PRE-INFECTIONAL AND POST-INFECTIONAL COMPOUNDS INCLUDING PHYTOALEXIN RESPONSE IN *TECTONA GRANDIS* L.

CHAPTER - III

PRE-INFECTIONAL AND POST-INFECTIONAL COMPOUNDS INCLUDING PHYTOALEXIN RESPONSE IN TECTONA GRANDIS L.

INTRODUCTION

Nearly 14 per cent of the land area of India is under forest cover. Of the various timbers from forests, Teak wood (Tectona grandis L.) occupies a most prominent position in the economy of India. Tectona grandis is a dry deciduous tall tree with straight, pale brown rough bark. Leaves elliptic or obovate, acute-acuminate 10-45x5-13 cm, coriaceous, rough and stellately tomentose. Flowers are white or pale blue in colour and are borne on terminal panicles, Fruits are sub globose, somewhat 4 lobed and stellately hairy drupes seeds are ellipsoid, oblong, glabrous and reticulate.

T. grandis is distributed widely in Gujarat, Maharashtra, Tamilnadu, West Bengal, Madhya Pradesh and Kerala. Teak wood is a class by itself and has a world wide reputation becuase of its characters. Teak is used for high class furniture, boat and ship building, construction of carriages and wagons, houses and bridges and as railway sleepers. The wood is also used for making cabinets, plywood and musical instruments. The timber is impregnated by a large quantity of resinous matter which fills up every pore of the wood. It does not warp, split or crack. A yellow dye obtained from the bark

of Tectona is used for staining baskets. Distillation of the wood yields a valuable tar oil, used as a substitute for linseed soil.

The bark of Tectona is a powerful astringent. The extract from the wood is given in dyspepsia and heartburns and as an anthelmintic. It is used as a local refrigerent and sedative. Its paste or that of sawdust is a soothing dressing for the inflammation and irritation caused by marking nut; it is also used for dispersing inflammatory swellings and for relieving headache and toothache. The flowers and seeds are diuretic. The oil extracted from the fruit is a hair tonic in that it promotes growth of hair and useful for the cure of skin itch (Dastur, 1977).

Earlier workers have reported the presence of quinones, lapachol, dehydrotectol and tectoquinone from the root (Joshi, et al.,1977), β-lapachone, dehydro-α-lapachone, tectol, 1,4,5,8-tetra-hydroxy-2-isopentadienyl anthraquinone (Agarwal et al.,1965) quinone A_6,A_7 (O-quinoid structure belonging to naphthalene series) from leaves (Scandermann and Simatupang, 1965), 2,5, diOH-1-1- OMe-3-methyl anthraquinone from cultures of stem tissue (Dhruwa et al.,1973) and saponins from the bark (Bhattacharjee and Das, 1969) of Tectona.

Compounds like Lapachonone (Sandermann and Dietrichs, 1959), tectol, tectoquinone and dehydrotectol (Rudman,1961)

and heart wood extractives (Puri,1967) have been reported to be toxic to parasitic microorganisms. The natural durability of the wood of T. grandis is mainly due to the substances present in them. The decay resistance in Teak was studied by Rudman and Dacosta (1959) and it was shown that the wood resistant to Coriolus versicolor loses its resistance when extracted with ether-methanol mixture. Changes in phenolic compounds of teak leaves induced by powdery mildew infection have also been reported (Karadge et al.,1980).

In the present work leaves of T. grandis was screened for pre-infectional antifungal compounds and post-infectional compounds including phytoalexins. Fusarium solani (Mart.) Sacc. was chosen as the non-pathogenic test fungus for the induction of phytoalexins. Though the fungus is not pathogenic to Tectona, it causes a number of diseases in plants like Pyrus domestica (Jamaluddin and Tandon, 1977), Vitis Vinifera (Lele et al.,1978) and Manihot esculantum (Sivaprakasam et al.,1977) etc.

MATERIALS AND METHODS

Leaves of Tectona grandis was collected from pavagadh hills, Gujarat. The procedures followed for the extraction, isolation and identification of compounds, pathogenicity tests, Bioassay tests and the drop diffusate technique is described in Chapter-2.

RESULTS

Curvularia clavata Jain (IMI 316691) was isolated from the infected leaves of Tectona grandis. Pathogenicity tests have confirmed that Curvularia clavata is pathogenic on leaves of T. grandis (Fig.1). This is a new host record in India.

The diseased lesions developed 6-7 days after inoculation of the fungus. At first, brown coloured spots developed, which later got enlarged to blackish brown patches. The symptoms were visible on both sides of the leaves but were more towards the upper side. In forests the leaf spot disease is seen during the months from July to September.

