PHYTOALEXINS OF *CASSIA FISTULA* LINN. AND *MORINDA TOMENTOSA* HEYNE

CHAPTER IV

PHYTOALEXINS OF *CASSIA FISTULA* LINN. AND *MORINDA TOMENTOSA* HEYNE

Cassia fistula and *Morinda tomentosa* are two economically important forest trees of Gujarat. Though some data on the diseases of these plants are available, no serious effort has been made to study the post-infectional changes or the phytoalexin synthesis occuring in these two trees. In the present study, an attempt is made to study these aspects of fungal infection.

1.1 *CASSIA FISTULA* LINN.

Cassia fistula is a treee found throughout the deciduous forests and plains of India ascending to the foothills of the Himalayas. This is one of the most beautiful of our indigenous trees and its adds colour to our hills during the drier and hotter periods of summer. It is a small medium sized open branched deciduous tree with smooth greenish grey bark turning to brown with age. Leaves 20-45 cm long, 8-20 x 3-55 cm ovate or elliptic, glabrous above and glabrescent beneath, subcoriaceous and petiolulate. The fresh leaves are often of a rich copper colour with a soft damp undersurface and remain pendulous and folded until fully grown leaves fall in March-April and the first half of May. It is nearly leafless at the time of flowering. Flowers bright to golden yellow in 20-40 cm

long pendulent branched racemes. The first splashes of golden coloured flowers appear as the last of the old leaves are shedding. Fruit pods are long and rounded, 30-60 cm in length and 7-10 cm in diameter and are dark brown.

The timber of Cassia is hard and durable, and is used in making carts and agricultural implements. The bark of the tree is used for tanning. The leaves are used as emollient and their juice or paste is useful against ring worms and for relief of dropsical swellings. For relief of rheumatism and facial paralysis, the leaves are rubbed into the affected parts.

The pulp from the pods are of great therapeutic value, it is a mild pleasant and safe purgative even for children and expectant mothers. It is best combined with other purgatives as a confection, as by itself it requires to be taken in doses from one to two ounces to produce any effect, It is official in the British Pharmacopoeia as an ingredient of senna (Dey, 1984). The pulp is applied round the navel of a patient suffering from flatulence to cause evauation. It is also mixed with linseed or almond oil to remove intestinal obstruction.

The flowers are given as decoction in certain stomaichic affections. They are also used as food by the hill tribes of India. The root is used as a tonic, febrifuge and a strong purgative. An alcholic extract of the root bark is used as a cure for black water fever. (Ventatachalam and Ratnagiriswiran, 1941).

The Cassia pulp has been shown to contain one percent of anthraquinone derivatives, 50% sugars, gums, colouring matter and a small amount of volatile matter (Trease, 1952). Three waxy substances mp (1) 52-53°C (ii) 64-68°C and (iii) 114-116°C, an anthraquinone derivative melting at 250°C and a major anthraquinone, Rhein (1,8-dihydroxy-3-carboxyl-anthraquinone) were the compounds isolated from Cassia pulp (Modi and Khorana, 1952). The antibacterial activity of Cassia pulp was also studied by patel and Patel (1956). Bordoloi et al., (1964) have reported that polyphenolic extracts of C. fistula inhibited the spore germination of Colletotrichem falcatum.

The root bark of C. fistula, beside tannins, contains phlobaphenes and oxyanthraquinones. The leaves of C. fistula contain glycoside of 1-8-dihydroxyanthraquinone derivatives, Sennoside A and Sennoside B (Kaji and Khorana, 1964). The stem bark of C. fistula contain a substance called as fistucacidin (racemic or meso-3,4,7,8,4' pentahydroxyflavan), (Vankateshwarlu and Rao, 1964).

1.2 MORINDA TOMENTOSA HEYNE

Morinda tomentosa is a small tree commonly found in the deciduous forests of Gujarat and other central and southern parts of India. It has a blackish-brown, rough, irregularly fissured bark. Leaves 2.4-6.5 x 3-15 cm and are elliptic-oblong or obovate-oblong and tomentose. Flowers are white in fleshy

globose heads. Fruits 2-3 cm across of coalescent drupes, bright and glaucous-green in colour. Seeds are oblong, rough and pale to dark brown in colour.

The heartwood of M.tomentosa is durable and is largely used for making furniture. The leaves are used as a tonic and febrifuge, with aromatics they are given in dysentry and diarrhoea. The leaf juice is used to heal sores, ulcers and wounds. The fruit is a deobstrent and the fruit is given in dysentry and asthma. A mixture of the charred unripe fruit and salt is effective for spongy gums (Dastur, 1977). A red dye is obtained from the roots of M. tomentosa and have been extensively used under the name 'Suranji' as dyestuff. The colouring matter is mainly found in the root bark and is developed in large quantities at the end of the 3rd or 4th year of the growth after which it gradually disappears.

