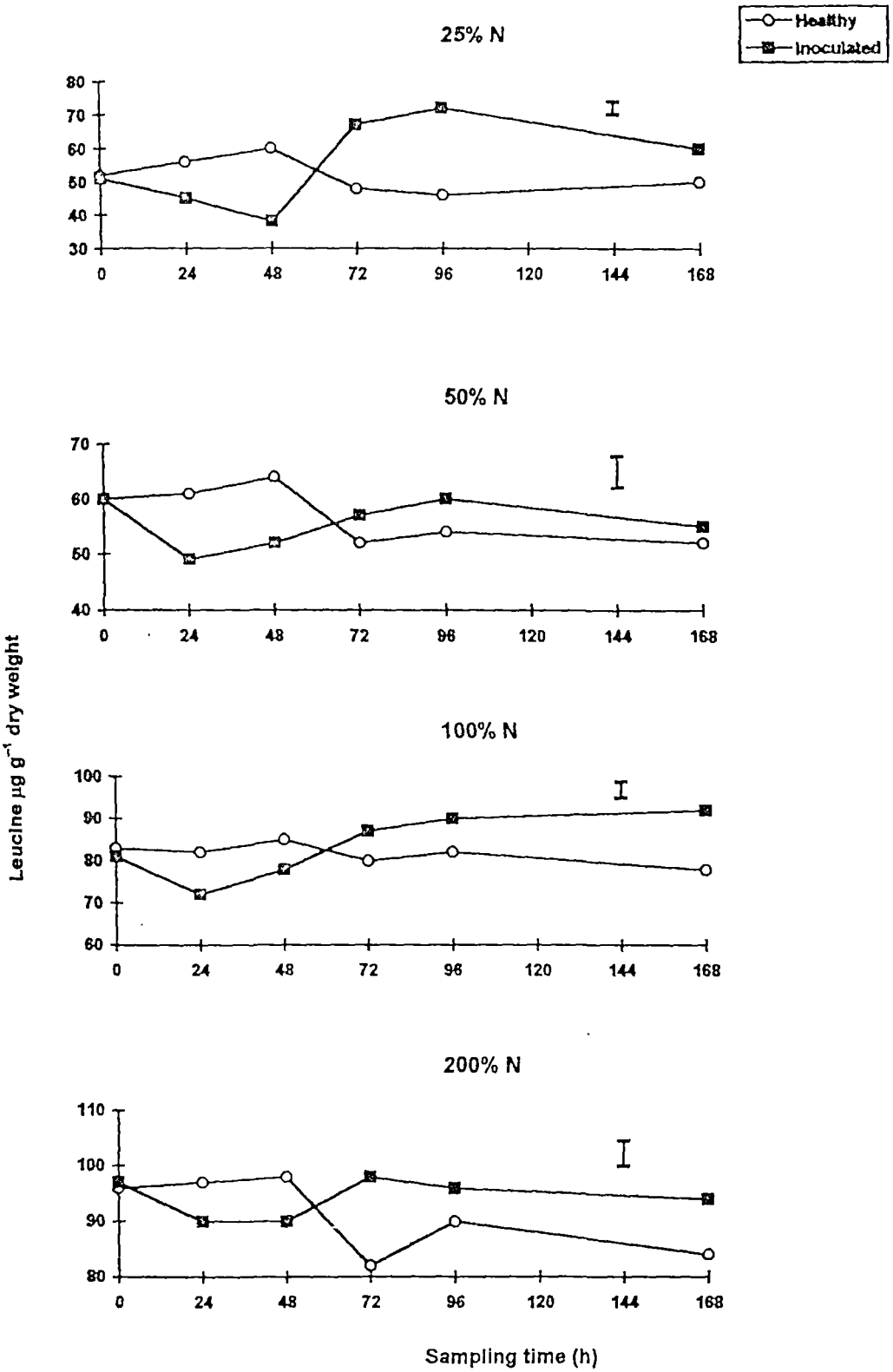


Figure 17 Changes in leucine in the healthy and C. *cassiicola* inoculated rubber seedlings at different levels of nitrogen

4.2.8.10 Cysteine (Figure 18)

Leaf extracts of rubber seedlings contained cysteine, the sulphur containing amino acid which increased with increase in nitrogenous fertilizer application. Level of this amino acid got reduced with increase in sampling period. At 25 and 50 per cent nitrogen level inoculation with *C. cassiicola* increased cysteine while at 100 and 200 per cent nitrogen it got reduced considerably.

4.2.8.11 Glutamic acid (Figure 19)

The content of glutamic acid markedly increased with increase in the application of nitrogenous fertilizer. Generally glutamic acid increased in the samplings of initial period and a reduction was recorded from 48 h of sampling period. *C. cassiicola* inoculation considerably increased the content of glutamic acid in all the fertilizer treatments throughout the sampling periods.

4.2.8.12 Arginine (Figure 20)

Application of graded levels of nitrogen significantly reduced the level of arginine. The difference in the arginine content from one sample to the next is very negligible. *C. cassiicola* inoculation increased the level of arginine in the treatments *viz.*, 25, 50 and 100 per cent nitrogen, however in the plants receiving double the recommended dose of nitrogen, the content of arginine reduced upon inoculation upto 48 h and thereafter it increased till the end of the experiment.

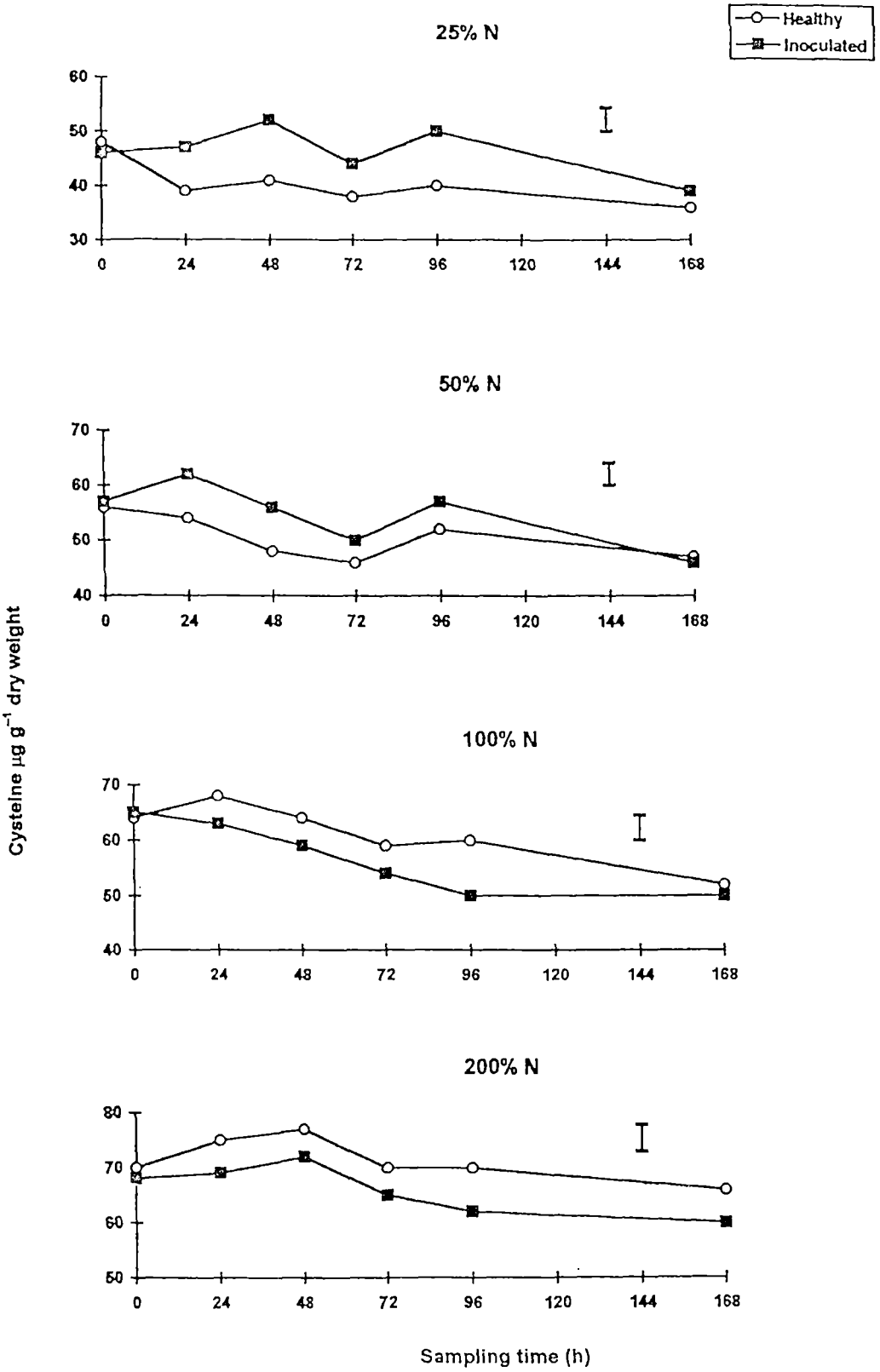


Figure 18 Changes in cysteine in the healthy and C. *cassiicola* inoculated rubber seedlings at different levels of nitrogen

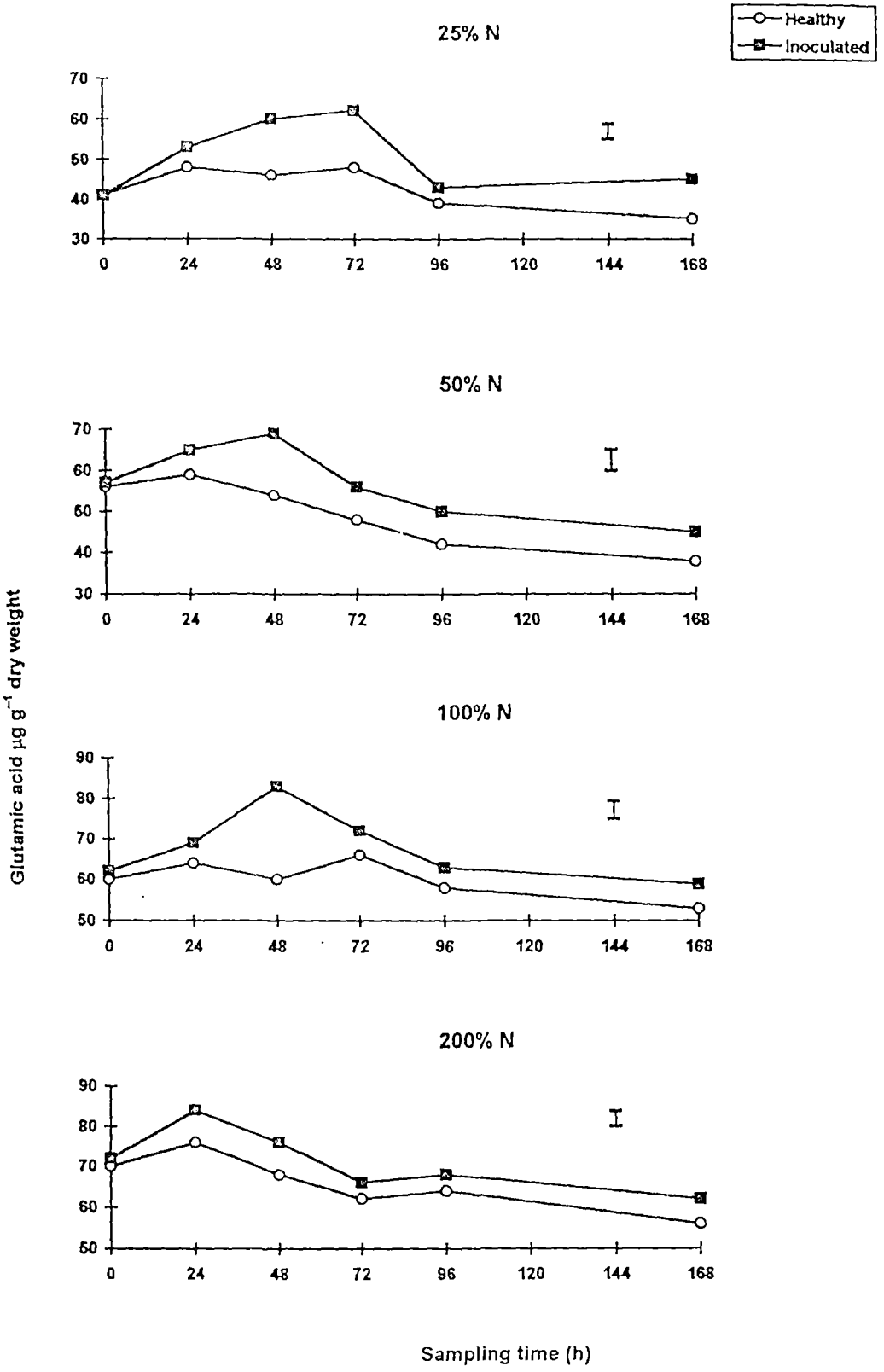


Figure 19 Changes in glutamic acid in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

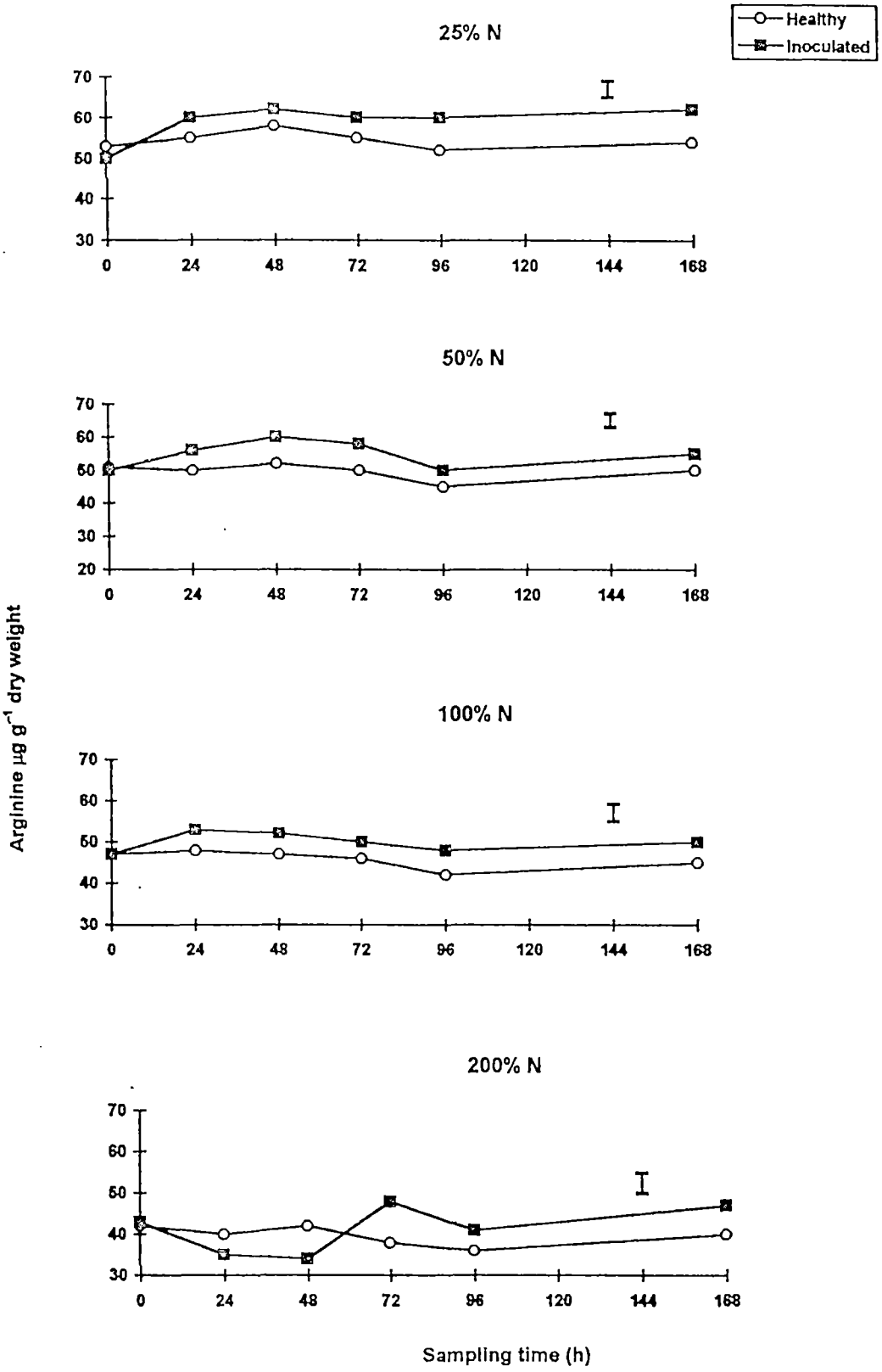


Figure 20 Changes in arginine in the healthy and C. *cassiicola* inoculated rubber seedlings at different levels of nitrogen

4.2.8.13 Histidine (Figure 21)

Increase in nitrogenous fertilizer application to rubber seedlings augmented the level of histidine. The level of histidine increased only in 24 h in all the nitrogen treatments and thereafter reduced with an increase in sampling time. Inoculation of rubber seedlings with *C. cassiicola* spores caused a reduction in the histidine content in all the levels of nitrogen up to 168 h.