SCREENING OF PRE-INFECTIONAL ANTIFUNGAL COMPOUNDS FROM LEAF EXTRACTS:

Of the many fractions collected, petroleum ether fraction (A) and water fraction (B) of the petroleum ether extract, chloroform fraction (C) and water fraction (D) of the chloroform extract, methanol fraction (E) and water fraction (F) of the methanol extract and the water fraction (G) of the water extract treated on both the pathogenic fungus C.clavata and the non-pathogenic fungus F. solani, the fractions A,D and E were found to exert no effect on the mycelial growth of Curvularia Sp. and Fusarium Sp. at all concentrations.

Fig. 1. Healthy (control) and diseased leaves of *Tectona grandis* Linn.

Fig.2 Spores of *Curvularia clavata* Jain.

FIG. 1

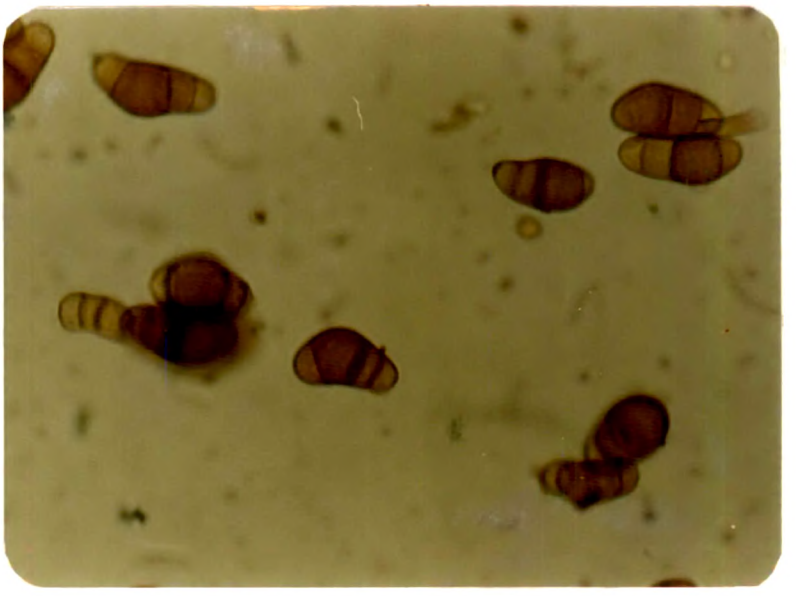

FIG. 2

Fractions C and F inhibited the growth of both Fusarium and Curvularia while fraction B inhibited only the former. Fraction C inhibited the growth of Curvularia Sp. at 10 ml dilution only (10 ml of the stock solution was added to the medium) while the same fraction inhibited the growth of F.solani at 5 and 10 ml dilution (Fig. 3).

Fraction F inhibited the mycelial growth of Curvularia Sp. and Fusarium Sp. at all dilutions (when 2, 5 and 10 ml of the stock solution was added). The per cent inhibition of colony diameter with respect to that of control of both the fungi are shown in Fig.4. Maximum inhibition of colony growth was noticed in 10 ml dilution in the case of both the pathogen and the non pathogen with each tested dilution, the mycelial growth of Curvularia Sp. was inhibited to a much greater extent than that of Fusarium Sp.

Fraction G stimulated or promoted the mycelial growth of both the pathogen and the non pathogen.

Fraction B severely inhibited the colony growth of the non-pathogen, F.solani (Fig.5). The PC of the fraction showed the presence of three simple phenols

(1) Rf-0.41 (in 15% acetic acid);brown in UV light, $\lambda \frac{Max}{MeOH}$ 333 nm

(2) Rf-0.51; brown in UV light; $\lambda \frac{Max}{MeOH}$ 340 nm and

(3) Rf-0.66; yellow in UV light; $\lambda \frac{Max}{MeOH}$ 265 , 330 nm

Fig.3. The effect of chloroform fraction(c) on the mycelial growth of Curvularia clavata (O—O) and Fusarium solani (•----•) as observed after seven days.

Fig.4. The effect of water fraction (F) of the methanol extract on the mycelial growth of Curvularia clavata (O — O) and Fusarium solani (•----•) as observed after seven days.