The compounds isolated from the heartwood of Morinda are (i) 5,7-dihydroxy,2-methylanthraquinone (ii) 1,5,6-trihydroxy-2-methylanthraquinone (morindone) (iii) Moridin (a glycoside of morindone (iv) 3-hydroxy-1-methoxy-2-methylanthraquinone (rubiadin-1-methyl-ether) (v) 1,3-dihydroxy-2-formyl-anthraquinone (nordammacanthal) (vi) 2-hydroxy-1-methoxy-2-formylanthraquinone and (vii) 2-hydroxy-1-methoxyanthraquinone (alizarin-1-methyl ether) (Murti et al., 1959).

In the present work both C. fistula and M.tomentosa were studied for post-infectional compounds and their

phytoalexin response. For inducing phytoalexins, pathogen isolated from the plants as also a non pathogen Fusarium Solani (Mart.) Sacc. were the test fungi used.

2. MATERIALS AND METHODS

Healthy and infected leaves of Cassia fistula and Morinda tomentosa were collected from the Botanical Garden, M.S. University Campus, Baroda. The diseased leaves were collected soon after the plants got infected. Fresh healthy leaves were used for the pathogenicity test and the drop diffusate studies.

The procedures followed for the extraction, isolation and identification of compounds, bioassay tests, pathogenicity tests and the drop diffusate technique is described in chapter 2 (Matherials and Methods).

3. RESULTS

Aspergillus niger van Tiegh. (IMI,316686) and Colletotrichium gleosporoides (Penzig) Penzig and Sacc. (IMI, 316690) were the fungi isolated from the infected leaves of Cassia fistula and Morinda tomentosa respectively. Pathogenicity tests confirmed that A. niger and C.gleosporoides were pathogenic on their respective host plants from where they were isolated. Fig.12 and Fig.13 show diseased leaves of Cassia fistula and M. tomentosa after artificial inoculation with spores of the respective pathogens.

Fig.12. Diseased leaves of Cassia fistula Linn.

Fig. 13. Diseased leaves of Morinda tomentosa Heyne

FIG. 12

FIG. 13

The leaf spot disease in Cassia fistula was found to in the months of January and February. The diseased lesions developed 6-8 days after inoculation with the pathogen. The symptoms were visible as irregular small black spots. The spots enlarged a little more in size 1-2 days after the first symptoms were seen. The diseased lesions were scattered on the upper side of the leaf.

In M. tomentosa, the leaf spot disease was noted in the months of October to December. The first signs of diseased lesions were seen 5-6 days after inoculation with C. gleosporides. The symptoms were seen as blackish brown, irregular, small and big patches. The visible symptoms were seen on both upper and lower surfaces, but more towards the tip of the leaf.

3.1 SCREENING OF POST-INFECTIONAL COMPOUNDS

The distribution of proanthocyanidins, iridoids, alkaloids, saponins and tannins, present in the healthy and infected leaves of C. fistual and M. tomentosa is presented in Table V. Saponins are absent from both the plants while alkaloids were present in both. Proanthocyanidins and tannins were seen only in C. fistula and iridoids in M. tomentosa.

The flavonoids and phenolic acids present in healthy and infected leaves of Cassia fistula and Morinda tomentosa are presented in Tables VI and VII. The flavonoids present in

healthy leaves of both the plants are methoxylated flavonols (3'4'-diOMe quercetin and 4'-OMe Kaempferol in M.tomentosa and 4'-OMe quercetin, 4'-OMe Kaempferol and 7,3'4'-tri OMe quercetin in C.fistula) The infected leaves of both these plants contained quercetin and Kaempferol, the demethylated derivatives of the above compounds. The concentration of the flavonoids in the infected leaves was much higher than those of the healthy leaves. Syringic, ferulic, vanillic and gentisic acids were present in both healthy and infected leaves of M.tomentosa. Syringic and p-Coumaric acids were present in healthy leaves of C. fistula while the infected leaves contained syringic and o-Coumaric acids.

When fresh healthy leaves of C.fistula were exposed to the pathogenic fungus, A niger the results were found to be similar to those obtained in vivo i.e. the flavonols, quercetin and Kaempferol were present in the diffusate (treated) while the control contained the methylated derivatives of the above compounds (Fig. 15)

The same pattern of results was seen in the case of M. tomentosa also,when healthy leaves were treated with spores of the pathogenic fungus C. gleosporoides.

Both the plants showed no qualitative difference in response when treated separately with spores of a common non pathogen F. solani.