4.2.8.14 Lysine (Figure 22)

Lysine content in rubber seedlings increased with an increase in the level of nitrogenous fertilizer application. Lysine content was maximum in 24 h of sampling and it gradually reduced in the subsequent sampling. Inoculation of *C. cassiicola* reduced the lysine content in the initial stages in 25 and 50 per cent nitrogen applied plants and thereafter it increased significantly. At 100 and 200 per cent of nitrogen level a gradual decrease in the lysine content was observed from 24 h onwards.

4.2.9 Changes in total nitrogen (Table 8)

Total nitrogen in rubber seedlings increased with an increase in the level of nitrogenous fertilizer application. The level of nitrogen in general decreased with an increase in sampling period except in the 24th h. Inoculation with *C. cassiicola* augmented the content of total nitrogen at 25, 50 and 100 per cent levels of nitrogen and the increase was significant during the initiation of the disease syndrome. However, at 200 per cent nitrogen, infection did not alter the total nitrogen content much.

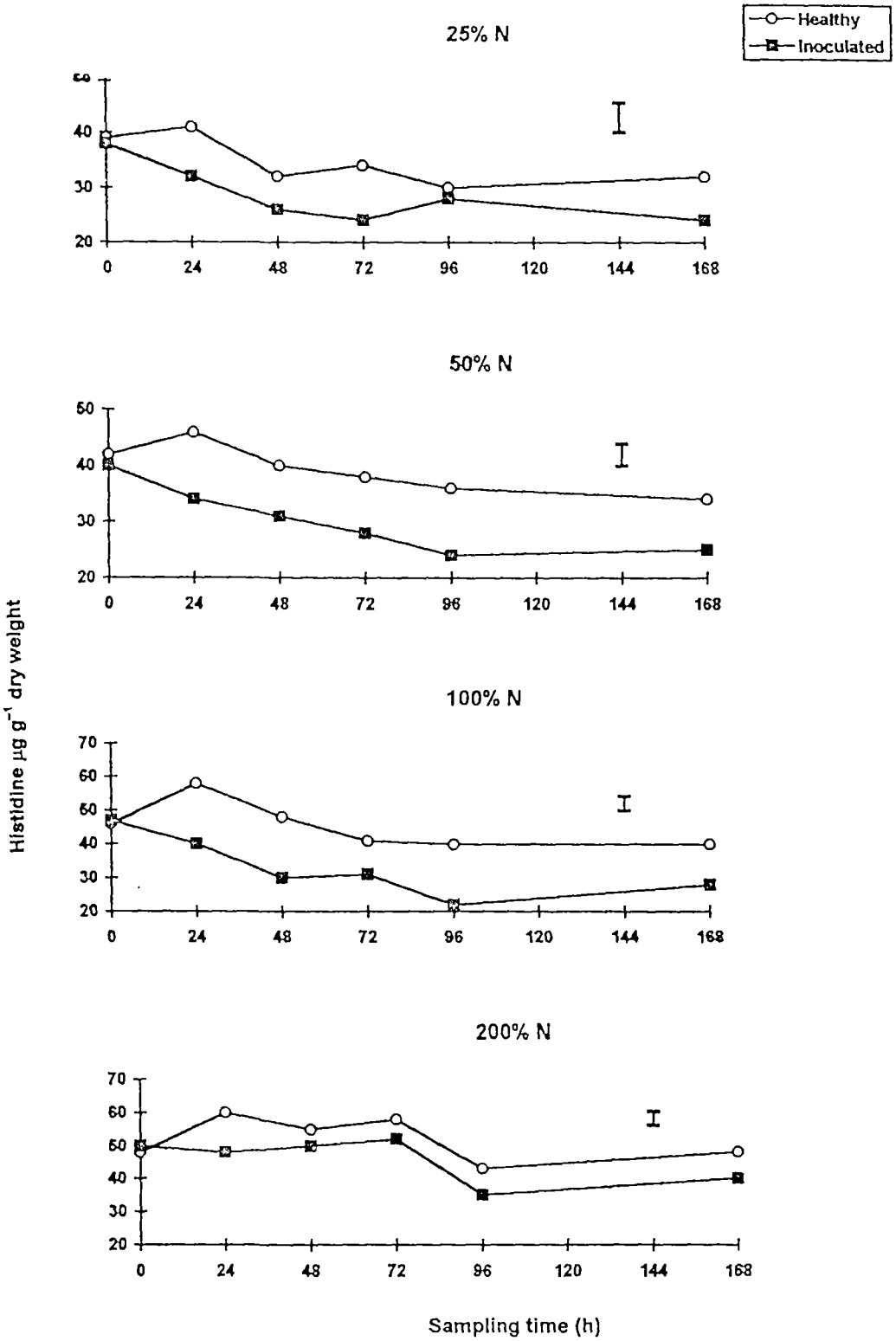


Figure 21 Changes in histidine in the healthy and C. *cassiicola* inoculated rubber seedlings at different levels of nitrogen

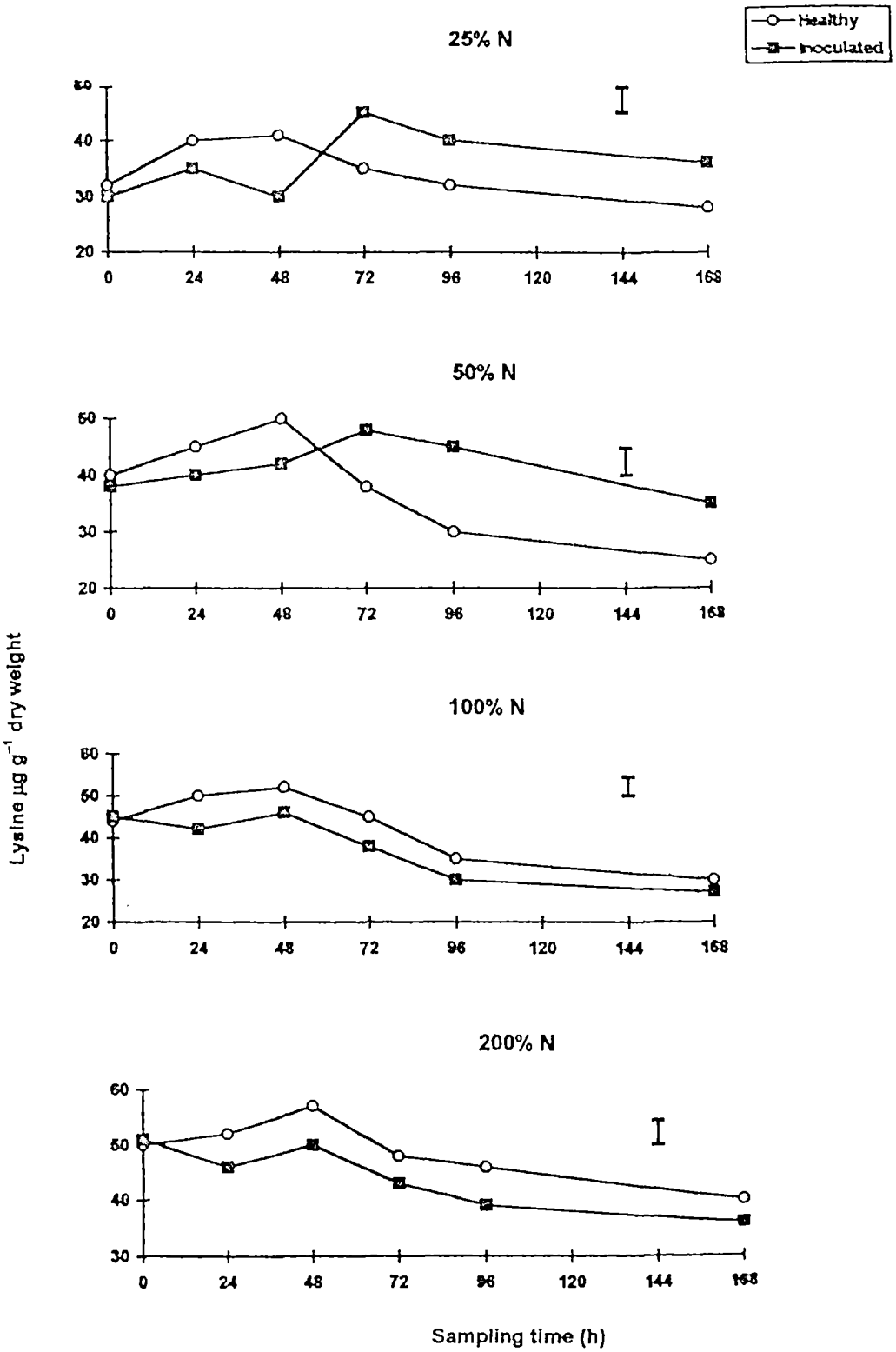


Figure 22 Changes in lysine in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

Table 8 **Changes in total nitrogen* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	28	27	24	25	22	20	24.33	29	32	36	30	30	23	29.33
50	30	31	26	24	25	22	26.25	31	40	30.5	28.5	29	24	31.08
100	35	38	30	29	26	24	30.33	36.5	42	40	34	28	22	33.75
200	38	40	34	34	30	29	34.17	39	45	46	36	28	25	36.58

CD (P=0.05) Interaction - 3.83
CD (P=0.05) Mean - 1.56

CD (P=0.05) Interaction - 4.12
CD (P=0.05) Mean - 1.68

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 3.67
50% N - 4.20
100% N - 3.81
200% N - 4.20

* mg g^{-100}

4.2.10 Changes in protein (Table 9)

Application of different levels of nitrogenous fertilizer increased the protein content in rubber seedlings. In general, protein content decreased with an increase in sampling time except in the 24th h. Inoculation caused an increase in the content of protein at all levels of nitrogenous fertilizer application.

4.2.11 Changes in ascorbic acid (Table 10)

The content of ascorbic acid increased with increase in the level of nitrogen. With an increase in sampling period, the level of ascorbic acid gradually decreased. *C. cassiicola* inoculation caused a significant reduction in the ascorbic acid content in the rubber seedlings treated with different levels of nitrogenous fertilizer. The extent of reduction of ascorbic acid was more in 25 and 50 per cent nitrogen treatments when compared to 100 and 200 per cent nitrogen treatments.

4.2.12 Changes in ascorbic acid oxidase (Table 11)

The increased levels of nitrogenous fertilizer application significantly reduced the activity of ascorbic acid oxidase. Sampling period did not alter the activity of this enzyme. *C. cassiicola* inoculation in plants treated with 25 and 50 per cent nitrogen resulted in an increased ascorbic acid oxidase activity and the increase was more in the initial period. While the activity of this enzyme upon inoculation was decreased in plants treated with 100 and 200 per cent nitrogen.

Table 9 **Changes in protein * in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	175	175	150	156	138	125	153.17	181	200	225	169	162	143	180
50	188	200	163	150	157	138	166	194	250	198	178	179	162	193.5
100	219	238	188	182	163	150	190	225	263	250	213	175	138	210.67
200	238	250	213	213	188	186	214.6	244	281	288	225	175	156	228.17

CD (P=0.05) Interaction - 4.55
CD (P=0.05) Mean - 1.86
CD (P=0.05)Interaction - 4.25
CD (P=0.05) Mean - 1.74

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 4.87
50% N - 4.53
100% N - 4.62
200% N - 4.36

* mg g^{-100}

Table 10 **Changes in ascorbic acid* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	250	265	292	288	275	265	277.5	282	198	224	246	264	225	223.17
50	301	289	295	290	310	280	294.17	298	228	206	195	210	240	229.5
100	320	312	310	296	310	290	306.33	322	296	250	255	240	280	273.83
200	332	340	326	310	308	302	319.67	330	318	314	286	240	274	293.67

CD (P=0.05) Interaction - 4.46
CD (P=0.05) Mean - 1.819
CD (P=0.05) Interaction - 4.69
CD (P=0.05) Mean - 1.914

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 4.79
50% N - 5.76
100% N - 4.62
200% N - 3.98

* mg g^{-100}

Table 11 **Changes in ascorbic acid oxidase* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time in (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	62	65	56	50	45	48	54.33	63	82	63	58	53	55	62.33
50	58	59	52	46	42	48	50.83	59	67	64	50	55	51	56.83
100	48	50	58	42	56	60	52.33	47	38	46	37	47	41	42.67
200	40	53	48	42	47	39	44.91	41	46	40	35	38	24	37.33

CD (P=0.05) Interaction - 4.48
CD (P=0.05) Mean - 1.83

CD (P=0.05) Interaction - 3.55
CD (P=0.05) Mean - 1.45

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 4.27
50% N - 3.67
100% N - 3.77
200% N - 4.89

* units/min.

4.2.13 Changes in peroxidase (Table 12)

Considerable peroxidase activity was recorded in rubber seedlings. The activity of this enzyme was accelerated by the increased levels of nitrogenous fertilizer application. The activity of this enzyme was inversely proportional to the sampling period. Inoculation of *C. cassiicola* augmented the activity of peroxidase considerably in all the levels of nitrogen application and the increase was much pronounced upto 72 h of inoculation. At 100 and 200 per cent nitrogen, the increase in the peroxidase activity upon *C. cassiicola* inoculation was comparatively less than lower levels of nitrogen.

4.2.14 Changes in polyphenol oxidase (Table 13)

Polyphenol oxidase activity in rubber seedlings decreased with increase in the level of nitrogenous fertilizer. A decrease in the enzyme activity was observed with increase in sampling periods. At 100 and 200 per cent nitrogen, the enzyme activity was significantly increased upto 48 h and thereafter the activity was not altered. *C. cassiicola* inoculation did not alter the PPO activity upto 72 h in the 25 and 50 per cent nitrogen and thereafter it was significantly reduced. At higher levels of nitrogen *viz.,* 100 and 200 per cent level, the PPO activity was increased upon inoculation.