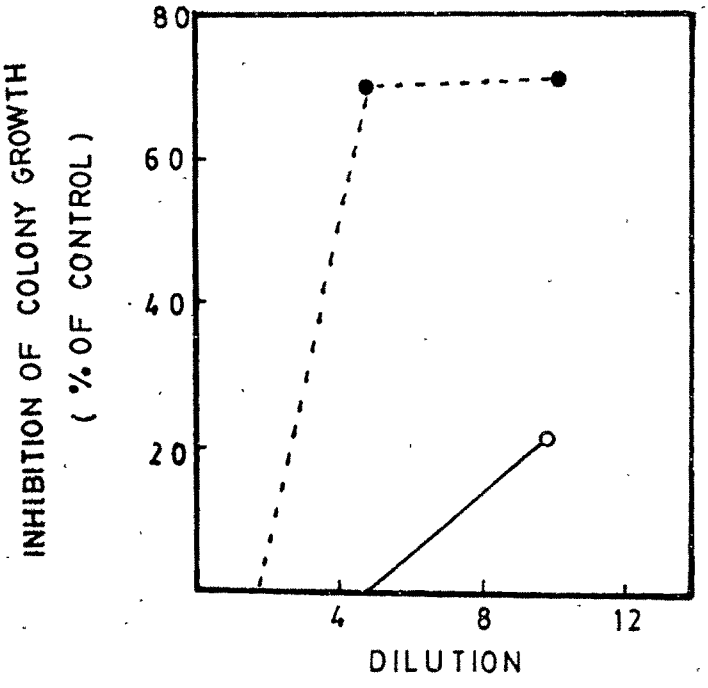
INHIBITION OF COLONY GROWTH
(% OF CONTROL)
80
60
40
20
4
8
12
DILUTION

FIG.3

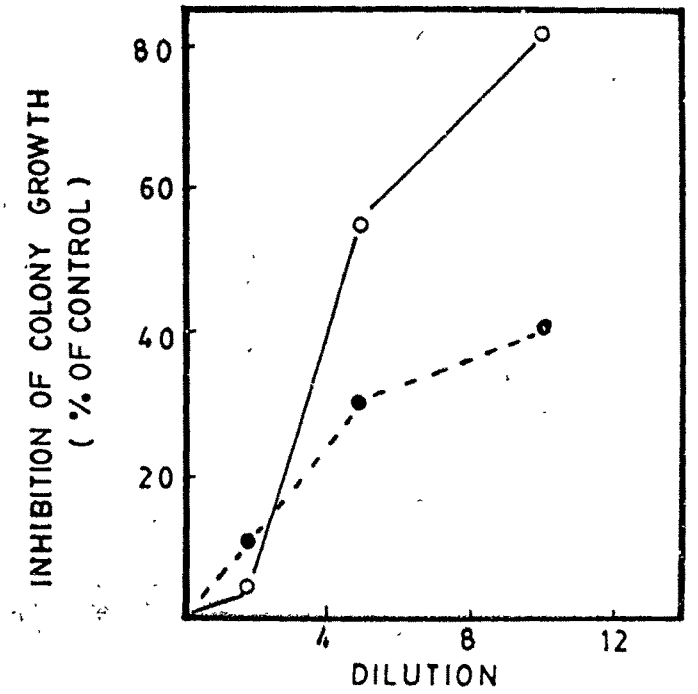
INHIBITION OF COLONY GROWTH
(% OF CONTROL)
80
60
40
20
4
8
12
DILUTION

FIG.4

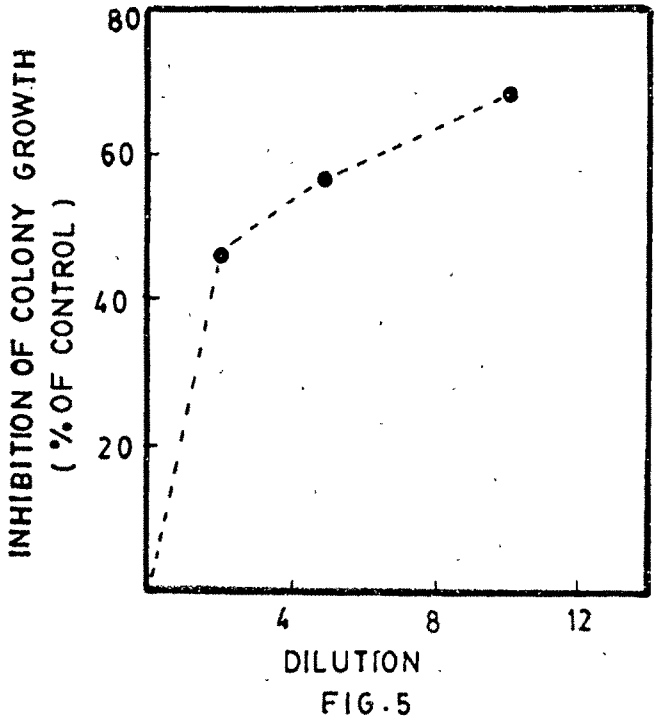
INHIBITION OF COLONY GROWTH
(% OF CONTROL)
80
60
40
20
4
8
12
DILUTION

FIG. 5

The effect of total phenols of F.solani is shown in Fig.6. Mycelial growth was strongly inhibited at all dilutions (2,5 and 10). Maximum inhibition of growth was noticed in 10 ml dilution.