Fig.14. Spores of Colletotrichum gleosporoides (Penzig) Penzig and Sacc.

Fig.15. A paper chromatogram showing compounds from diffusates of the leaves of Cassia fistula treated with spores of Aspergillus niger. Control (left) and treated (right).

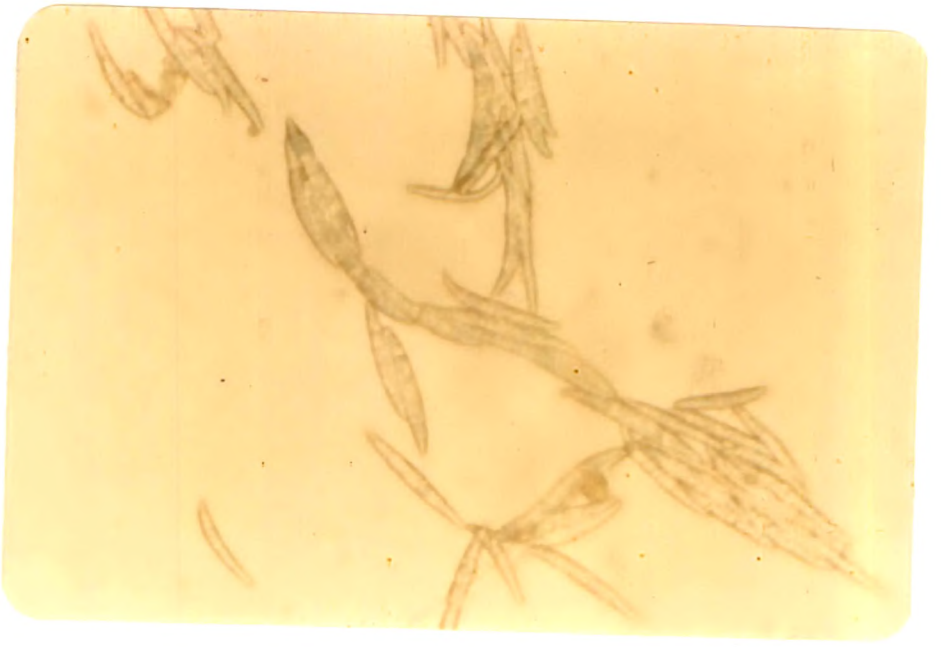


FIG .14

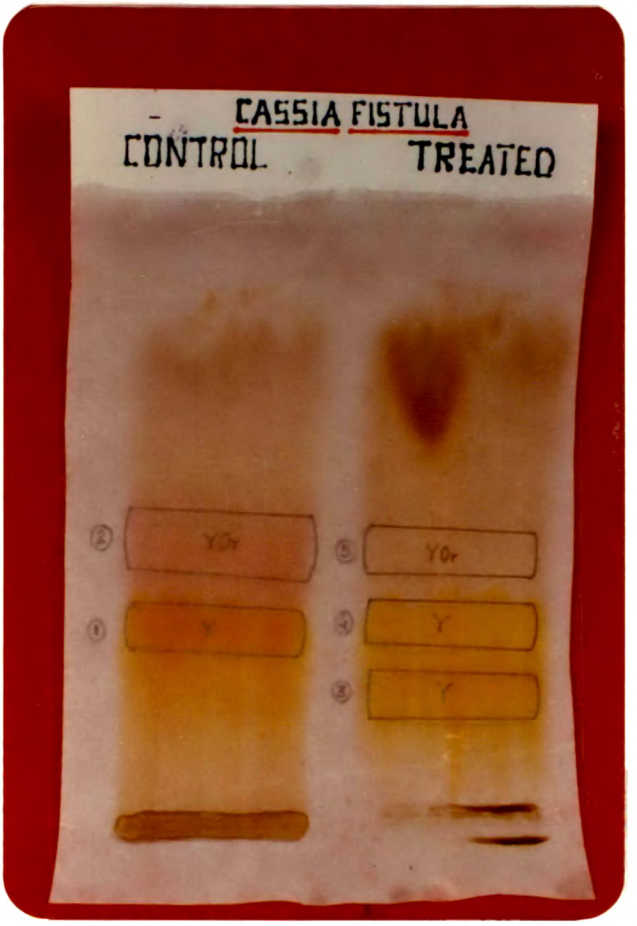


FIG .15

3.2 BIOASSAY TESTS

Quercetin, one of the compounds induced by the fungus was tested for its antifungal activity against both the pathogen, A. niger and C. gleosporoides. Quercetin inhibited the spore germination and germ tube elongation of A. niger and C. gleosporoides (Fig.16 and Fig.17) and also their mycelial growth at different concentrations. A maximum of 49 per cent inhibition of spore germination and 35 per cent inhibition of germ tube elongation were noted at 500 ppm in the case of A. niger while in C. gleosporoides a maximum of 32 per cent inhibition of spore germination and 53 per cent inhibition of germ tube elongation were seen at the same concentration. Quercetin inhibited the mycelial growth of both the fungi at 100 and 500 ppm (Fig.18 and Fig.19).