4.2.15 Changes in phenylalanine ammonia-lyase (Table 14)

Phenylalanine ammonia-lyase (PAL) activity got reduced with an increase in nitrogenous fertilizer. The reduction was much conspicuous from 50 to 200 per cent nitrogen. This enzyme activity was found to increase with an increase in sampling period. *C. cassiicola* inoculation significantly increased the activity of PAL in plants treated with 25 and 50 per cent nitrogen. At higher nitrogen level *i.e.,* 100 and 200 per cent nitrogen, the infection caused a reduction in PAL activity.

Table 12 **Changes in peroxidase * in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	312	340	346	358	286	275	319.5	315	395	406	402	322	295	355.8
50	340	321	330	318	302	320	321.83	345	363	390	363	316	332	351.5
100	386	366	372	334	328	320	350.16	388	379	402	348	342	338	366.17
200	416	392	370	340	350	296	360.66	420	415	385	364	363	299	374.33

CD (P=0.05) Interaction - 4.65
CD (P=0.05) Mean - 1.90

CD (P=0.05) Interaction - 4.38
CD (P=0.05) Mean - 1.79

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 4.62
50% N - 4.78
100% N - 5.19
200% N - 4.45

* units/min.

Table 13 **Changes in polyphenol oxidase* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	239	242	222	212	198	189	216.87	238	240	219	214	180	182	212.6
50	228	217	211	186	173	180	199.17	230	214	209	182	168	170	195.5
100	206	214	206	180	165	176	191.16	204	221	230	236	206	181	213.17
200	192	201	198	194	160	169	185.67	195	212	219	221	185	197	204.83

CD (P=0.05) Interaction - 4.39 CD (P=0.05) Interaction - 4.89

CD (P=0.05) Mean - 1.79 CD (P=0.05) Mean - 2.0

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 4.47

50% N - 5.26

100% N - 4.79

200% N - 3.80

* units/min.

Table 14 **Changes in phenylalanine ammonia-lyase* in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	8	8.8	9.4	9.2	10.6	6.2	8.7	8.1	10.8	11.0	10.5	11.2	11.7	10.55
50	7.4	6.9	7.5	8.2	8.0	9.2	7.86	8.5	8.4	9.5	11.5	11.8	12.2	10.32
100	6.2	7.8	7.2	7.0	7.4	6.8	7.06	6.3	5.7	5.25	6.8	6.6	6.0	6.11
200	5.4	6.1	5.5	7.3	8.4	7.5	6.7	5.0	4.6	4.2	6.0	7.8	6.5	5.68

CD (P=0.05) Interaction - 0.31
CD (P=0.05) Mean - 0.13
CD (P=0.05) Interaction - 0.41
CD (P=0.05) Mean - 0.17

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 0.48
50% N - 0.43
100% N - 0.42
200% N - 0.33

* µg cinnamic acid formed/h/g/fresh weight

4.2.16 Changes in tyrosine ammonia lyase (Table 15)

Nitrogenous fertilizer application did not alter the level of tyrosine ammonia lyase activity in rubber seedlings upto 50 per cent nitrogen and further increase of nitrogen upto 200 per cent significantly decreased this enzyme activity. As the sampling period increased, there was a marginal increase in the activity of TAL. *C. cassiicola* inoculation increased the activity of TAL in plants receiving 25 and 50 per cent nitrogen. However in 100 and 200 per cent nitrogen treated plants, inoculation of *C. cassiicola* considerably reduced the activity of TAL.

4.3 Effect of nitrogen on the *in vitro* production of hydrolytic enzymes

4.3.1 Cellulases (Table 16)

C. cassiicola grew well on filter paper enriched sugar free Czapek's broth medium producing cellulase. The production of C_1 and C_x was observed in the culture filtrate. Both these fractions of cellulase (C_1 and C_x) enzymes were increased with an increased level of nitrogen in the medium upto 1500 ppm.

Table 15 **Changes in tyrosine ammonia-lyase in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen**

Nitrogen level	Healthy plants							Inoculated plants						
	Sampling time (h)						Mean	Sampling time (h)						Mean
	0	24	48	72	96	168		0	24	48	72	96	168	
25	3.6	4.2	6.0	5.4	5.6	4.9	4.95	3.7	6.5	6.9	7.1	6.8	5.4	6.07
50	4.2	4.1	4.6	5.75	6.5	4.9	5.01	4.3	4.8	5.5	5.9	6.2	4.1	5.13
100	1.9	3.1	4.2	3.6	3.3	3.6	3.28	2.1	2.5	3.3	2.7	2.5	2.2	2.55
200	2.4	2.65	2.7	2.9	3.2	3.6	2.908	2.5	2.4	1.8	2.2	2.6	2.7	2.37

CD (P=0.05) Interaction - 0.36
CD (P=0.05) Mean - 0.148
CD (P=0.05) Interaction - 0.37
CD (P=0.05) Mean - 0.15

CD (P=0.05) for interaction between healthy and inoculated plants at

25% N - 0.36
50% N - 0.41
100% N - 0.42
200% N - 0.45

* µg *p*. coumaric acid formed/h/g/fresh weight

Table 16 **Effect of nitrogen on biomass and production of cellulase (C_1 and C_x) of *C. cassiicola***

Nitrogen ppm.	Biomass (mg/50 ml)	C_1*	C_x**
0	170	8	17
250	210	16	22
500	236	20	28
750	258	24	34
1000	264	29	39
1250	280	33	42
1500	288	37	48
1750	290	34	49
2000	293	34	49
CD (P=0.05)	6.45	3.0	4.0

* Units

** Per cent loss in viscosity of carboxymethyl cellulose

4.3.2 β-glucosidase

C. cassiicola when grown on salicin enriched Czapek's medium, grew well and produced β-glucosidase, the level of which increased upto 1250 ppm and further increase in nitrogen level did not show appreciable change.

Table 17 **Effect of nitrogen on biomass and production of β-glucosidase of *C. cassiicola***

Nitrogen ppm.	Biomass mg/50 ml	β-glucosidase production*
0	86	98
250	114	236
500	138	389
750	146	585
1000	162	620
1250	180	640
1500	182	646
1750	185	648
2000	190	650
CD (P=0.05)	6.0	6.85

* Reducing sugars released (glucose equivalents)µg/ml of the reaction mixture

4.3.3 Pectinolytic enzymes

In pectin enriched Czapek's broth medium, *C. cassiicola* grew well and produced the enzymes. The growth increased with increase in nitrogen upto 1500 ppm. The pathogen produced protopectinase (PP) polygalacturonase (PG) pectin *trans*-eliminase (PTE) and polygalactunate *trans*-eliminase, and all these enzymes increased with increase in the level of nitrogen in the medium upto 750 ppm.

Table 18 **Effect of nitrogen on biomass and production of pectionlytic enzymes of *C. cassiicola***

Nitrogen ppm.	Biomass mg/50 ml	Protopecti-nase*	Polygalact-uronase**	Pectin *trans*-eliminase**	Polygalactu-ronate *trans*-eliminase**
0	110	5	46.4	18.4	14.8
250	135	10	48.2	19.2	15.9
500	148	15	54.5	20.6	17.6
750	169	20	56.2	24.2	18.8
1000	172	20	57.0	24.8	19.2
1250	192	20	57.2	25.4	20.8
1500	200	20	58.0	26.0	21.4
1750	198	25	58.2	26.0	21.6
2000	204	25	59.0	26.4	21.2
CD (P=0.05)	6.0	4.0	0.8	0.7	0.8

* Maceration expressed in units of 5
** Per cent loss in viscosity

4.3.4 Protease

C. cassiicola grew on casein enriched broth medium at different levels of nitrogen and produced protease enzyme. The growth as well as the

concentration of the enzyme in the medium increased with an increase in nitrogen level upto 1750 ppm.

Table 19 Effect of nitrogen on biomass and production of protease of *C. cassiicola*

Nitrogen ppm.	Biomass mg/50 ml	Protease production µg/ml
0	94	84
250	108	136
500	120	142
750	126	150
1000	132	161
1250	138	170
1500	145	177
1750	200	183
2000	200	194
CD (P=0.05)	5.2	5.0

4.4 *In vitro* studies on toxin production by *C. cassiicola*

4.4.1 Effect of nitrogen on toxin production in modified Czapek's medium

Effect of graded levels of nitrogen on biomass and toxin production by C. *cassiicola* was tested in liquid culture. The biomass production increased with increase in nitrogen upto 1750 ppm and further increase in nitrogen did not affect the growth of the pathogen. The toxin production as exhibited by the inhibition of *B. subtilis* also increased with increase in nitrogen level in the

medium. The increase was much appreciable upto 750 ppm of nitrogen addition.

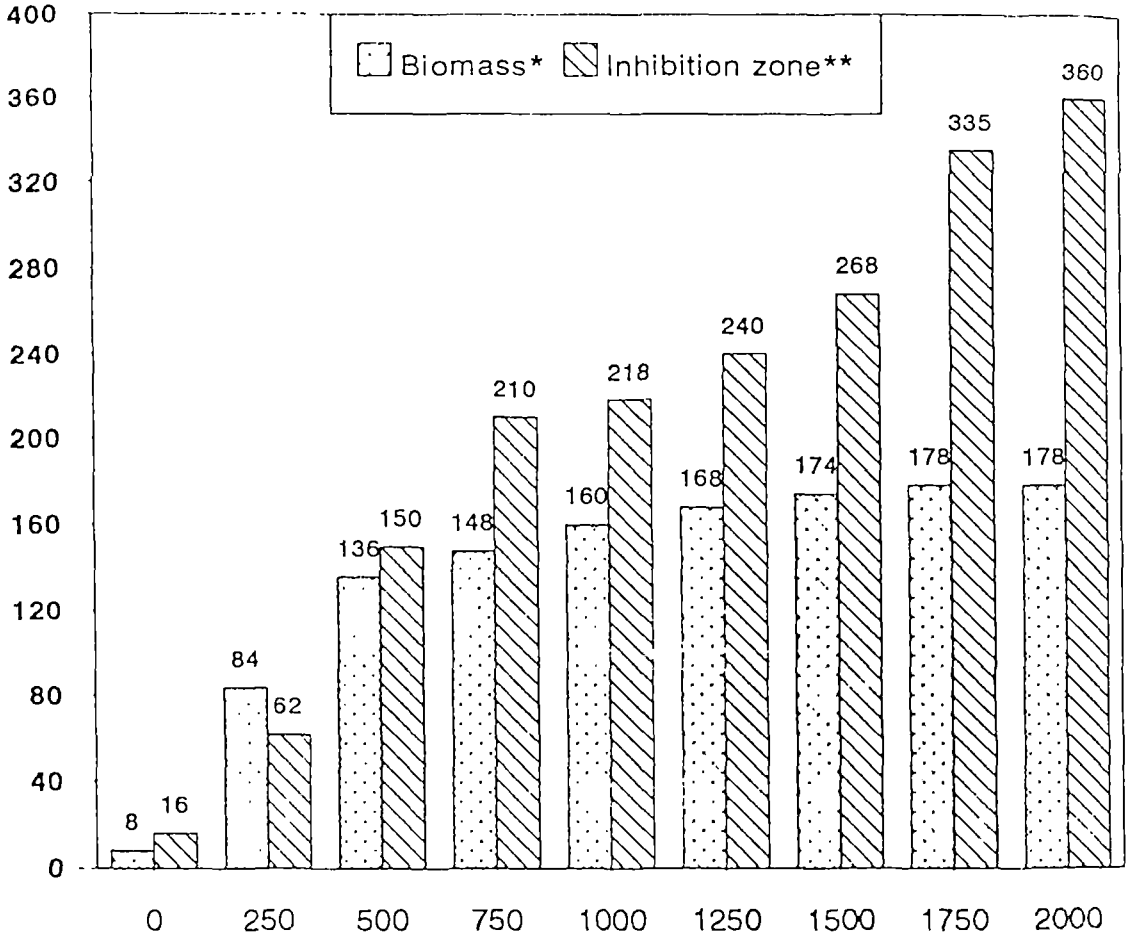


Figure 23 Effect of nitrogen on toxin production by *C. cassiicola* in modified Czapek's medium

* mg/50 ml
**mm^2

4.4.2 Toxin production by *C. cassiicola* in leaf extracts of rubber seedlings grown at different levels of nitrogen

C. cassiicola grew well in the leaf extracts of rubber seedlings applied with graded levels of nitrogen and the growth increased with increase in nitrogen. Application of increased levels of nitrogen to rubber seedlings increased the toxin production by the fungus in the leaf extracts of respective treatments. Extracts from uninoculated plants fertilized with different levels of nitrogen did not inhibit the growth of *B. subtilis*.

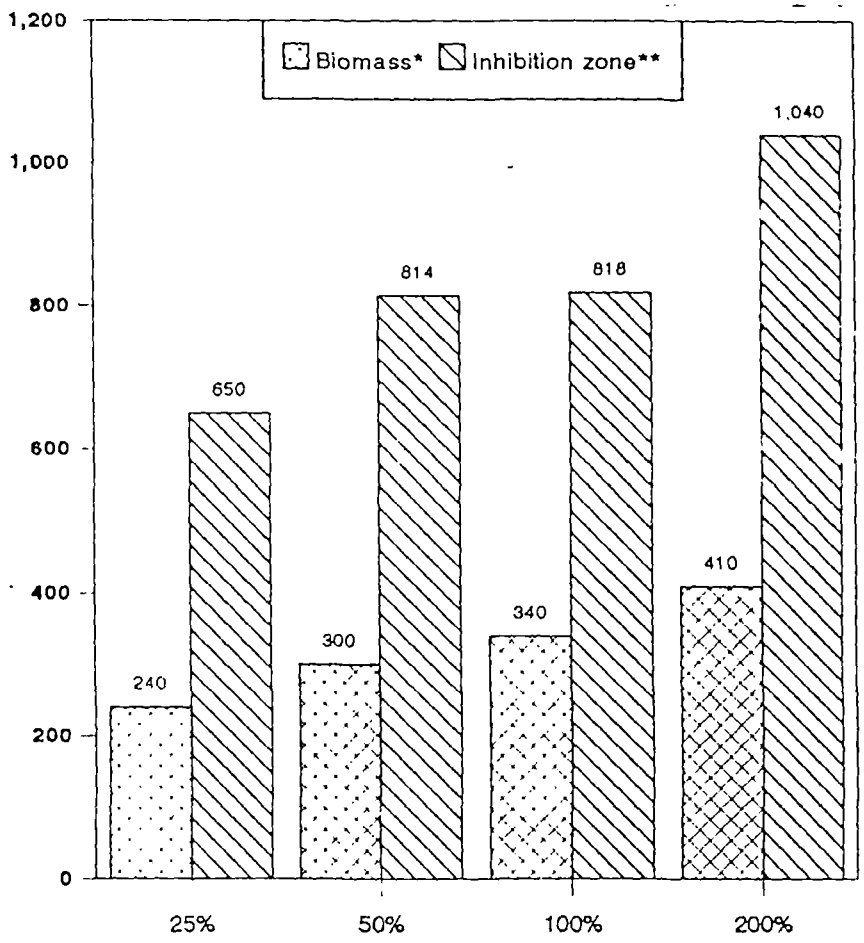


Figure 24 Effect of different levels of nitrogen application to rubber seedlings on toxin production in the leaf extracts by *C. cassiicola*

* mg/50 ml
**mm^2

4.4.3 Effect of phenolic acids on the action of toxins of *C. cassiicola* (Table 20)

Incorporation of phenolic acids at 0.002, 0.004, 0.008 and 0.01 M concentration into the culture extracts of *C. cassiicola* reduced the activity of toxins contained in them. Increase in the concentration of phenolic acids reduced the action of toxins. At 0.01 M level chlorogenic acid, ferulic acid and *p.* coumaric acid completely neutralised the activity of toxins which was indicated by the full growth of *B. subtilis*. Protocatechuic acid, *p.* hydroxy benzoic acid and vanillic acid inactivated the toxin(s) of *C. cassiicola* in the extracts of culture filtrate to the extent of 93.05, 91.1 and 82.78 per cent respectively at the maximum concentration tested (0.01M). On the other hand, caffeic acid and cinnamic acid inactivated the toxin(s) only up to 69.44 and 50 per cent respectively.