SCREENING OF POST-INFECTIONAL COMPOUNDS

The distribution of proanthocyanidins, iridoids, alkaloids, saponins and tannins in healthy and infected leaves of T.grandis is presented in Table-III. Saponins were absent , while tannins, proanthocyanidins, iridoids and alkaloids were present in both the healthy and infected leaves of Tectona. Analysis of sugars, amino acids and steroids showed no qualitative changes between healthy and infected leaves.

The distribution of various phenolics in healthy and infected leaves of T.grandis is presented in Table IV. Both healthy and infected leaves contained vanillic, melilotic and gentisic acids and quinones. It was found that the fungus infected leaves of Tectona contained ferulic acid, 3',4'-dimethoxyquercetin, coumarins and a number of simple phenols instead of the 4'-OMe apigenin, luteolin, syringic and synapic acids in the healthy leaves.

INDUCTION OF PHYTOALEXINS

There was no significant chemical difference between the diffusates of control and treated leaves, when the healthy leaves were treated with the spore suspension of the pathogen.

C. clavata, whereas the diffusate with the spores of the non pathogen, F.solani contained p-hydroxybenzoic acid, which was absent in the diffusate maintained as control. TLC bioassay showed dense mycelial growth of F.solani in plates containing diffusates of control when compared to minimum growth of mycelia in the TLC plates with diffusates of the fungal treated leaves.

Mycelial growth assay of F.solani with p-hydroxybenzoic acid showed that F.solani was strongly inhibited at all concentrations (Fig. 7). Maximum inhibition was noted at 1000 ppm. Inhibition zone of the mycelial growth increased with increase in concentration (Fig. 8). Spore germination and germ tube elongation of F. solani were also inhibited by p-hydroxybenzoic acid at 300, 500 and 1000 ppm concentration (Fig. 9). A maximum of 85 per cent inhibition of spore germination and 64 per cent inhibition of germ tube elongation were noted at 1000 ppm.

p-Hydroxybenzoic acid inhibited the mycelial growth, spore germination and germ tube growth also of C. clavata at all concentrations. (Figs. 10 and 11). Maximum inhibition of mycelial growth was noted at 1000 ppm. A maximum of 58 percent inhibition of spore germination and 63 per cent inhibition of germ tube elongation was seen at 1000 ppm.

DISCUSSION

Of the different fractions of leaf extracts of T.grandis fractions C and F inhibited the growth of the pathogen and the

Fig.6. The effect of total phenols from leaves of Tectona grandis on the mycelial growth of Fusarium solani. The plates were incubated at 25° ± 2°C and observed after six days. (Dilutions: 1, 2, 5, 10; E-Control).

Fig.7. The effect of p-hydroxybenzoic acid on the mycelial growth of Fusarium solani. The plates were incubated at 25° ± 2°C for a period of seven days. Left (control) ; Right (Treatments : 300 ppm; 500 ppm and 1000 ppm)