Maximum inhibition of mycelial growth of both fungi were seen at 500 ppm. Periodic measurements revealed that after a period of 4 days of incubation at 25 ± 2°C, the inhibition zone was greater in case of both the fungi than the later period of incubation (from 4 to 10 days). During the period between the 4th and the 10th day of incubation, the fungal colony in the plates treated with quercetin of both fungi grew more rapidly then their initial rate. The hyphal density was also lesser. Fig.20 andFig.21 shows the per cent inhibition of the mycelial growth of A. niger and C. gleosporoides by different concentrations of quercetin measured every 2 days.

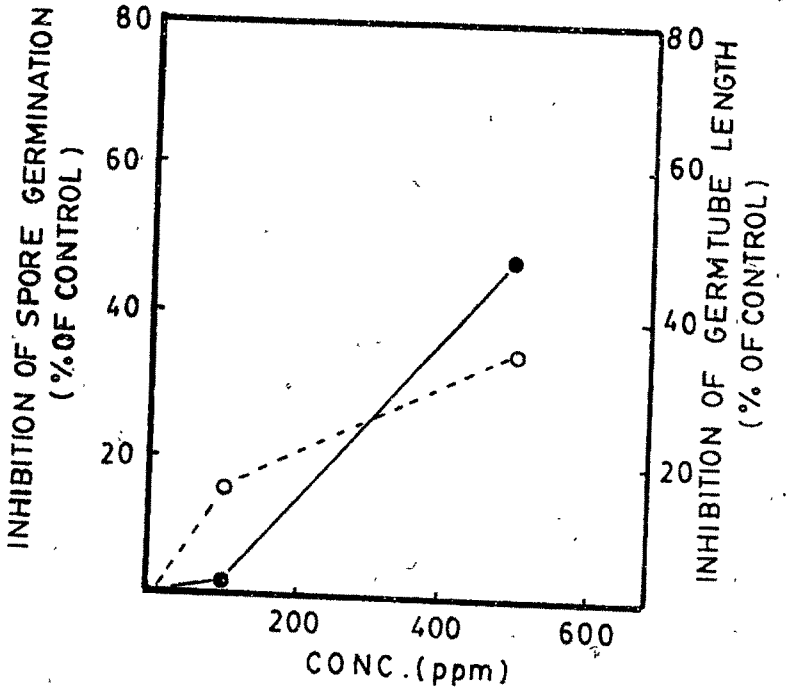


Fig.16. The effect of quercetin on spore germination (•——•) and germtube elongation (O----O) of Aspergillus niger after 24 hours of incubation at 25° ± 2°C.