4.5 Changes in amino nitrogen, total sugars and total phenols in the leaf leachates of healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen (Figure 25)

The leaf leachates of rubber plants contained amino nitrogen, total sugars and total phenols in detectable quantities. An increase in the level of nitrogenous fertilizer from 25 per cent to 200 per cent of the recommended dose, increased the content of amino nitrogen and total sugars. In the leaf leachates of inoculated plants, the amino nitrogen content was more than that of healthy plants whereas the total sugar content was less in all the levels of nitrogen.

The content of total phenols in the leachates of rubber plants grown under different levels of nitrogen decreased with increase in nitrogen. Leaf leachates of plants grown at 25 and 50 per cent nitrogen upon inoculation with *C. cassiicola* recorded an increased phenol level. On the contrary leachates of inoculated plant grown at 100 and 200 per cent nitrogen level contained less total phenols.

Table 20 **Effect of phenolic acids on the inactivation of toxin(s) of *C. cassiicola***

Phenolic acid	Concentration (M)	Inhibition (mm^2)	Percentage of reduction in the inhibition zone over the control
Control	—	(360)	—
Chlorogenic acid	.002	45	87.50
	.004	21	94.16
	.008	15	95.83
	.010	0	—
Ferulic acid	.002	120	66.66
	.004	74	79.44
	.008	20	94.44
	.010	0	—
p. hydroxybenzoic acid	.002	160	55.56
	.004	84	76.67
	.008	58	83.88
	.010	32	91.10
Protocatechuic acid	.002	180	50
	.004	120	66.67
	.008	42	88.33
	.010	25	93.05
Vanillic acid	.002	210	41.67
	.004	160	55.56
	.008	120	66.67
	.010	62	82.78
p. coumaric acid	.002	92	74.44
	.004	40	88.89
	.008	10	97.22
	.010	0	—
Caffeic acid	.002	260	27.78
	.004	205	43.05
	.008	160	55.56
	.010	110	69.44
Cinnamic acid	.002	310	13.88
	.004	240	33.33
	.008	210	41.66
	.010	180	50.00

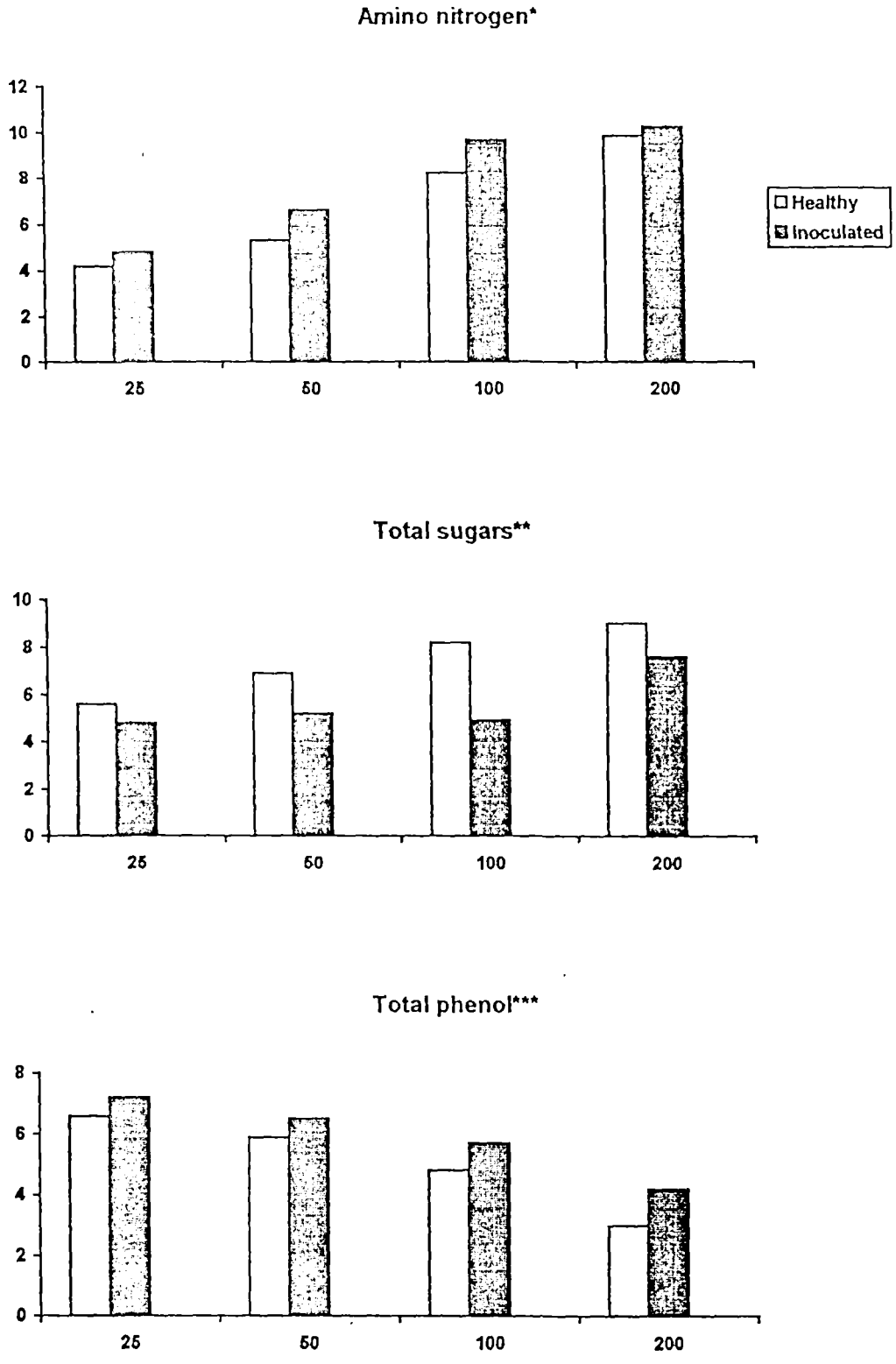


Figure 25 Changes in amino nitrogen, total sugars and total phenols in the leaf leachates of healthy and *C. cassiicola* inoculated rubber seedlings at different levels (% of recommended dose) of nitrogen

* $\mu g/cm^2$ in glutamic acid equivalents

4.6 Changes in the population of phyllosphere microorganisms in the healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

Rubber seedlings grown under pot culture conditions in the glass house harboured bacteria, fungi and yeasts.

4.6.1 Bacteria

With the increase in the level of nitrogenous fertilizer application, the population of bacteria increased in rubber seedlings. *C. cassiicola* inoculation significantly increased the population of phyllosphere bacteria at all levels of nitrogen.

4.6.2 Filamentous fungi

The population of filamentous fungi reduced due to application of an increased level of nitrogen. The difference in fungal population in 50 and 100 per cent of nitrogen level was very negligible. Inoculation with *C. cassiicola* led to significant increase in the level of fungal population compared to healthy ones.

4.6.3 Yeasts

The population of yeasts increased up to 50 per cent nitrogen application and further increase in nitrogen resulted in the reduction of yeasts. *C. cassiicola* inoculation significantly increased the population of yeasts in all the levels of nitrogen.

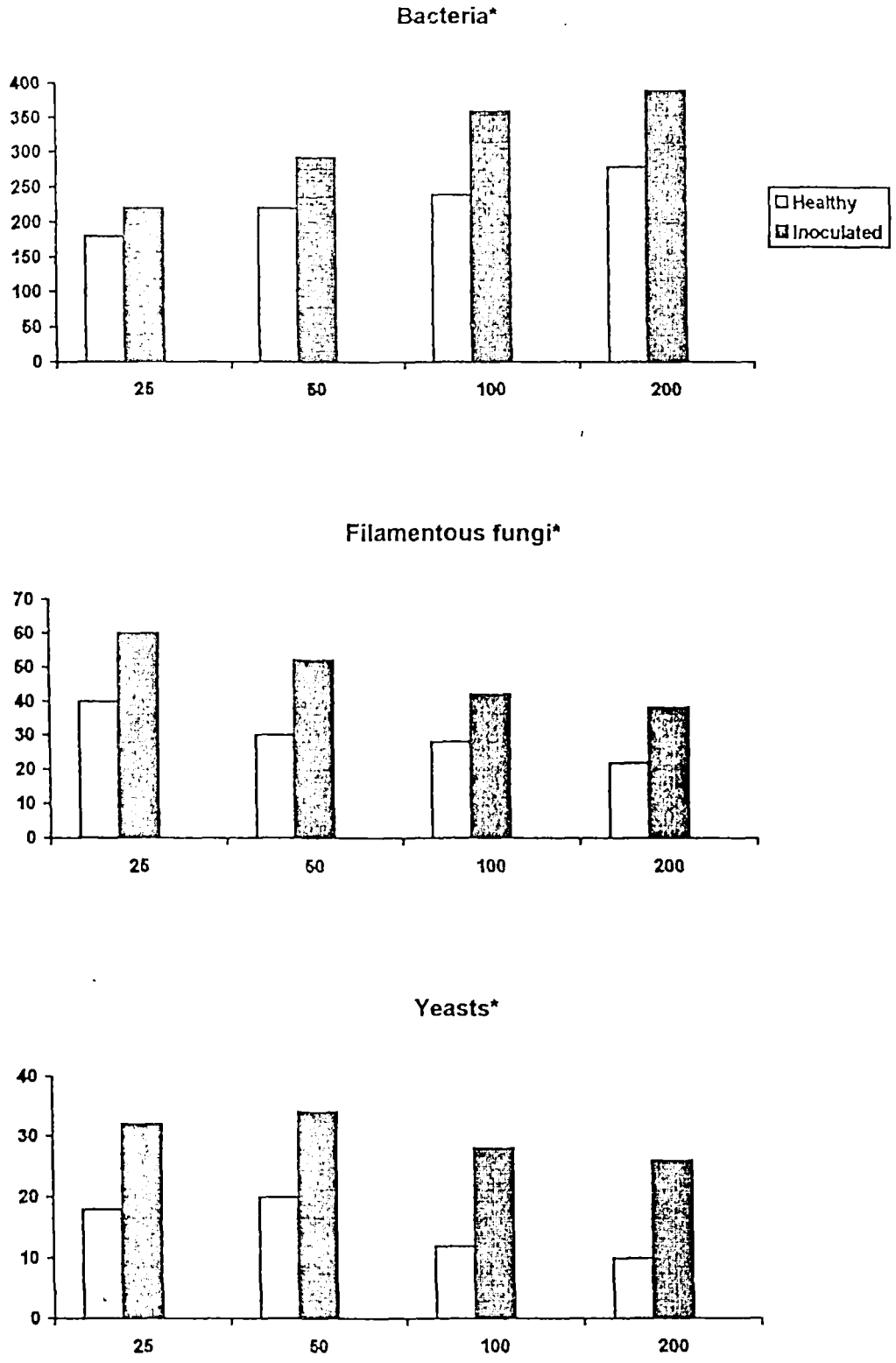


Figure 26 Changes in the population of phyllosphere microorganisms in healthy and *C. cassiicola* inoculated rubber seedlings at different levels (% of recommended dose) of nitrogen

* No./cm^2

4.7 Percentage of phyllosphere bacteria and yeasts at different levels of nitrogen inducing germination and appressoria formation in *C. cassiicola*

The studies on the effect of bacteria and yeasts from leaves of rubber seedlings applied with different levels of nitrogen on the germination and appressoria formation revealed that they had no effect on spore germination. On the other hand they induced the appressoria formation in *C. cassiicola*. The bacteria and yeasts inducing appressoria formation increased with an increase in the level of nitrogen. The number of bacteria inducing appressoria formation was more than yeasts.

Table 21 **Percentage of bacteria and yeasts inducing appressoria formation in *Hevea* leaves fertilized with different levels of nitrogen**

Nitrogen level (Per cent of recommended dose)	Bacteria	Yeasts
25	38	8
50	44	10
100	48	22
200	54	32

Chapter 5

DISCUSSION

Corynespora leaf spot disease was first reported in India in 1958 on a mild scale in seedlings nurseries. Thereafter, the disease has attained a significant position due to its devastating nature. In the recent past, the pathogen started severely attacking many high yielding clones in mature plantations. The appearance of this disease on RRII 105 is of much significance as majority of the area is planted with this clone.

The recommended nitrogenous fertilizer for rubber seedling nurseries is 500 kg N per ha (Abdul Kalam *et al.*, 1975). In order to get maximum buddable plants for green budding as well as brown budding, rubber growers tend to apply higher dose of nitrogenous fertilizers indiscriminately for rapid growth of plants. Mineral nutrition greatly influences the physiology of plants and consequently the resistance towards disease (Gaumann, 1950; Vidhyasekaran, 1988). In the present study excessive application of nitrogenous fertilizer led to severe incidence of *Corynespora* leaf spot disease in rubber seedlings. It is, however, imperative on our part to use the optimum level of nitrogenous fertilizers for the successive cultivation of rubber plants.

Effect of graded levels of nitrogen on the incidence of *Corynespora* leaf spot disease

The well known reaction type employed by plant pathologist is in a sense the integrated expression of various symptoms on the host due to pathogenic invasion. The symptom expression in rubber seedlings applied with different levels of nitrogen and C. *cassiicola* inoculation varied widely. In plants receiving recommended levels of nitrogenous fertilizer and above, typical leaf spots of C. *cassiicola* developed. At low levels of nitrogen *i.e.,* 25 and 50 per cent of the recommended dose, the spots were mostly of pin head size. Appearance of pin head spots due to pathogenic invasion is the characteristic symptom of disease resistance (Mahadevan, 1991). Rajalakshmy *et al.* (1979) also reported a higher *Corynespora* leaf spot disease incidence in rubber seedlings in the presence of higher nitrogenous fertilizer and less incidence in its absence. Predisposing effect of nitrogenous fertilizer was observed in black gram (*Vigna mungo*) to C. *cassiicola* (Nagalakshmy and Subha Rao, 1995). Since indiscriminative application of nitrogenous fertilizers favoured the disease the prudent approach of disease management is application of optimum level of this fertilizer.

A number of theses has been postulated for the disease susceptibility upon application of higher dose of nitrogenous fertilizers. In rice, Prasad and Regunathan (1972) showed that the blast disease incidence was proportional to nitrogen fertilization. They also stated that abundant nitrogen supply might increase both soluble amino acid content and sugars which creates better nutritional conditions to the pathogen resulting in increased susceptibility to infection. The other reasons that can be attributed for high susceptibility due to heavy nitrogen fertilization are luxuriant and succulent

growth and thin cuticle development with consequent reduction in mechanical resistance to pathogenic invasion (Ou, 1972; Mahadevan, 1991). The results in the present study clearly indicated that rubber seedlings succumb to *Corynespora* leaf spot disease by the application of increased levels of nitrogen. Rapid browning around lesions suggested the hypersensitivity of the infected tissue and is characteristic of resistant reactions (Ohata *et al.*, 1963). Hypersensitivity reaction is a defence mechanism operative in the incompatible host parasite relationship and by such reaction the host plants resist the great majority of invading pathogens (Muller, 1959; Vidhyasekaran, 1988). Prolonged symbiosis in susceptible (compatible or congenial) tissues and rapid hypersensitive death of resistant (incompatible or incongenial) host cells are characteristic of many other host parasite interactions (Mahadevan, 1991).