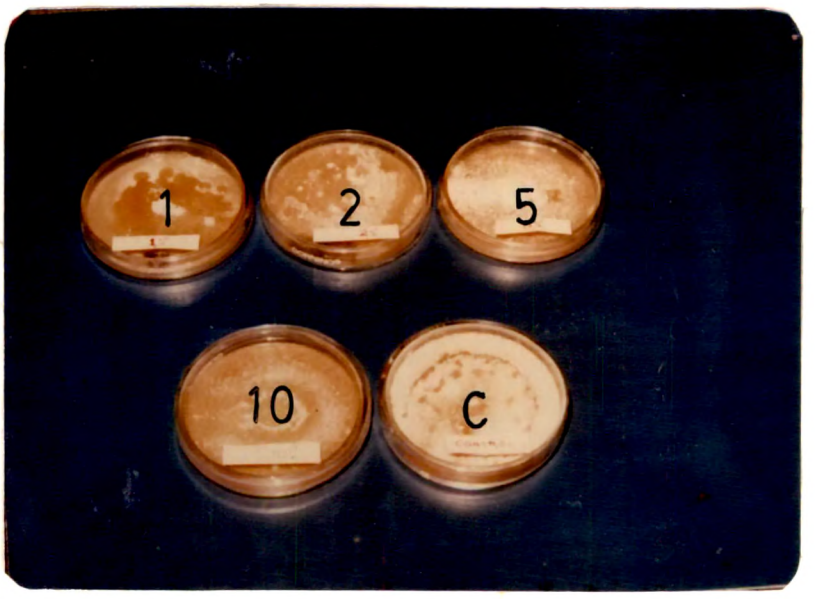

FIG.6

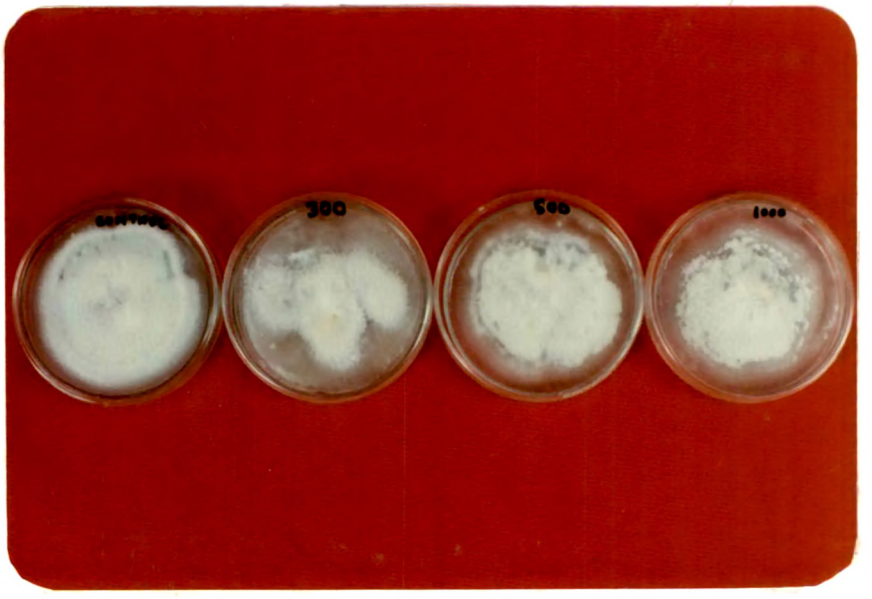

FIG.7

Fig.8. The effect of p-hydroxybenzoic acid on the mycelial growth of Fusarium solani. Days after inoculation: 2 days (•---•), 4 days (⊙——⊙) and 7 days (O——O).

Fig.9. The effect of p-hydroxybenzoic acid on spore germination (O---O) and germ tube length (•——•) of Fusarium solani after 8 hours of incubation at 25° ± 2°C.

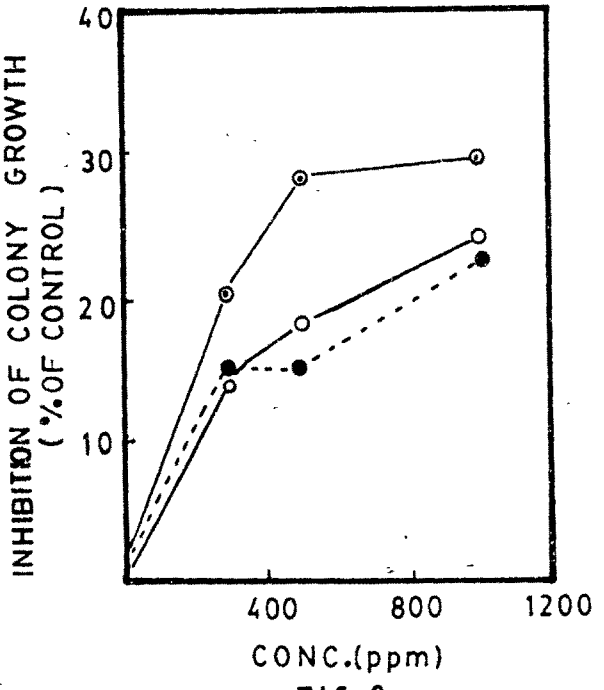
INHIBITION OF COLONY GROWTH
(% OF CONTROL)
40
30
20
10
400
800
1200
CONC.(ppm)