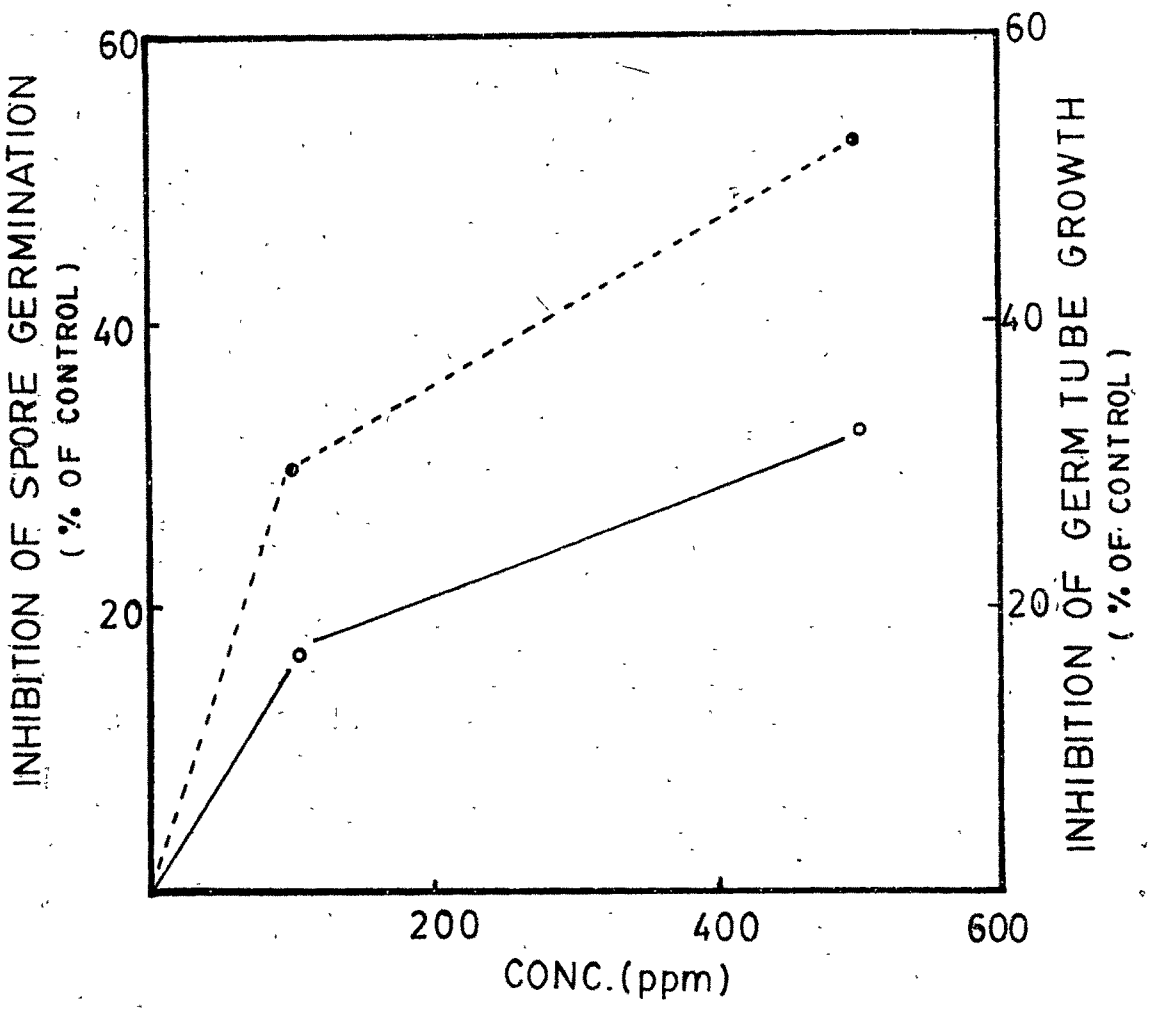


Fig.17. The effect of quercetin on spore germination (O——O) and germtube elongation (•——•) of Colletotrichum gleosporoides after a incubation period of 24 hours at 25° ± 2°C.

Fig.18. The effect of quercetin on the mycelial growth of Aspergillus niger. The plates were incubated at 25° ± 2°C for 10 days. Control (left), Treatments: 100 ppm, 500 ppm (Right).

Fig.19. The effect of quercetin on the mycelial growth of Colletotrichum gleosporoides. The plates were incubated at 25° ± 2°C for 10 days. Control (left) Treatments: 100 ppm, 500 ppm, (Right).