Changes in Phenolic compounds

Higher plants contain a vast array of phenolic compounds like anthocyanins, leucoanthocyanins, anthoxanthins, hydroxybenzoic acids, flavonoids, glycosides, sugar esters of hydroxybenzoic acids and coumarin derivatives (Kuc, 1963; Goodman *et al.*, 1967; Vidhyasekaran, 1988). Phenolic compounds in the plant tissues play an important role in resistance against plant pathogens (Rubin and Artsikhovskaya, 1963; Vidhyasekaran, 1988).

Phenols, as well as their oxidation products quinones, mediated by polyphenol oxidase and peroxidase (Thomson, 1964) are highly inhibitory to microorganisms (Farkas and Kiraly, 1962; Kuc, 1963; Vidhyasekaran, 1997). The sulfhydryl group of many of the enzymes is inhibited by quinones which may lead to the blockage of metabolic processes of host or pathogen

(Vidhyasekaran, 1997). Subsequent oxidation of quinones leads to the formation of melanoid pigments. Lesion caused by *C. cassiicola* are brown in colour and presumably contain melanoid pigments.

Results of the present investigation revealed that increased application of nitrogen altered the phenolic content in rubber seedling s. The phenolic content was high in plants receiving less dose of nitrogen and it decreased with increase in the level of nitrogen. Kiraly (1964) reported that wheat (*Triticum vulgare*) when heavily manured become highly susceptible to stem rust (*Puccinia graminis* Pers. var *tritici* Eriks and Henn). He attributed it to the reduced phenolic content of the heavily manured plants. The plant tissues which are rich in amino nitrogen became more susceptible to infection. Increased levels of nitrogen application increases the amino nitrogen content of the plants. The excess of amino nitrogen might reduce the toxicity of phenols and render the plants more susceptible (Ohata *et al.*, 1966; Sridhar and Mahadevan, 1979). It may, therefore be said that the decreased phenol level coupled with enhanced amino nitrogen content, due to large doses of nitrogenous fertilizer may lead to the break down of toxicity of phenols which is ultimately expressed in cellular susceptibility.

In plants at low nitrogen level, the amino acids available might be insufficient for the inactivation of phenolics and hence disease resistant reaction to pathogenic invasion. The other possible reasons for the accumulation of phenols may be the release of bound phenolics due to enhanced β-glucosidase (Goodman *et al.*, 1967). The present study revealed that sugars are less in plants receiving lower levels of nitrogen. Since sugars are the building blocks of phenolics, some of the sugars might have been

utilized in the synthesis of phenols which is more in plants applied with lower levels of nitrogen (Muralidhar, 1982). The enhanced activity of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) in the plants receiving low levels of nitrogen might have also contributed for the augmented phenolic reserve (Glazner, 1982).

As observed in rice plants, (Mohanty *et al.*, 1982). *C. cassiicola* inoculation significantly altered the phenolic content of rubber seedlings grown under different levels of nitrogenous fertilizer. In plants receiving higher levels of nitrogen, the phenolic content was significantly decreased throughout the experiment. Accumulation of phenols has taken place due to infection by *C. cassiicola* in plants applied with lower levels of nitrogen. Accumulation of phenolics was much pronounced during the initial stage in plants receiving 25 and 50 per cent recommended dose of nitrogen. Earlier studies with rice infected by *P. oryzae* suggested a more or less pronounced accumulation of phenols in the resistant and decreased concentrations in the susceptible varieties (Prasad and Regunathan, 1972). In many host pathogen interactions, it is not the pre-formed phenolics but the phenolics synthesized after infections which are related to disease resistance. The speed with which phenolics are synthesized also determines the disease resistance (Matta *et al.*, 1969) which confirmed the finding of the present study. In the present study, only small pin point lesions were observed in plants applied with lower levels of nitrogen. Browning of cells in response to pathogenic invasion was reported to be the symptom of resistant reaction in plants (Sakamoto, 1950). Suzuki *et al.* (1953) found that such infected tissues contain greater amounts of chlorogenic acid when browning reaction sets in the infected cells. Oku (1965) reported that the resistance of rice to

Cochliobolus miyabeanus infection was due to the oxidation of phenols by the polyphenol oxidase (PPO) of the pathogen. Both resistant and susceptible *Hevea* plants were reported to have PPO. In the present study also, PPO activity in rubber seedlings is observed and the change due to inoculation differ at lower as well as higher levels of nitrogenous fertilizer application. At 25 and 50 per cent nitrogen, *C. cassiicola* inoculation did not alter the PPO activity up to 72 h and thereafter significant decrease was observed. At higher level of nitrogen the PPO activity increased upon inoculation. PPO activity seemed to be suppressed after inoculation in lower levels of nitrogen.

While studying the resistance of *Hevea* leaves to *Microcyclus ulei*, Hashim *et al.* (1980) suggested PPO activities might have been suppressed after inoculation with the pathogen. Melouk and Horner (1972) also detected lower PPO activities in diseased peppermint infected with *Phoma strassera*. They attributed the lowering of PPO activity to continuous contact between the enzyme and the oxidised substrate which inhibited the enzyme activity.

Peroxidase (PO) is another enzyme responsible for the oxidation of phenols (Prasad and Regunathan, 1972). In the present study, PO increased irrespective of nitrogen level in all the inoculated plants. However, the increase was significant during the initial stages in plants receiving lower levels of nitrogen. Increased PO activity increases oxidation of phenols and as the oxidation products of polyphenols are toxic, the spread of the pathogens were kept at check. The pin point brown lesions of plants under 25 and 50 per cent nitrogen are suggestive of such a resistant reaction. The reduced activity of PPO in plants receiving 25 and 50 per cent nitrogen could

be due to increased oxidation of phenols by PO which in turn inhibited the PPO activity as suggested by Melouk and Horner (1972).

While studying the disease resistance, mere estimation of total phenols may not reflect the change or alterations of individual phenols. Hence estimation of individual phenols only can provide a direct evidence of disease susceptibility by the application of nitrogen in plants before and after infection. In the present study, healthy rubber seedling contained 14 spots in the chromatogram which gave a positive reaction to various reagents of phenols. Eight phenolic acids *viz.*, *p.* hydroxybenzoic acid, ferulic acid, *p.* coumaric acid, vanillic acid, caffeic acid, chlorogenic acid, protocatechuic acid and cinnamic acid were identified. Six more unidentified spots were noted in the extracts of healthy plants under all levels of nitrogen treatment. In the infected plants one more spot was observed which was also not identified. Prasad *et al.* (1972) and Sridhar and Ou (1974) also observed an additional phenolic spot in *P. oryzae* inoculated rice plants. They suggested the appearance of additional phenolic compound may be due to host parasite interaction. The quantity of this phenolic acids varied widely due to different levels of nitrogenous fertilizer application, sampling time and inoculation with *C. cassiicola.*

Application of increased level of nitrogen reduced the content of *p.* coumaric acid, vanillic acid, chlorogenic acid, cinnamic acid, caffeic acid, *p.* hydroxybenzoic acid and ferulic acid. Accumulation of phenolic acids in plants is an indication of disease resistance (Mahadevan, 1991; Vidhyasekaran, 1997).

Present study clearly showed that nitrogenous fertilizer application induced susceptibility in rubber plants by reducing phenolic acids. Post infectional increase is also reported earlier to confer resistance in plants against various fungal diseases (Vidhyasekaran, 1997). Ohata *et al.* (1966) found ferulic acid and *p.* coumaric acid increased as much as 5 times of the healthy rice tissues due to *P. oryzae* inoculation. The same were more and rapidly observed in resistant plants. In the present study, phenolic acids like *p.* hydroxybenzoic acid and vanillic acid increased due to infection at low nitrogen levels and decreased at high nitrogen levels. The increased phenolic acid due to *C. cassiicola* infection in plants receiving low nitrogen may also inhibit the enlargement of lesions due to their fungitoxic activities (Ohata *et al.*, 1966). Koti Reddy (1975) also reported that phenolic acids mixtures were highly fungitoxic. Besides, phenolic acids were also found to inhibit the activity of fungal enzymes (Koti Reddy, 1970). It is thus clear that the application of reduced level of nitrogen leads to induced resistance to *C. cassiicola* in rubber plants.

The augmented accumulation of phenolic compounds in the infected tissues might be due to one or more of the following mechanisms.

Cell walls of higher plants contain phenolic glycosides in the inactive form and not utilized by the plants, but are more soluble than phenols *per se* and in response to pathogenic invasion, the inactive glucosides are hydrolysed by glucosidase of the pathogen and/or host and the phenols released from the conjugated phenolics might inhibit the pathogen (Pridham, 1965). Several of the plant pathogens were also found to possess the faculty

of β-glucosidase system (Flood and Kirkham, 1960; Anthoni Raj, 1974; Vidhyasekaran, 1997).

Higher plants are also known to synthesise phenolic compounds via the shikimic acid and acetate pathways (Kosuge, 1969; Stafford, 1974). The intermediary compounds of carbohydrate metabolism via., erythrose phosphate and phosphoenol pyruvate are used up for the synthesis of shikimic acid. Shikimic acid thus synthesised in this pathway forms, through several intermediates; prephenic acid which subsequently get converted to either phenylalanine or tyrosine. The intermediates of the phenylalanine and tyrosine pools serve as precursors for various aromatic compounds (Goodman *et al.*, 1967). Head to tail condensation of acetate unit appears to be involved in the synthesis of polyacetic acid and it is suggested that some benzoid compounds may also be formed from polyacetic acids (Goodman *et al.*, 1967). Probably melonyl coenzyme–A and acetyl coenzyme–A are involved as intermediates for the synthesis of ring A of the iso-flavone, pisatin etc. (Hadwiger, 1966; Kosuge, 1969). It is probable that both shikimic acid and acetate pathways may operate together towards the synthesis of phenolic compounds (Kosuge, 1969; Vidhyasekaran, 1988) (Figure 27). The results obtained, in the present study revealed that inoculation with *C. cassiicola* tends to decrease the sugar level of the tissues suggesting the possibility of phenols being synthesised at least in part from the sugars as suggested by Uritani (1961).

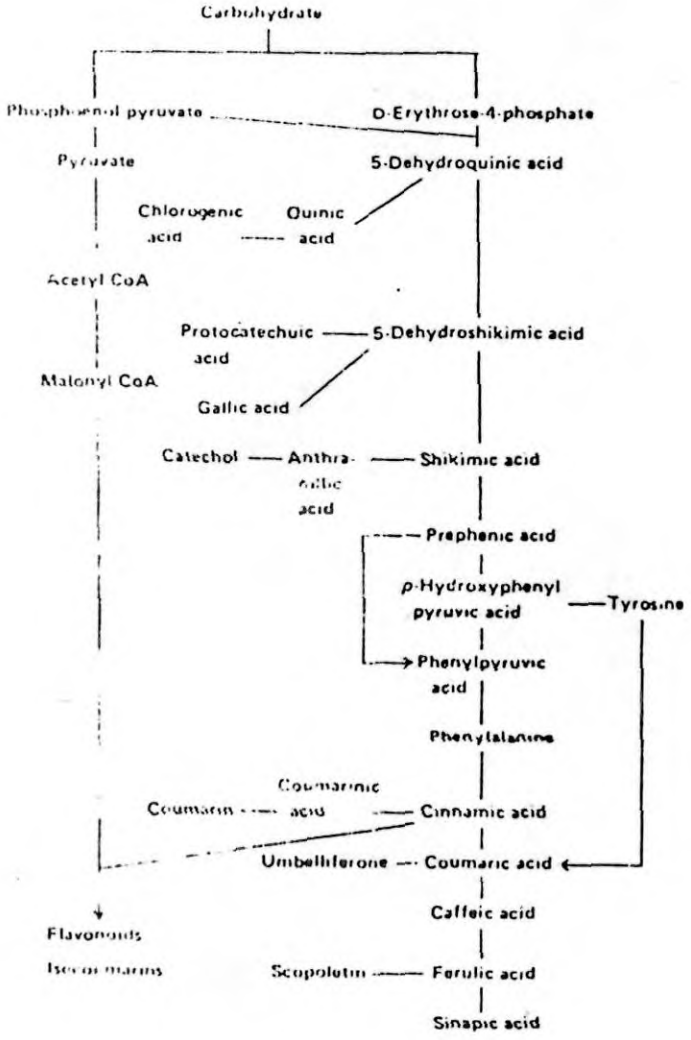
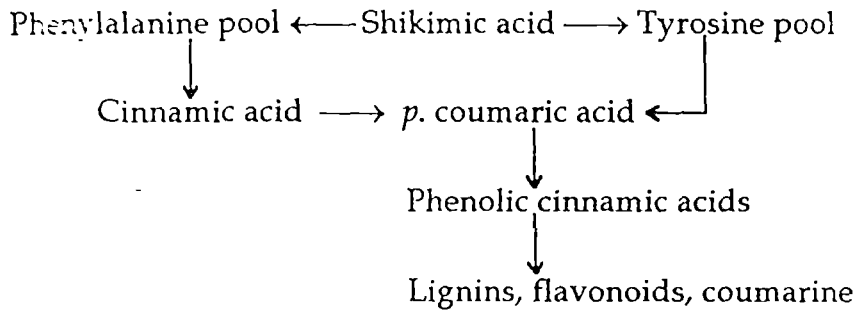


Figure 27 Biosynthesis of phenolics

Phenols might also accumulate due to interconversion of aromatic compounds. Deamination of phenylalanine by PAL leads to the formation of cinnamic acid and its hydroxylation further leads to the formation of other cinnamic acid derivatives like *p.* coumaric, caffeic and ferulic acids. Similarly TAL catalysis the conversion of L. tyrosine to *p.* coumaric acid (Goodman *et al.*, 1967; Vance *et al.*, 1980). The present study also shows that *C. cassiicola*

inoculation causes an increase in PAL and TAL in plants applied with lower levels of nitrogen.



The decrease in phenolic constituents observed on inoculation might be due to (1) the formation of higher classes of compounds than the precursors and incorporation into lignins on entering into biochemical pathways after the rings are split oxidatively (Thomson, 1964); (2) the polymerisation into melanin (Thomson, 1964) and/or (3) utilization by the pathogen (Farkas and Kiraly, 1962). *C. cassiicola* inoculation leads to an increased activity of PO which may remove polyphenols and the synthesis of polyphenols may be less as evidenced by the low activity of PAL and TAL in plants receiving higher levels of nitrogen.

The depletion of sugars upon inoculation and the augmented level of amino acids in plants inoculated with *C. cassiicola* may also be a factor for the reduction in phenols. Due to low levels of sugars, the synthesis of phenols might be reduced (Sridhar and Mahadevan, 1979) and higher levels of amino acids might have inactivated the phenols as described earlier.