FIG.8

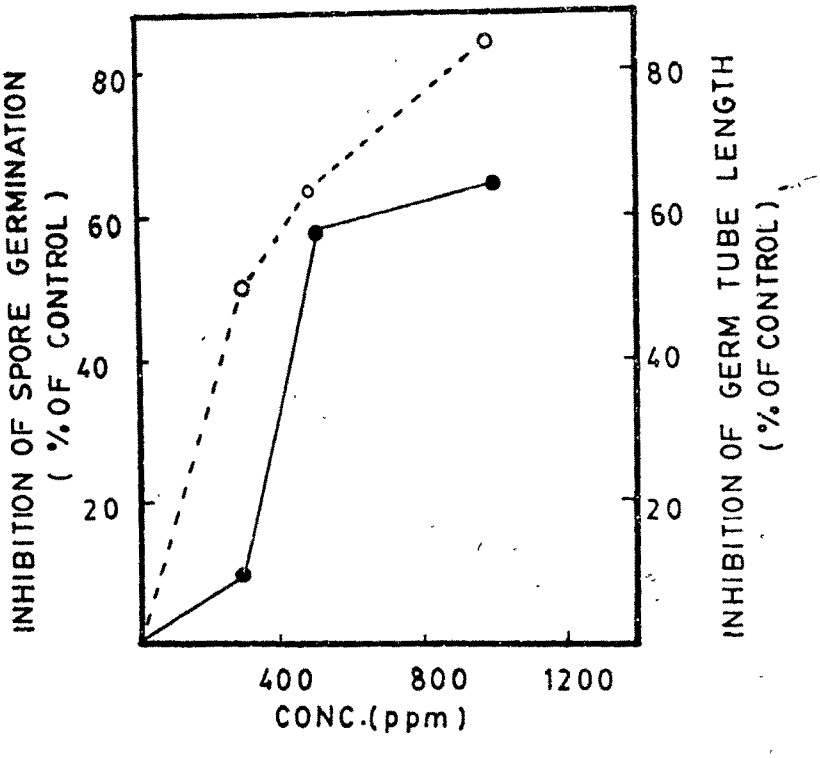
INHIBITION OF SPORE GERMINATION
(% OF CONTROL)
80
60
40
20
INHIBITION OF GERM TUBE LENGTH
(% OF CONTROL)
80
60
40
20
400
800
1200
CONC.(ppm)

FIG. 9

Fig.10. The effect of p-hydroxybenzoic acid on mycelial growth of Curvularia clavata. Days after inoculation: 3 days (O—— O), 6 days (•——•).

Fig.11. The effect of p-hydroxybenzoic acid on spore germination (O-----O) and germ tube length (•—— •) of Curvularia clavata after 6 hours of incubation at 25° ± °C.

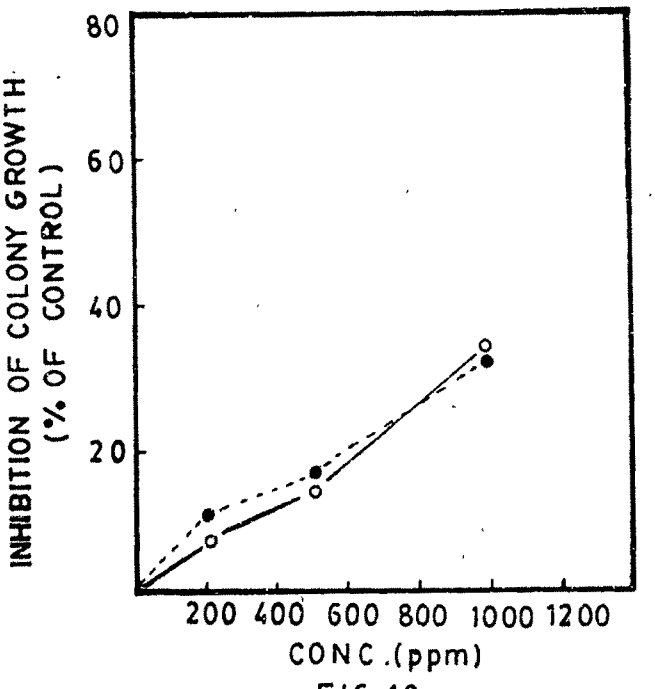
INHIBITION OF COLONY GROWTH
(% OF CONTROL)
80
60
40
20
200 400 600 800 1000 1200
CONC.(ppm)