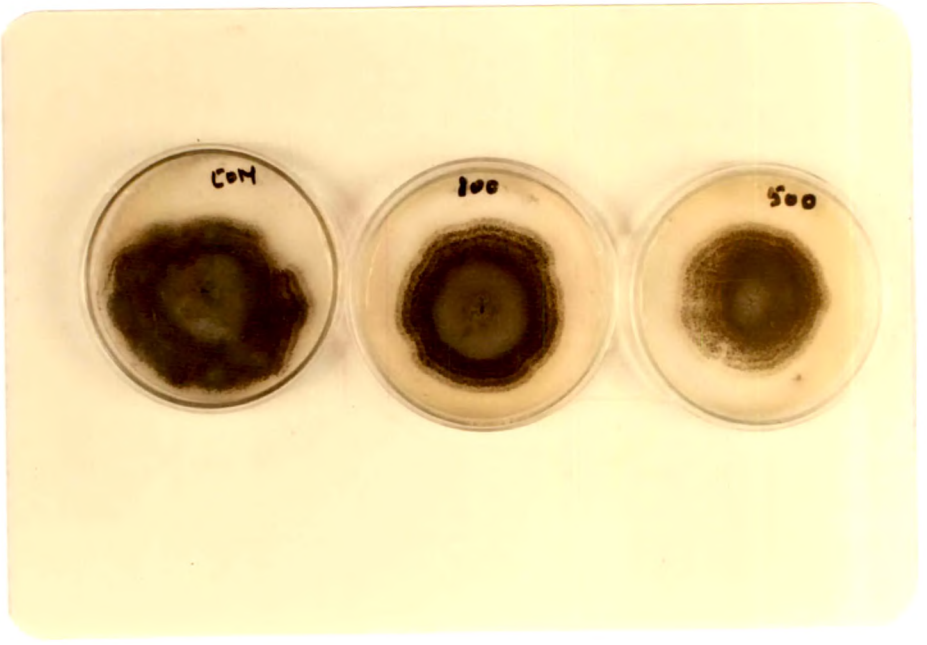


FIG. 18

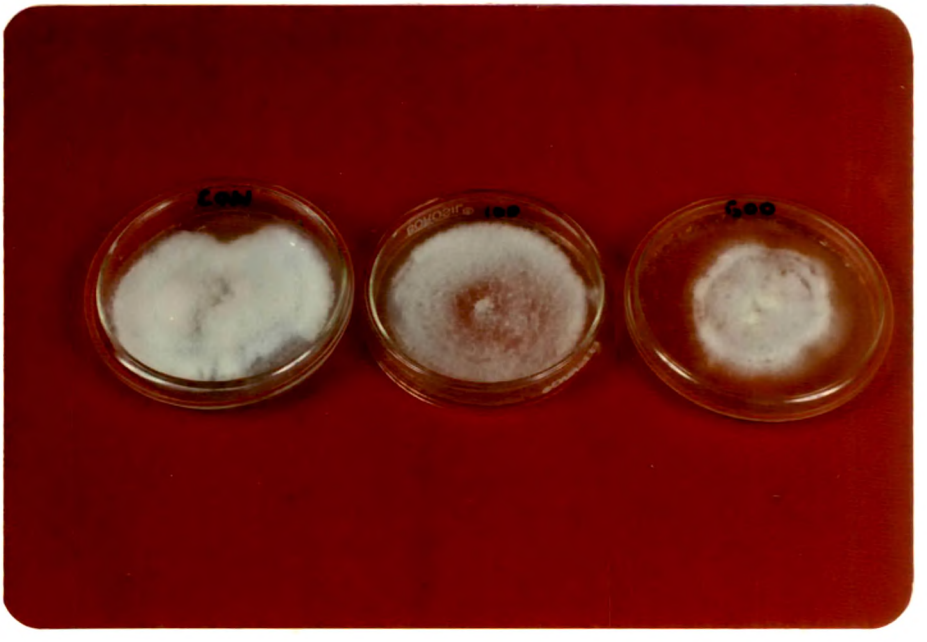


FIG. 19

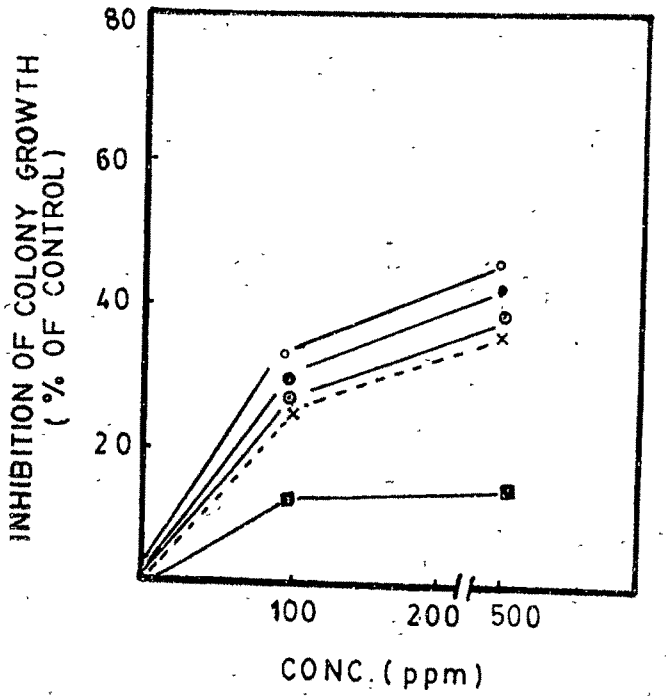


Fig.20. The effect of quercetin on the mycelial growth of Aspergillus niger. Days after inoculation: 2 days (■—■), 4 days (O—— O), 6 days (●—●), 8days (⊙—⊙), 10 days (x---x).