The studies of Prasad and Regunathan (1972) have revealed that *P. oryzae* utilized several phenolic compounds like vanillic, *p.* coumaric, ferulic, salicylic, chlorogenic, protocatechuic *p.*hydroxybenzoic, caffeic and

quinic acids and pyrogallol, arbutin and D. salicin as carbon source at lower concentrations. However, at higher concentrations they were inhibitory to the growth of the fungus. The present study also revealed the presence of 14 phenolic compounds in rubber seedlings and it is probable that the reduction in the amount of phenolic compounds in the diseased tissues observed might be due to the utilisation by the pathogen also. Wakimoto *et al.* (1960) obtained evidences for the utilization of various phenolic compounds like phloroglucinol, protocatechuic acid and rutin at lower concentration by *P. oryzae.*

Carbohydrates

It has been well established that carbohydrate metabolism of plants is altered due to pathogenic invasion (Uritani, 1963; Sridhar and Mahadevan, 1979). Pathogenesis itself is an interaction between the pathogen and carbohydrates of the host and it is the carbohydrate that determines the pathogens ability to produce enzymes (Albersheim *et al.*, 1969). It is well known that the levels of carbohydrate in the plant tissue to a large extent influences the disease development (Goodenough and Kempton, 1977). The results of the present study revealed that application of nitrogen and *C. cassiicola* inoculation significantly altered the level of reducing and non-reducing sugars and starch content in rubber seedlings. Application of increased levels of nitrogenous fertilizer augmented the content of reducing sugars while considerable reduction in non-reducing sugars and starch content were recorded. The increase in soluble sugars upon nitrogenous fertilizer application in rice as observed in the present study was recorded (Prasad and Regunathan, 1972). *C. cassiicola* inoculation decreased the

reducing sugars, and non-reducing sugars irrespective of different levels of nitrogenous fertilizer application. Severe incidence of the disease in plants having higher sugars indicate that *Corynespora* disease of rubber is a high sugar disease as suggested by Horsfall and Dimond (1957).

It is a common phenomena of reduced incidence of high sugar disease in plants having low levels of sugars due to inadequate supply of nitrogen (Kothandaraman, 1984). The reduction in sugar content in infected plants may be due to (i) parasitic utilization; (ii) conversion to non-carbohydrates by the host and/or (iii) inhibition of carbohydrate synthesis of the host in the presence of pathogen (Sridhar, 1969; Prasad and Regunathan, 1972). High amounts of reducing and non-reducing sugars have been recorded in many plants susceptible to several pathogens (Reddy and Sridhar, 1975).

Carbohydrate form the major substrate for respiration of higher plants (Hackett, 1959). It is hence probable that the decrease in reducing sugar level of inoculated plants observed in the present study may also be due to the diversion of a part of sugars for the augmented respiration.

Application of nitrogen at high levels decreased the non-reducing sugars which was much pronounced at 100 and 200 per cent nitrogen application. Disease resistance in plants is reported to be due to higher levels of non-reducing sugars (Sridhar, 1969).

C. cassiicola inoculation led to a reduction in non-reducing sugars in all the fertilizer treatments. The decrease in level of non-reducing sugars may be due to the transformation of the same to reducing sugars (Asada, 1957 and 1962) to meet accelerated synthesis of phenols–a response observed in plants

applied with lesser dose of nitrogen and to meet the requirement for the augmented level of respiration. A reduction of both reducing and non-reducing sugars due to pathogenic invasion were observed by many workers in various crops (Ramakrishnan, 1966; Sindhan and Parashar, 1996).

Increased application of nitrogenous fertilizer and *C. cassiicola* inoculation significantly altered the level of starch in rubber seedlings. Nitrogen application in general reduced the starch content in all the nitrogen levels. There was a reduction in sugar content upon inoculation while an increase in starch content was observed in all the levels of nitrogen upon infection by *C. cassiicola*. Accumulation of starch in rice leaves around the lesions caused by *C. miyabeanus* was observed by Tanaka and Akai (1960) and they attributed it to the possible inhibition of the enzyme β amylase in infected tissues. Similar phenomenon is also likely to take place in rubber plants infected with *C. cassiicola*. The accumulation of starch in the inoculated leaves may be also due to derangements in the translocation of starch caused by the pathogen and its metabolites or due to stimulated synthetic processes. It is therefore, probable that a higher starch level may play a role in disease resistance against *C. cassiicola*.

Nitrogenous compounds

It is proved beyond doubt that nitrogen metabolism of the host plants is altered in response to pathogenic invasion (Goodman *et al.*, 1967; Hwang *et al.*, 1983). Amino acids and sugars of host plants form the main building blocks for cell synthesis of the pathogen. Tissues containing high reserve of soluble sugars and nitrogen are, therefore, generally prone to pathogenic invasion.

Increase in amino nitrogen has been reported in rice leaves (Nayudu *et al.*, 1979) and in *Poa pratensis* (Robinson and Hodges, 1981) due to nitrogen application. Higher the amino nitrogen content of the tissues the more has been the disease susceptibility. Appa Rao (1964) stated that the residual nitrogen consisting of ammoniacal, amino and amide nitrogen increased susceptibility of rice plants to various diseases. The severity of *Corynespora* leaf spot disease in rubber under the influence of enhanced nitrogen level in tissues observed in the present study upholds the above views.

C. cassiicola inoculation altered the amino nitrogen content of rubber plants fertilized with graded levels of nitrogen. At all levels of nitrogen amino nitrogen level reduced initially upon inoculation and as the disease advances there was a significant increase. The increase in the level of soluble nitrogen in response to pathogenic invasion was recorded in rice (Ramakrishnan, 1966) and in cucurbits (Singh and Chohan, 1977). Such a change is attributed to degradation of structural proteins of the host by the protolytic activity of the pathogens (McCombs and Winstead, 1964). In the present study, it is proved that *C. cassiicola* produces protease in culture and it is therefore possible that during disease development some of the amino acids might have been derived from protein hydrolysis (Otani, 1955). Alterations in the host's synthetic and degradative pathways may be the reason for the initial reduction in the level of amino nitrogen observed in this study (Fowdon, 1965). The reduction in amino nitrogen upon *C. cassiicola* inoculation could be attributed to the utilization of amino nitrogen by the pathogen as observed by Shishiyama *et al.* (1969) in rice plants. The common phenomenon in many kinds of plants is the accumulation of amino acids and their amides with the excessive application of nitrogen fertilizer

(Tanaka, 1963). The results of the present study revealed that application of graded levels of nitrogen increased contents of asparagine, glutamine, glutamic acid, phenylalanine, aspartic acid, tyrosine, leucine, methionine, cysteine and histidine while the content of glycine was not altered much. Fungi in general have the preference for amino acid for their growth and function (Otsuka *et al.*, 1963; Vidhyasekaran, 1988). It is probable that the increased susceptibility of rubber seedlings to *Corynespora* leaf disease in response to nitrogen fertilization may be due to the accumulation of asparagine and glutamine which ultimately favour the development of the fungus as suggested by Tanaka and Katsuki (1952) and Palfi (1965). However higher amino nitrogen content of the tissues might have decreased the toxicity of phenol as discussed earlier thereby increasing the susceptibility of the tissues.

One of the interesting observation in the present study is the failure of nitrogenous fertilizer in significantly increasing the content of glycine and arginine. These amino acids especially arginine is reported to have a positive influence in disease resistance. Allen and Orth (1941) reported the inhibitory effect of arginine on spore germination of *Phytophthora*. Tanaka and Katsuki (1952) have reported that arginine and glycine are less effective in promoting the growth of *P. oryzae*. The present study also points to the favourable role of arginine in the resistance of rubber seedlings to *C. cassiicola* under low nitrogen level.

C. cassiicola inoculation, in general, increased the level of glutamic acid, glutamine, aspartic acid, asparagine, glycine and alanine in rubber seedlings irrespective of the level of nitrogenous fertilizer application. The level of

methionine and histidine decreased upon infection in plants under all the nitrogen level and the decrease is less in plants at 25 per cent nitrogen level. The content of glycine at higher nitrogen levels decreased upon infection and increased at low nitrogen levels. But phenylalanine and tyrosine increased in plants applied with high levels of nitrogen and decreased in plants applied with low levels of nitrogen. The augmentation of amino acids during pathogenesis is not a rare occurrence (Hrushovetz, 1954). The increase in amino acid in host tissues due to infection may be due to *de novo* synthesis by the host/pathogen *per se* (Rohringer, 1957). It is also possible that some of the amino acids might have come from the break-down of the host proteins as discussed earlier. The increase in cysteine, glutamic acid and aspartic acid in resistant plants following infection is reported to play an important role in internal defence mechanisms (Chattopadhyay and Bera, 1978) and this explains the possible disease resistant mechanisms under low level of nitrogen in rubber seedlings.

Augmentation of aromatic amino acids (phenylalanine and tyrosine) may be due to the reduced activity of respective lyases. Sadasivan (1968) and Vidhyasekaran (1988) found that phenylalanine and tyrosine are responsible for the disease resistance of *P. oryzae* and *Drechslera oryzae*. While studying amino acids of wheat, Fuchs and Rohringer (1955) found that histidine, leucine and asparagine present in the healthy plants were absent in plants infected with *P. graminis*.

Aromatic amino acids decreased quantitatively in plants receiving lower levels of nitrogen while they increased in plants with high levels of nitrogen due to *C. cassiicola* inoculation. Simultaneously the PAL and TAL

increased upon inoculation with *C. cassiicola* in plants receiving low levels of nitrogen and reduced in plants with high levels of nitrogen. Therefore the augmented levels of phenolic reserve in plants receiving low levels of nitrogen may be due to the enhanced activity of PAL ase and TAL ase and the aromatic amino acids have provided the aromatic rings of phenolic acids (Goodman *et al.*, 1967).

It is shown by several workers that nitrogenous fertilizers augmented the total nitrogen content of the tissues which ultimately enhanced the susceptibility of the plants to diseases (Tokunaga *et al.*, 1966; Rajalakshmy *et al.*, 1979). In the present study enhanced nitrogen application increased total nitrogen content of rubber seedlings a precursor for various nitrogen compounds in plants. *C. cassiicola* inoculation in general increased the total nitrogen and crude protein content of the plants grown at different levels of nitrogen. Post infectional accumulation of total nitrogen was observed in many host parasite combination (Rubin and Artsikhovskaya, 1963). The increase in total nitrogen due to infection was pronounced in plants at 25 per cent nitrogen level. The increase in total nitrogen and crude protein content of infected tissues may be due to (i) enhanced amino acid synthesis (ii) increased protein synthesis either by host or pathogen or cumulative synthesis (Tomiyama, 1963; Farkas and Stahmann, 1966) and (iii) increased synthesis of more enzyme proteins (Stahmann, 1967; Uritani *et al.*, 1967).

Ascorbic acid and ascorbic acid oxidase

The innate resistance of plants depends largely upon the organic reducing agent, ascorbic acid which is found to play a significant role in several host parasite combination (Sharma *et al.*, 1975). Ascorbic acid

reduces the toxic quinones to less toxic phenols (Uritani and Lechika, 1953) by serving as hydrogen donors and thus either suppressing or reversing the action of polyphenol oxidase (Henz, 1956). The redox potential of the host is altered by ascorbic acid (Goodman *et al.*, 1967). Since ascorbic acid level is largely influenced by ascorbic acid oxidase this enzyme plays a role in disease resistance (Aulakh and Grover, 1970). In the present study application of graded levels of nitrogen found to significantly increase the ascorbic acid level. Prasad and Regunathan (1972) also reported an increase in the ascorbic acid level in rice plants due to nitrogen application. Higher levels of ascorbic acid in plants showing resistant reaction to pathogenic invasion than in the ones showing susceptible reaction was well established (Pushpanadhan, 1957). It is possible that in the resistant plants, the ascorbic acid (the high energy substrate) might be readily utilized for the synthesis of toxic substances against the pathogen (Kalyanasundaram, 1952).

C. cassiicola inoculation significantly reduced the level of ascorbic acid in all the nitrogen level. The decrease in the level of ascorbic acid may be due to the oxidation by the oxidative enzyme or its participation in reducing the quinones to phenols. In the present study, it has been observed that the activity of ascorbic acid oxidase decreased in plants applied with 100 and 200 per cent nitrogen and increased in plants with 25 and 50 per cent nitrogen up on infection with C. *cassiicola*. Therefore the decrease in the level of ascorbic acid in plants applied with higher nitrogen may be due to the participation of the same to toxic quinones to less toxic phenols. However, in plants applied with lower levels of nitrogen, the ascorbic acid oxidase may be responsible for the reduced level of ascorbic acid upon inoculation with C. *cassiicola*. C. *cassiicola* inoculation of plants grown with higher levels of nitrogen

reduced the activity of ascorbic acid oxidase while at lower levels of nitrogen it increased. Toxins elaborated by *P. oryzae* was reported to inhibit ascorbic acid oxidase activity (Tamari *et al.*, 1963) and hence the toxins produced by *C. cassiicola* might also be responsible for the inhibitory action against ascorbic acid.

Peroxidases and polyphenol oxidases

Quinones and other oxidised phenols inhibit microorganisms and their enzymes. These reactions are correlated with defence mechanism of infected plants (Mahadevan, 1966). The enzyme system of the plant is altered due to invasion of pathogen. Changes in oxidative enzymes like PO, PPO and AAO in infected plants have been reported by several investigators (Uritani *et al.*, 1967; Vidhyasekaran, 1988). Phenol oxidation in the infected tissue is mediated by enzymes produced by the infecting organism and the host. PO, an iron containing enzyme has been found to play a vital role in the terminal oxidation of substances (Farkas and Kiraly, 1958). Many investigators have also observed a close correlation between disease resistance and PO activity (Goodman *et al.*, 1967. Breton *et al.* (1996) observed a significantly higher PO activity for the clone GT1 resistant to *C. cassiicola.* Alteration of PO activity due to nitrogen fertilization in rice has been reported by Sridhar (1969) and Prasad and Regunathan (1972). In the present study the activity of PO increased with increase in nitrogen level. The results of this study are in agreement with the finding of Prasad and Regunathan (1972). At low level of nitrogen application the activity of PO triggered during the initial stage of *C. cassiicola* inoculation. Similar increase in PO activity in *P. oryzae* was reported by Toyoda and Suzuki (1960). Tomiyama (1963) stated that the

oxidative enzymes are more active in the resistant variety than the susceptible one. Johnson and Cunningham (1971) detected 109 per cent increase in PO activity in resistant wheat variety compared to 20 to 40 per cent in susceptible variety. Augmentation in PO activity in infected resistant plants may be attributed to (i) development of new isozymes (Sridhar, 1978); (ii) alteration in growth regulator metabolism and (iii) increase in phenolic level (Iwata *et al.*, 1981).

PPO of rubber seedlings was reduced due to increase in nitrogenous fertilizer application. The decreased activity of PPO due to increased nitrogen application could be attributed to the enhanced level of ascorbic acid as discussed earlier. The activity of PPO decreased upon infection at lower level of nitrogen and increased at higher level of nitrogen. The decrease of PPO activity in plants showing resistance upon infection and increase in susceptible plants are well established (Sridhar, 1978; Kuc, 1966).

Interestingly corresponding to PO activity phenols level in infected tissues also got altered. This may evidently mean that in the resistant plants the oxidation of phenolics by PO is carried out at a relatively faster rate leading to the accumulation of phenol oxidation products, the quinones which exert inhibitory effect on the pathogen. Quinones also undergo polymerisation which leads to the formation of melanoid pigment characteristics of lesions caused by *C. cassiicola* under low nitrogen level.