FIG.10

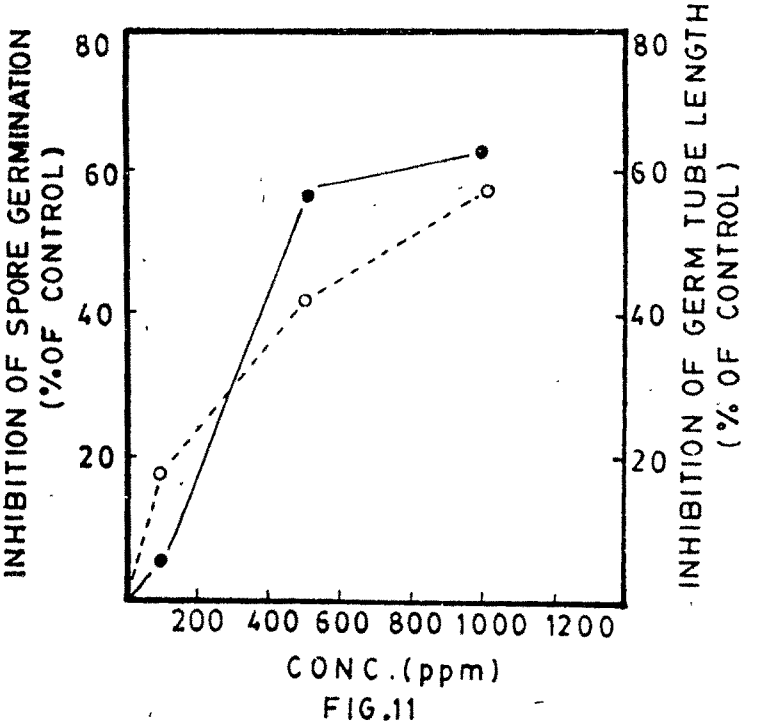
INHIBITION OF SPORE GERMINATION
(%OF CONTROL)
INHIBITION OF GERM TUBE LENGTH
(% OF CONTROL)
80
60
40
20
200 400 600 800 1000 1200
CONC.(ppm)

FIG.11

non-pathogen at 10 ml dilution showing that the fractions contain antifungal compounds. Even though *in vitro* studies showed the presence of such antifungal compounds, these substances do not prevent the pathogen from infecting the plant, suggesting that these compounds may not be present in appreciable amounts to prevent infection or that these compounds may not be toxic at *in vivo* conditions. This supports Tomiyama's suggestion (1963) that the toxic compounds extracted from the cells may not always play the same role or may not possess the characters in a living cell as *in vitro*.

The fact that fraction B containing three simple phenols strongly inhibited the growth of the non-pathogen proves that it is these phenols which give resistance to the plant against *F.solani*. This indicates the presence of preinfective antifungal phenols in *T. grandis*. Similar pre-infective phenols are already reported from plants such as *Hordeum vulgare*, *Amaranthus tricolor*, *Populus hybrida* and *Solanum tuberosum* (Ludwig *et al.*,1960; Chandramohan *et al.*,1967; Glattes, 1971; Swamination and Kochler,1976).

In vitro studies in *Tectona* grandis have shown that the phenols from the leaves do not restrict the growth of the pathogen even though phenols from plants are known to be toxic. Thus *in vivo* the pathogem overcomes the resistance brought about by the phenols in the plant may be by detoxifying the phenols rendering the plant susceptible to

to the pathogen. A similar situation is also seen in Solanum and Thuja. The potato peel contains as much as 0.3 to 0.6 mg/g of dry tissue of solanine (Lampitt et al., 1943). These concentrations are much more than necessary to inhibit parasitic fungi (Mc Kee, 1959), but does not do so. Furthermore, in the heartwood of Thuja plicata, the fungitoxic thujaplicins, present to an extent of 4-5%, nevertheless cannot prevent the disease. Similar results have also been reported by a few other investigators (Wahloroos and Virtanen, 1969; Stoessl and Unwin, 1969).

Fraction G stimulated growth of both the pathogen and the nonpathogen indicating that the compounds from the fraction helped in promoting the growth of the fungi. Similar stimulation is exhibited when the juice of oat seedlings was applied to Helminthosporum avenae and Ustilago avenae, parasites of oat. It is to be noted that the same extract was inhibitive to the spore germination of all the fungi tested which were nonpathogens of oat Ustilago tritici, U. nuda, U. zeae Helminthosporium oryzae and H. turcium (Tomiyama et al., 1952).

It is clearly evident that fungal infection brings about a number of changes in the chemical constitution of plants.

The replacement of syringic and sinapic acids by ferulic acid is significant. Ferulic acid is known for its antifungal activity against Poria weirii (Li et al., 1972) and Fusicocum amygdali (Borys and Childers, 1964) and imparting

resistance in wheat varieties (Chigrin and Rozuru, 1969) and inactivation of tungro virus *in vivo* (Sridhar *et al.*,1979).