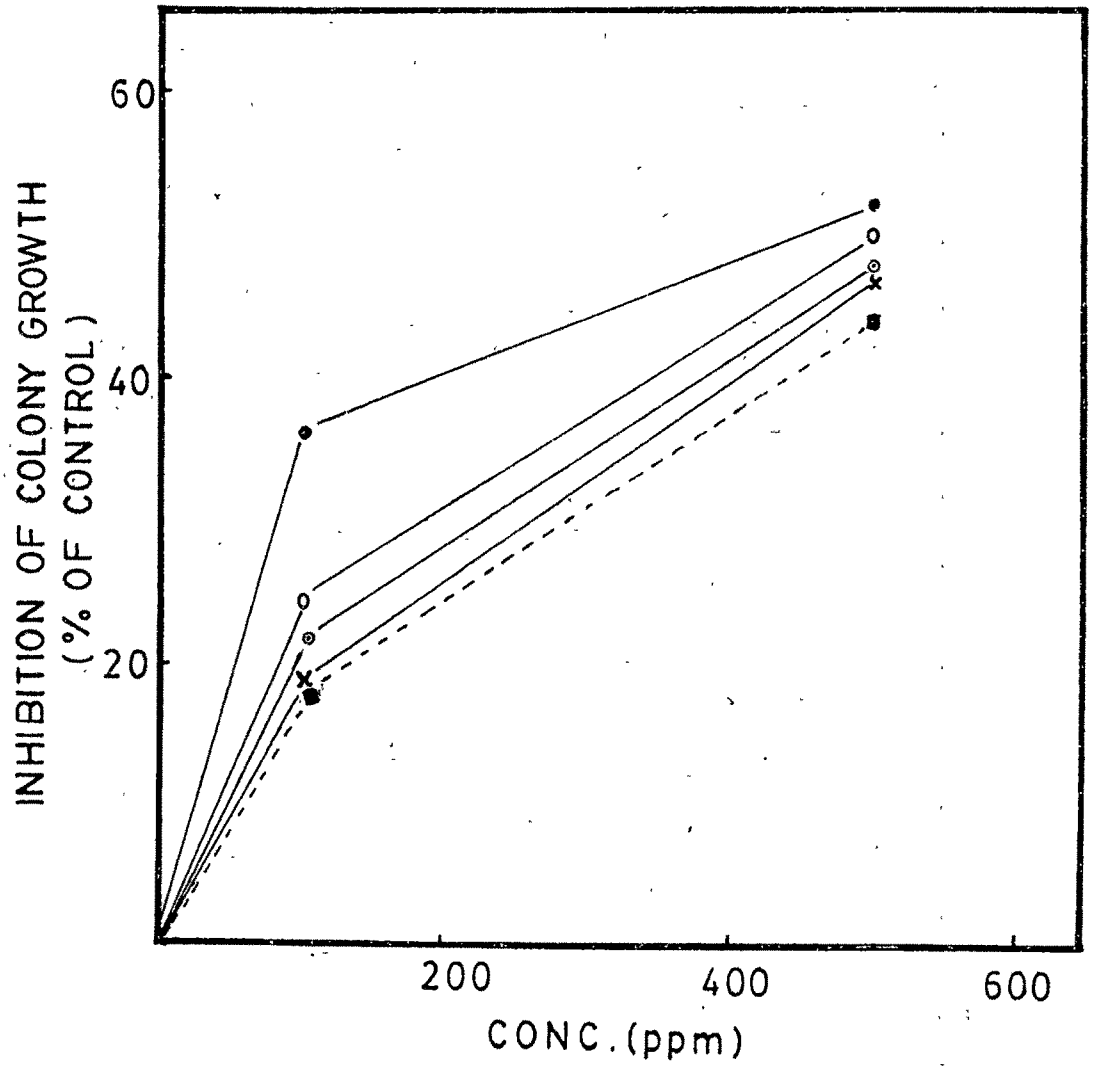
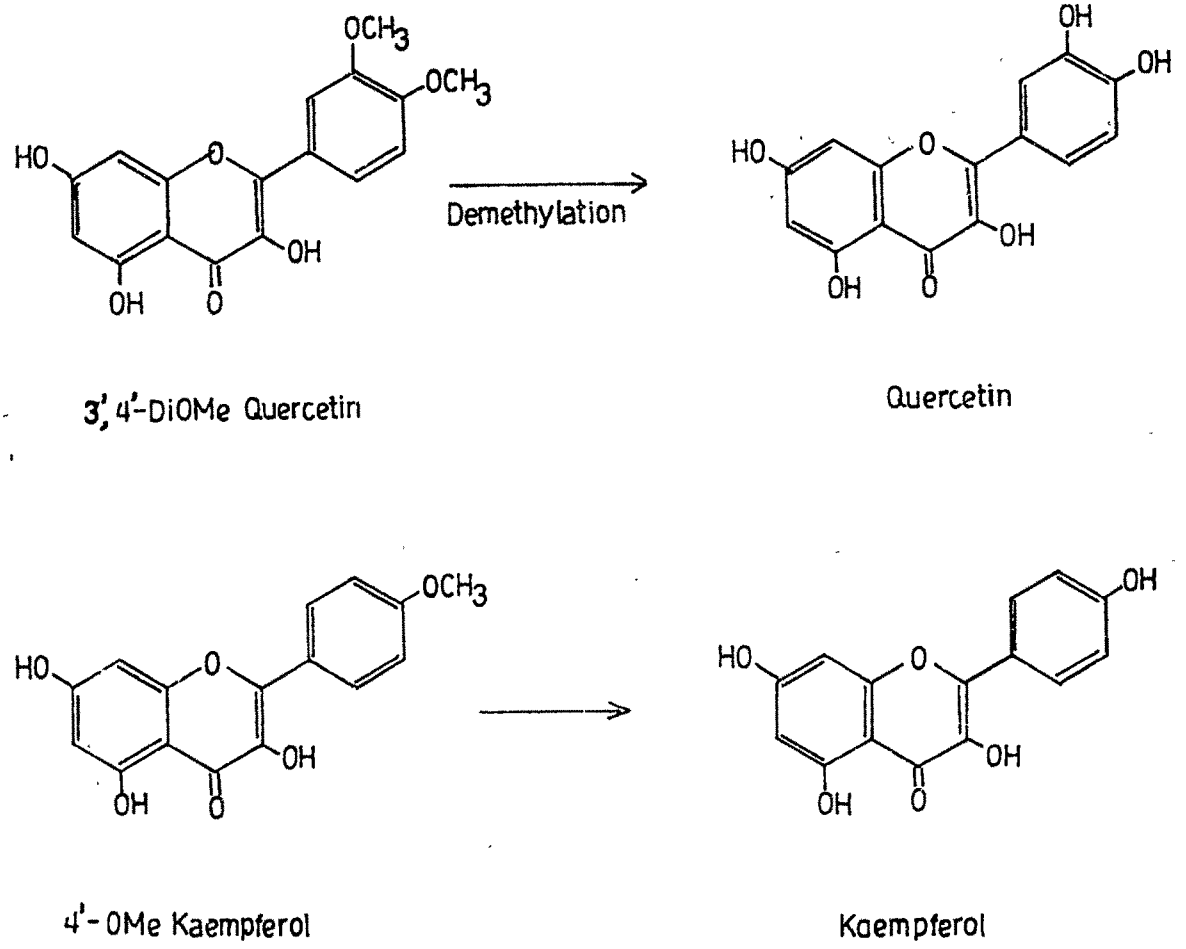