Lyases

Phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) are reported to deaminate the respective amino acids (Neish, 1961;

Green *et al.*, 1975) and appear to be an important mechanism in higher plants for the synthesis of various aromatic substances and consequently linked with disease resistance (Young *et al.*, 1966; Vance *et al.*, 1980). Neish (1961) detected the activity of PAL in rice plants. Sadasivan (1968) suggested the role of phenylalanine and tyrosine in the disease resistance mechanism of rice varieties against *D. oryzae* and *P. oryzae.*

The present investigation revealed that application of increased levels of nitrogen reduced the activity of both PAL and TAL in rubber plants while low nitrogen levels enhanced the activity of this enzyme. The reduction in the activity of lyase enzymes due to nitrogen application was also reported by Matsuyama and Dimond (1973) in rice. Enhanced activity of lyase enzymes is common in resistant plants (Yamamoto and Nakao, 1976). The enhanced activity of the lyase enzymes might have accumulated phenols in plants applied with less nitrogen as observed in the present study, at the expense of phenylalanine and tyrosine.

Pathogenic infection in plants causes a shift in the activity of PAL and TAL (Hiroshi *et al.*, 1978; Vance *et al.*, 1980). In the present study, *C. cassiicola* caused an augmented activity of the lyase enzymes with a corresponding reduction in the phenylalanine and tyrosine in plants receiving low level of nitrogen. However in plants receiving high nitrogen, infection caused a reduction in these two enzymes. Such a reduced activity of lyase enzymes in compatible reaction was reported by Purushothaman (1974).

Vance *et al.* (1980) reviewed the mechanism of biosynthesis of phenols and their role in disease resistance. TAL catalyses the conversion of phenylalanine to cinnamic acid. Cinnamic acid is hydroxylated at para

position by cinnamic acid-4-hydroxylase to form *p.* coumaric acid; however, *p.* coumaric acid may also be formed by the deamination of tyrosine catalysed by TAL. *p.* coumaric acid formed is further hydroxylated by *p.* coumaric acid hydroxylase to give caffeic acid (Kosuge, 1969). C-methyl transferase then methylates caffeic acid to ferulic acid. These phenolic acids through a series of enzymatic reactions lead to the formation of lignin.

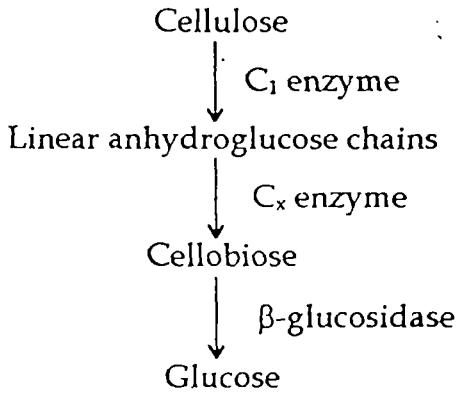
The decreased activity of PAL and TAL due to infection in plants receiving higher levels of nitrogen, might be due to the existence's of a lyase inactive system in these plants. Cahill and McComb (1992) reported the swtiching-off of PAL genes as an early step in the developments of pathogen in susceptable plants.

Hydrolytic enzymes

Enzyme production is a pre-requisite for the establishment of pathogenesis. The genetic make up of the plant pathogen as well as the nutrients supporting the growth determine the capacity of production of enzyme. Degradation of cell wall host tissues at some stages or other is involved in pathogenesis by most of the pathogen (Wood, 1960; Goodman, 1967).

Major composition of cell wall of the plants involve cellulose, hemicellulose, pectin and proteins. For the successful establishment of plant pathogens they should break the barriers formed by these compounds. Both physical and chemical changes of cell wall predispose the plants to various diseases (Vidhyasekaran, 1997). Interesting results were observed in the present study when different levels of nitrogen was studied *in vitro* on the

growth and production of cellulolytic enzymes of *C. cassiicola*. This fungus was found to produce C_1, C_x and β-glucosidase under *in vitro* condition. Komarajah and Reddy (1988) also reported the production of cellulases by *C. cassiicola*. Cellulose is the major cell wall polysaccharide and is composed of glucose units in chain configuration β-1,4 glycosidic bonds. The degradation of cell wall by cellulolytic enzymes is well established (Yazdi *et al.*, 1990; Vidhyasekaran, 1997). The favourable effect of added nitrogen in the medium on the growth and production of enzymes by *C. cassiicola* indicates that the disease severity in rubber seedlings is due to the availability of nitrogenous compounds in the tissues of plants receiving higher doses of nitrogen. Many plant pathogens are known to degrade cellulose by either of the enzymes C_1, C_x and β-glucosidase (Reese and Levinson, 1952). They also suggested the possible pathways of the cellulose hydrolysis leading to glucose.



The result of the present study clearly indicate that cellulolytic enzymes are increased by higher dose of nitrogen involved in severity of disease development in rubber seedlings by *C. cassiicola*.

In the present study, *C. cassiicola* was found to produce pectinolytic enzymes *viz.*, protopectinase, polygalacturonase, polygalacturonate

trans-eliminase and pectin *trans*-eliminase *in vitro*. The production of this enzymes increased with increase in the level of nitrogen in the medium up to 750 ppm and further increase in the level of nitrogen failed to increase the enzyme production significantly. The increased activity of pectinolytic enzymes with increase in nitrogen level of medium up to 750 ppm indicate that the increased nitrogen level in rubber seedlings receiving high dose of nitrogen is one of the reason for disease severity. Vidhyasekaran (1997) reviewed the entire role of pectinolytic enzyme on disease development and showed that all the pectinolytic enzymes are involved in sequence and synergetically in disintegrating the tissues and establishing the plant pathogenesis.

Proteolytic enzymes

C. cassiicola also produced proteolytic enzymes which increased with increase in nitrogen in the medium up to 1250 ppm. Structural proteins are also important compounds of the cell wall which are reported to be degraded by the action of proteolytic enzymes (Hislop *et al.*, 1982; Movahedi and Heale, 1990 a, b). The analysis of the tissue of rubber seedlings under graded nitrogen indicate that the total nitrogen content was more in plants receiving high levels of nitrogen. Such enhanced levels of nitrogen in tissues might have favoured the activity of protease leading to the establishment of *C. cassiicola*. The *in vitro* studies on hydrolytic enzymes production by *C. cassiicola* under the influence of nitrogen in the medium indicate that these enzymes are increased by nitrogen level and their involvement in disease syndrome.

Toxin production by the pathogen and inactivation by phenolic acids

Plant pathogens in general are reported to elaborate toxins and antimetabolites in culture medium and in plant tissues. Such toxins are associated with the developing plant disease syndrome (Oku, 1967; Mahadevan, 1991). *C. cassiicola* inoculation produced toxin which inhibited the bacteria. *B. subtilis* and the quantity of toxin increased with nitrogen up to 750 ppm. The production of toxin by *C. cassiicola* in various crops (Sarma *et al.*, 1975; Onesirosan *et al.*, 1975) and in rubber (Lyanagae, 1986; Breton *et al.*, 1997) is well established. The capacity to elaborate these toxins depends upon the nutrient status of culture fluid and plant tissues (Kothandaraman, 1984).

Toxin production was augmented in leaf extracts obtained from rubber plants grown under increased levels of nitrogen. The plants receiving high levels of nitrogenous fertilizer contain more of sugars and amino acids and less phenols as evidenced from the present study. Prasad and Regunathan (1972) found that toxin production in *P. oryzae* is encouraged by amino acids as well as reducing sugars. The higher levels of various amino acids and reducing sugars accompanied by the lower levels of phenols might have resulted in the maximum production of toxin. The detoxification of pathogen produced toxin which plays an important role in disease production is one of the defence mechanism exhibited by higher plants to protect themselves from the invasion of pathogens (Rubin and Arzichowskaya, 1953). Plant phenolics detoxified the toxin produced by *C. cassiicola* and the extent of detoxification differed with different phenolics. Chlorogenic acid, ferulic acid and *p.* coumaric acid completely detoxified at 0.01M concentration. Other

phenolics have comparatively lesser activity. The least activity was recorded in caffeic acid and cinnamic acid treatments. Rubber seedlings contain all the phenols studied for toxin inactivation, the concentration of which decreased with increase in nitrogen fertilization. It was also found that ferulic acid, *p.* coumaric acid and chlorogenic acid reduced the toxicity of the toxin in *C. cassiicola* filtrate effectively. Therefore it is possible for the enhanced level of phenolic acids to detoxify the toxins. Chlorogenic acid contain caffeic acid and quinic acid moieties with a total of 5 hydroxy groups. The enhanced detoxifying property of chlorogenic acid might possibly due to this structural differences. Hence, the alteration in the phenolic reserve in rubber plants due to the application of nitrogen might directly be involved in susceptibility or resistance of rubber plants against *C. cassiicola.*

Changes in phenols, amino nitrogen and total sugars in the leaf leachates of healthy and *C. cassiicola* inoculated rubber seedlings at different levels of nitrogen

Leaching out of organic and inorganic nutrients by plants up on contact with moisture is a well known phenomena (Morgan and Tukey, 1964). The organic substances include free sugars, pectic substances, sugar alcohols, amino acids (Tukey and Romberger, 1959), vitamins (Wasicky, 1958), alkaloids (Tukey, 1971) and phenolic substances (Kozel and Tukey, 1968; Nicholson *et al.*, 1989). In the present investigation organic constituents like sugars amino nitrogen and phenols were detected in the leaf exudates of rubber seedlings raised at graded levels of nitrogen. The levels of sugars and amino nitrogen were high in leaf leachates of plants receiving higher dose of nitrogen while the level of phenols got reduced by increased nitrogen application. *C. cassiicola* inoculation augmented amino acids and decreased

the sugars in the leachates of leaves of all the nitrogen treatment. However phenol content of the leachates in diseased plants under low nitrogen was more while at high nitrogen level the reverse was observed.

Variation in the biochemical constituent of leaf leachates of rice was showing different levels of resistance was reported by Mohanty and Gangopadhyay (1981). The amount of nutrient substances diffusing into the water drop on leaf surface depends on the permeability of cell wall (Rubin and Artsikhovskaya, 1963) and nitrogen level affects the composition of leaf exudation and bring out an effect similar to that of host constituents (Huber and Watson, 1974). Tukey (1971) also rightly pointed out that the nutrient composition of leaf leachates is the reflection of nutrient status of plants. The observed changes in the level of sugars, amino acids and phenols in the leachates of plants receiving graded levels of nitrogenous fertilizer might be due to the corresponding changes in the composition of these compounds in the tissues. Observing such changes, Purushothaman *et al.* (1976) suggested that the augmented levels of sugars and amino acids in the leachates of rice plants receiving nitrogenous fertilizer might be due to the increase in the permeability of the cell wall. They also noted that excess nitrogen application led to elongation of cell walls and increases membrane permeability.

C. cassiicola inoculation led to biochemical changes and such changes are reflected in the leachates of the leaves. This finding is in confirmity with the observation made by Goto *et al.* (1979) who reported an increase in the surface fluids of citrus leaves due to infection by *Xanthomonas citri.* The present finding clearly indicated that the biochemicals present in the leaf leachates also play a role in disease resistance/susceptibility under the

influence of nitrogenous fertilizer applied to plants and *C. cassiicola* inoculation.

Phyllosphere microflora

In nature all the parts of a plant harbour a variety of microorganisms which include bacteria, fungi, yeasts and actinomycetes (Ruinen, 1956; Jenson, 1971; Clark, 1976) and these populations are altered by many factors. The factors that alter the physiology and biochemical constituents, external structures and health of plants lead to changes in the phyllosphere microorganisms. Unlike soil microorganisms, the phyllosphere microorganisms solely depend for the nutrients on the outer surface of leaves (Last, 1955; Sarkar and Samaddar, 1982). Any change in the nutrients of leaf surface exudate naturally influences the microbial population. In the present study, rubber leaves were found to harbour more bacteria followed by filamentous fungi and yeasts, and similar observations were made by Kerling (1958) in *Beta vulgaris* and Hislop and Cox (1969) in rice. Increase in the application of nitrogenous fertiliser favoured the growth of bacteria under the influence of leaf exudates containing more sugar and amino acids. At the same time the fungal population was brought down by the increase in nitrogen application. This could be due to the reduced generation time of bacteria compared to fungi (Salle, 1974). The production of toxic metabolites might also played a role in the reduction of fungal population. These results are on par with the findings of Purushothaman *et al.* (1976) and Kothandaraman (1984) who observed higher population of bacteria in plants applied with higher level of nitrogen in rice. Yeasts population in leachates of rubber plants increased with increase in nitrogen fertilizer up to 50

per cent of recommended dose and further increase led to decrease of the same. Irrespective of nitrogenous level increased yeasts population was recorded upon *C. cassiicola* inoculation. Last (1955) found that less population of yeasts in the exudation of plants showing resistance to various disease as observed in the present study. The enhanced level of bacterial population in the phyllosphere of plants is an indication of susceptibility of such plants to diseases as reported by Chandrakumar and Balasubramanian (1981). The enhanced microbial population due to increased level of nitrogenous fertilizer is attributed to the availability of nutrients in leaf exudates of such plants (Dickinson, 1976) as found in this investigation. The phyllosphere microorganisms may also alter the composition of nutrients available on the leaf surface by their activity on the cell walls of plants (Blakeman, 1971; Bhattacharyya and Purkayastha, 1983). The enhanced sugar and amino nitrogen level coupled with reduced phenolic content in leaf leachates due to nitrogen application might have augmented population of bacteria. The reduction in the population of filamentous fungi in leaves of plants under low levels of nitrogen may be due to the enhanced activity of bacteria, which compete for nutrients with fungi and the production of toxic metabolites. At lower nitrogen level rubber leaves recorded less population of bacteria with increased level of phenols. Such a reduction in bacterial population is attributed to the inhibitory activity of phenolic reserves (Amstrong *et al.*, 1943).

C. cassiicola inoculation in general increased the population of bacteria, filamentous fungi and yeasts in the phyllosphere of plants applied with increased levels nitrogen. The enhanced microbial population due to disease development is a well-known phenomenon in plant kingdom (Stadelmann

and Schwinn, 1979). The increased fungal and bacterial population might be due to the increased level of nutrients and the decay of leaf tissues up on infection by pathogen in plants receiving higher dose of nitrogenous fertilizers. The reduced microbial population in plants receiving low levels of nitrogen may be due to low level of sugars as revealed in the biochemical analysis of leaf leachates in low levels of nitrogen treatment.

Interaction of phyllosphere microorganisms

Before getting established plant pathogens must pass through stages like spore germination and entry into the tissues. At these stages, they have to interact with saprophytes and other pathogens which influence the infection of plants by pathogens (Mahadevan, 1975). The germination of the spores is influenced by the nutrients in the infection site (Godfrey, 1976; Grover and Batra, 1967). In the present study, the yeasts and bacteria had no effect on germination of spores on *C. cassiicola* indicating that phyllosphere microorganisms do not influence the spore germination.

Appressoria formation is the pre-requisite for the penetration into plant tissues. The bacteria and yeasts influencing appressoria formation in *C. cassiicola* increased with increase in nitrogenous fertilizer. Such an increase is attributed to the nutrient levels of the exudates of plants (Blakeman and Barbery, 1977) and the increase in the population of bacteria and yeasts.