The production of the flavonol 3',4'-diOMe quercetin in place of 4'-OMe apigenin and luteolin is also a significant change. This indicates a change in biosynthetic pathway in which flavonols, with more phenolic hydroxyls which can act as better antifungal agents are produced. The role of flavonols in disease resistance of cotton is already proved. *Verticillum* resistant terminal leaves of cotton plants possess higher concentration of constitutive flavonols and synthesise more flavonols than other susceptible leaves in response to infection (Howell *et al.*,1976). Flavonoids also accumulate more rapidly in resistant than susceptible corn cultivars in response to infection by *Colletotrichum* (Hammerschmidt and Nicholson, 1977).

The production of p-hydroxybenzoic acid in the diffusate (treated) of the plant in response to spores of *F. solani* is quite noteworthy. It is also interesting to note that the phenolic acid is produced in response to the non-pathogen and not the pathogen. This clearly indicates that plants offer more resistance to the non pathogen than to the pathogen. Studies of *Trifolium* have shown that in non pathogen infected tissue the phytoalexin always occured in high amounts where *T. pratense* was inocuted with *Sclerstium trifolium* (pathogen) and *Botrytis cinerea* (non pathogen) (Debner and Smith, 1976).

The production of phytoalexins by a non pathogenic fungus is seen in potatoes also, where rishitin, a phytoalexin is produced by a incompatible race of phytophthora infestans and that no rishitin production occurs in potatoes infected by a compatible race.

The strong inhibition of mycelial growth, spore germination and germ tube growth of F. solani and C.clavata by p-hydroxybenzoic acid further proves the antifungal activity of this compound. The immediate production of p-hydroxybenzoic acid as a response to fungal attack and its antifungal activity leads to the conclusion that p-hydroxybenzoic acid could be called a phytoalexin. Incidentally p-hydroxybenzoic acid is also reported to be an intermediate in the production of a fungicide. (Merck index). It would be interesting to correlate the report of benzoic acid, as a phytoalexin, in apples infected by Nectria galligena Bres with this. Furthermore, p-hydroxybenzoic acid was also found to produce in Nectria infected apples (Swinburne,1971).

Though p-hydroxybenzoic acid occurs in plants as a component of lignin, its role in imparting resistance to the plant is already evidenced by the studies on carrot slices using Botrytis cinerea. In this plant, infection leads to the production of antifungal compounds like p-hydroxybenzoic acid and 6-methoxymellin (Harding and Heale, 1980,1981). The production of more p-hydroxybenzoic acid indicate more

lignification. Lignification either directly or indirectly would also form a potential barrier to infection. (Kolattukudy, 1975; Henderson and Friend, 1979). The importance of lignification is seen in Phytophthora infestans - potato interaction (Friend, 1976; Henderson and Friend, 1979) and also in disease resistance and immunization of non-solanaceous plants. (Hammerschmidt and Kuc, 1980; Ride, 1980, Vance et al., 1980). Asada et al., (1972) also reported the production of phenolic acids in the roots of Japanese raddish infected by Pernospora parasitica. They emphasized the importance of these phenolic acids in the defence mechanism of roots, especially the formation of lignin.

p-hydroxybenzoic acid was also found to be more toxic to the non-pathogen than the pathogen. Increased toxicity to the non-pathogen is also evidenced by the investigations on the antimicrobial spectra of phytoalexins, pisatin and phaseollin (Cruckshank, 1962; Cruickshank and Perrin, 1971; Van etten, 1973). Similar results are reported by Nonaka and Yasui (1966) who examined the toxicity of ipomeamarone, the main furanoterpenoid phytoalexin in sweet potato, to six fungi; Ceratocystis fimbriata, three strains of Fusarium solani, Gibberella zeae and Piricularis oryzae and found that, ipomeamorone was more toxic to non pathogenic fungi than to the pathogenic ones.

Table III The distributions of Proanthocyanidins, iridoids, alkaloids, saponins and tannins in healthy and fungal infected leaves of Tectona grandis.

Tectona grandis	1	2	3	4	5
Healthy leaves	+	+	+	.	+
Infected leaves	+	+	+	.	+

1. Proanthocyanidins 2. Iridoids 3. Alkaloids
4. Saponins 5. Tannins

Table IV : The distribution of phenolics in the healthy and fungal infected leaves of *Tectona grandis*.

Tectona grandis	1	2	3	4	5	6	7	8	9	10	11	12
Healthy leaves	+	+			+	+	+	+	+		•	+
Infected leaves			+	+			+	+	+	+	+	+

1. 4'-OMe apigenin 2. Luteolin 3. 3'4'-dimethoxyquercetin
4. Ferulic acid 5. Syringic acid 6. Sinapic acid
7. Vanillic acid 8. Melilotic acid 9. Gentisic acid
10. Coumarins 11. Simple phenols 12. Quinones