The present study clearly revealed that the application of increased levels of nitrogen fertilizer to rubber seedlings creates a favourable condition for infection by *C. cassiicola* by augmenting sugars, amino nitrogen, ascorbic acid, oxidative enzymes and phyllosphere microorganisms which induce

appressoria formation in *C. cassiicola* and by decreasing the phenolic reserves, ascorbic acid oxidase and lyase enzymes. The post infectional changes in the above constituents in plants applied with nitrogen were found to favour disease development. Nitrogen level in tissues of rubber plants also favoured the toxin production by *C. cassiicola*. The reduced production of cell wall splitting enzymes, cellulases, pectinases and protease of pathogen in plants receiving low nitrogen levels and the reverse in these activities at higher nitrogen levels is attributed to the resistance/susceptibility of rubber seedlings to *C. cassiicola*.

Chapter 6

SUMMARY

A pot culture study was carried out to find out the effect of graded levels of nitrogenous fertilizers on the incidence of *Corynespora* leaf spot in rubber seedlings. The rubber seedlings were inoculated with the spore suspension of *C. cassiicola* after 70 days of establishment. The development of disease symptom in the inoculated plants was recorded on eighth day of inoculation. The leaf samples collected at 0, 24, 48, 72, 96 and 168 h after inoculation were quantitatively analysed for total phenols, O. D. phenols, reducing and non-reducing sugars, starch, amino acids, total nitrogen and ascorbic acid. The leaves were also used for the qualitative and quantitative determination of phenolic acids and amino acids. The activity of oxidative enzymes (PPO, PO and AAO) and lyase (PAL and TAL) was also estimated in the above samples. The effect of nitrogen on the *in vitro* production of hydrolytic enzymes (cellulases, β-glucosidase, pectinolytic enzymes and protease) and toxin(s) was studied. Further, the changes in leaf leachate composition and phyllosphere microbial population in rubber plants fertilized with different levels of nitrogen and the effect of phyllosphere microorganisms on the appressoria formation of *C. cassiicola* were also investigated. The results obtained were summarised below.

The severity of *Corynespora* disease increased with increase in nitrogen level. At low nitrogen levels, the disease index was less while at higher

nitrogen level, the disease index was high with bigger spots and a major portion of the leaves damaged, leading to leaf fall.

Application of increased levels of nitrogen reduced both total and O. D. phenols and the reduction was much pronounced at the 200 per cent of the recommended dose. At low levels of nitrogen, C. *cassiicola* inoculation increased the content of the phenols while at higher levels of nitrogen, it was reduced. Increasing the level of nitrogen reduced the content of *p.* coumaric acid, vanillic acid, caffeic acid, chlorogenic acid, cinnamic acid and protocatechuic acid while the content of *p.* hydroxybenzoic acid and ferulic acid was not altered in the lower levels of nitrogen, but its contents got reduced at higher levels of nitrogen. C. *cassiicola* inoculation augmented the contents of *p.* hydroxy benzoic acid, vanillic acid and caffeic acid at lower levels of nitrogen and reduced at higher levels. Inoculation also caused an increase in protocatechuic acid at lower levels while at higher levels, the increase was noticed only in the initial stages of disease development. The contents of ferulic acid, *p.* coumaric acid and chlorogenic acid increased during the initial period of disease development at lower levels of nitrogen. At higher levels, the content of ferulic acid was reduced whereas the contents of *p.* coumaric acid and chlorogenic acid were not significantly altered. Cinnamic acid contents increased during the initial period of inoculation irrespective of nitrogen treatment and thereafter it was not significantly altered.

The content of reducing sugars was increased by the increased application of nitrogenous fertilizer while non-reducing sugars decreased. In general, the content of all the sugars decreased upon inoculation with

C. cassiicola. Increased levels of nitrogen reduced the starch content while *C. cassiicola* inoculation increased the starch reserve in the leaf tissues.

The content of amino nitrogen in rubber leaves increased due to graded levels of nitrogen. *C. cassiicola* inoculation augmented the level of nitrogen with a reduction upto 24 h in plants receiving nitrogen at 25, 50 and 100 per cent of recommended dose. Such an increase upon *C. cassiicola* inoculation in plant receiving 200 per cent nitrogen is not much appreciable. Rubber seedlings contained maximum contents of asparagine followed by glutamine, phenylalanine, methionine, tyrosine, aspartic acid, alanine leucine, cysteine, glutamic acid, arginine, histidine, lysine and glycine. Application of graded levels of nitrogen augmented the content of these amino acids except glycine and arginine. Increased level of nitrogen did not alter the glycine content while arginine content got reduced. *C. cassiicola* inoculation augmented the content of asparagine, glutamine and glutamic acid at all levels of nitrogen. Alanine and leucine content also increased in the later stages of disease development. Cysteine and glycine increased in 25 and 50 per cent nitrogen whereas lysine increased in 25 per cent nitrogen treated plants only. Phenylalanine and tyrosine increased in higher levels of nitrogen while they got reduced in lower levels due to infection. Histidine and methionine in rubber seedlings applied with graded levels of nitrogen decreased due to infection.

Increased application of nitrogen increased the total nitrogen content which represent the protein level in rubber seedlings. Both total nitrogen and protein increased upon infection with *C. cassiicola* in plants under all fertilizer treatments.

The content of ascorbic acid increased significantly with increase in nitrogen level. *C. cassiicola* inoculation reduced the content of ascorbic acid in plants at all level of nitrogen however, the reduction was more at 25 and 50 per cent nitrogen treatments.

Activity of ascorbic acid oxidase was not much altered with increase in the level of nitrogen upto 50 per cent of the recommended dose while further increase in nitrogen resulted in a reduction in the activity of this enzyme. *C. cassiicola* inoculation increased the activity of ascorbic acid oxidase in plants receiving nitrogen upto 50 per cent whereas a reduction in ascorbic acid concent at 100 and 200 per cent nitrogen level was observed.

Polyphenol oxidase activity in rubber seedlings decreased with increase in the level of nitrogenous fertilizer. *C. cassiicola* inoculation increased the enzyme activity in the initial periods and decreased in the later periods of inoculation at lower levels of nitrogen. At 100 and 200 per cent nitrogen, the PPO activity increased upon inoculation throughout the sampling period.

Application of increased level of nitrogen enhanced the activity of peroxidase. *C. cassiicola* inoculation led to a significant increase in PO activity at all levels of nitrogen. However, at 100 and 200 per cent nitrogen application, the increase in peroxidase activity was less when compared to lower levels.

The activities of lyases PAL and TAL were reduced due to application of increased levels of nitrogen. *C. cassiicola* inoculation increased the activities

of PAL and TAL at 25 and 50 per cent nitrogen and a decrease was noticed in 100 and 200 per cent nitrogen levels.

C. cassiicola produced cellulases and protease in culture. The production of cellulases increased with the increase in nitrogen upto 1500 ppm. The production of β-glucosidase increased upto 1250 ppm and thereafter no appreciable change was observed. An increase in the production of protease was also observed with increase in nitrogen level upto 1750 ppm. The pathogen produced protopectinase (PP), polygalacturonase (PG), pectin transeliminase (PTE) in the pectin enriched medium and all these enzymes increased with increase in the level of nitrogen upto 750 ppm.

The production of toxin(s) by *C. cassiicola* increased due to increase in nitrogen level in the medium and the increase was much pronounced upto 750 ppm nitrogen. *C. cassiicola* when cultured in the leaf extracts of rubber seedlings grown at different levels of nitrogen produced toxin(s) and toxin production increased due to increase in the level of nitrogen. Chlorogenic acid and *p.* coumaric acid completely neutralised the toxin(s) of *C. cassiicola* at 0.01 M level but the effect of caffeic acid and cinnamic acid in neutralising the toxin(s) of *C. cassiicola* was relatively less when compared to ferulic acid, *p.* coumaric acid and chlorogenic acid.

The leaf leachates of rubber plants contained total phenols, amino nitrogen and total sugars. The content of total phenols in the leaf leachates decreased with increase in nitrogen. In the inoculated plants the content of total phenols increased in 25 and 50 per cent nitrogen treatment while 100 and 200 per cent nitrogen treated plants contained less total phenols. Amino nitrogen and total sugars increased with increase in nitrogen level. In the

leachates of inoculated plants the amino nitrogen content was more in all the levels of nitrogen whereas total sugars content was less.

The population of bacteria and yeasts on the leaves of rubber seedlings increased whereas the population of fungi got reduced due to the application of increased levels of nitrogen. *C. cassiicola* inoculation led to an increase in the population of these phyllosphere microorganisms.

Phyllosphere bacteria and yeasts had no effect on the germination of *C. cassiicola* spores. However they induced the appressoria formation in *C. cassiicola*. The bacteria and yeasts inducing appressoria formation increased with increase in nitrogen level.

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Annexure 1

1. Modified Czapek's Broth Medium

Dipotassium hydrogen phosphate	1.0 g
Magnesium sulphate	0.5 g
Potassium chloride	0.5 g
Ferrous sulphate	0.01 g
Sucrose	30.0 g
Distilled water	1.0 litre
pH	6.8 to 7.2

2. Nutrient glucose agar

Beef extract	3.0 g
Bacto peptone	5.0 g
Glucose	5.0 g
Sodium chloride	5.0 g
Distilled water	1.0 litre
Agar	15.0 g
pH	6.8 to 7.2

Basal medium for phyllosphere microorganisms (Leben, 1972)

Peptone	10.0 g
Casein hydrolysate	1.0 g
Glucose	5.0 g
Agar	20.0 g
Distilled water	1.0 litre
pH	6.5 to 6.8

For the culturing of bacteria, 50 mg of cycloheximide per litre were added to the basal medium before autoclaving and 50 mg of 2.35-triphenyl-2H tetrazolium chloride per litre were added to the cooled medium after autoclaving and before pouring into the plates.

In making the fungal medium 100 mg chlorotetracycline hydrochloride per litre were added to the cooled basal medium before pouring the medium into the plates.

In the preparation of medium for enumeration of yeasts, the cooled fungal medium was adjusted with 0.1 N sulphuric acid to pH 4.4 to 4.8 before pouring in to the plate. All media were sterilized at 20 psi for 15 min.

Annexure 2

1. Reagent 'A'

In 800 ml of the glass distilled water, 25 g of unhydrous sodium carbonate, 25 g of sodium potassium tartarate (Rochelle salt) 20 g of sodium bicarbonate and 200 g of unhydrous sodium sulphate were dissolved and diluted to 1 litre with glass distilled water.

2. Copper Reagent 'B'

To 100 ml of glass distilled water, 15 g of copper sulphate and 1 to 2 drops of concentrated sulphuric acid were added.

3. Arsenomolybdate colour reagent

To 450 ml of glass distilled water, 25 g of ammonium molybdate, 21 ml of con. sulphuric acid and 3 g of sodium arsenate dissolved in 25 ml of glass distilled water were added and the mixture was kept in an incubator at 37°C for 48 h. The reagent was stored in a glass stoppered brown bottle.

4. Citrate buffer

Exactly 21 g of pure citric acid was dissolved in 200 ml of 1N sodium hydroxide in a standard flask and the volume was raised to 500 ml with glass distilled water.

5. Ninhydrin reagent

To 500 ml of the citrate buffer at pH 5.0, 800 mg of hydrated stannous chloride was added. The solution was mixed with 20 g of recrystalised ninhydrin, dissolved in 500 ml methyl cellosolve. Fresh reagents was prepared on the day of use.

6. **Diluent solution**

Equal volume of glass distilled water and n-propanol were mixed and used.

7. **Di azotized sulphanilic acid reagent**

Twenty five g of sulphanilic acid was dissolved in 125 ml of 10 per cent sodium hydroxide and the solution was cooled. To this, 100 ml of 10 per cent sodium nitrite were added. The resulting solution was added dropwise through a separating funnel into ice cold hydrochloric acid. (40 ml concentrated acid in 20 ml distilled water) while stirring. The precipitated diazonium salt was filtered through Whatman No. 42 filter paper and washed successively with ice cold water ethanol and ether. The salt was finally air dried and stored in a brown bottle in a refrigerator (Waldi, 1965). For spray, 0.1 g of the diazonium salt was dissolved in 20 ml of 20 per cent sodium carbonate and used immediately.

8. **Tetraazotized benzidine**

Solution A

Five g of benzidine were mixed with 14 ml of concentrated hydrochloric acid and diluted in 1000 ml with distilled water.

Solution B

Ten g of sodium nitrite were dissolved in 100 ml of distilled water

Solution B was added to solution 'A' in equal proportions just before use (Randerath, 1964).

9. **Ferric chloride-Potassium ferricyanide**

One g each of ferric chloride and potassium ferricyanide were dissolved in 100 ml of distilled water separately and mixed in equal proportions before spraying (Dawson *et al.*, 1969).

10. Alkaline silver nitrate

Solution A

Five g of silver nitrate were dissolved in 50 ml of distilled water and diluted to 1000 ml with acetone.

Solution B

To 100 ml of distilled water 5 g of sodium hydroxide were added and diluted to 1 litre with ethanol.

The chromatographic plate was dipped in solution A, dried and dipped in solution B. The paper was finally washed with ammonia and water to clear the background (Dawson *et al.*, 1969).

11. Ferric chloride

A 6 per cent ferric chloride stock solution was prepared with distilled water and diluted to represent 2 per cent before use (Reio, 1958).

12. Sodium molybdate

A 0.1M sodium molybdate solution was prepared by dissolving 24.198 g in a litre of distilled water (Dawson *et al.*, 1969).

13. Copper sulphate

A two per cent copper sulphate solution in distilled water was first sprayed followed by dilute ammonia on chromatogram (Reio, 1958).

14. Indophenol reagent

To 150 ml of glass distilled water 50 mg of sodium 2,6-dichlorophenol indophenol were added, warmed gently on a water bath till the dye dissolved and 42 mg of sodium bicarbonate added. The mixture was cooled and made up to 200 ml in a standard flask with glass distilled water. The reagent was stored in a dark place at 2°C and used within one week.

15. Standardisation of the indophenol reagent

The indophenol reagent was standardised before use. Five ml of the standard ascorbic acid, solution containing 2 mg per ml were taken in white porcelain dish and titrated against the indophenol dye until the solution changed to pink colour which persisted at least for 15 seconds.

16. Sodium phosphate buffer (Dawson *et al.*, 1969)

0.2 M solutions of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) (A) and sodium hydrogen phosphate ($NaH_2PO_4.2H_2O$) (B) were prepared separately. The two solutions were mixed in the following proportion and diluted with distilled water to get the buffer of required pH value

pH	Solution A ml	Solution B ml	Molar concentration	Dilution
6.2	18.5	81.5	0.2	--
6.5	32.0	68.0	0.05	Diluted to 400 ml with distilled water
6.5	32.0	68.0	0.01	Diluted to 200 ml with distilled water
7.0	61.0	39.0	0.2	--
7.1	66.5	33.5	0.1	Diluted to 200 ml with distilled water

17. Sodium acetate-acetic acid buffer (Dawson *et al.*, 1969)

Stock solutions of sodium acetate 0.2M (A) and acetic acid 0.2M (B) were prepared. To prepare buffers of pH 5.1, 5.2 and 5.6 the solutions A and B were mixed in the proportions given below. Fresh buffers were prepared every time and used.

pH	Solution A	Solution B
5.1	74.5	25.5
5.2	79.0	21.0
5.6	91.0	9.0

18. **Borate buffer** (Dawson *et al.*, 1969)

Stock solution of 0.1M sodium borate (A) and 0.4M hydrochloric acid (B) were prepared separately in distilled water. To prepare desired pH of the buffer they were mixed as given below. Fresh buffers were prepared every time before use.

pH	Solution A ml	Solution B ml	Molar concentration	Dilution
8.6	13.5	50.0	0.1	Diluted to 100 ml with distilled water
8.8	9.4	60.0	0.1	Diluted to 100 ml with distilled water