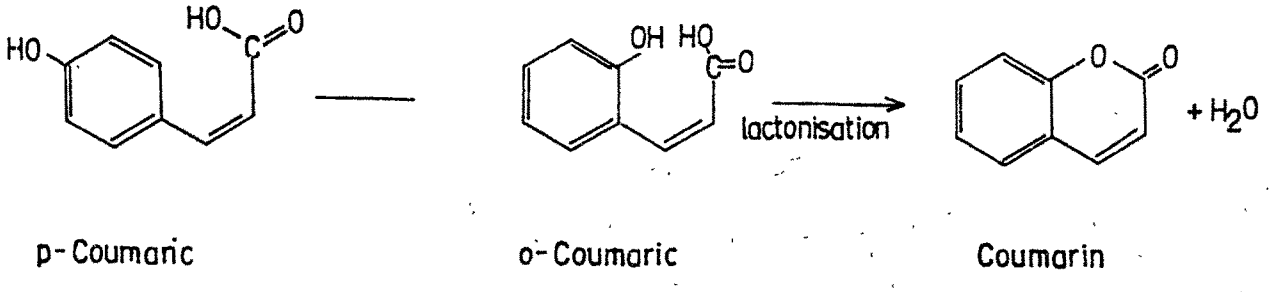
Fig.21. The effect of quercetin on the mycelial growth of Colletotrichum gleosporoides. Days after inoculation: 2 days (•—•), 4 days (O——O) 6 days (⊙——⊙), 8 days (×——×), 10 days (■-□□□■).

Fig.22: A. Demethylation of methylated compounds leading to the production of hydroxylated compounds, quercetin and kaempferol.

B. Isomerisation of p-coumaric to o-coumaric acid, o-coumaric on lactonisation produces a coumarin.


OCH3
OCH3
HO
O
OH
OH
O
Demethylation
OH
OH
HO
O
OH
OH
O
3', 4'-DiOMe Quercetin
Quercetin
OCH3
HO
O
OH
OH
O
OH
HO
O
OH
OH
O
4'-OMe Kaempferol
Kaempferol

A


HO
HO
C=O
OH HO
C=O
lactonisation
O
O
+ H2O
p-Coumaric
o-Coumaric
Coumarin

B

FIG. 22

4. DISCUSSION

The results clearly indicate that the fungal infection brought about a change in the chemical constitution of both, C. fistula and M.tomentosa. The in vitro studies with the help of drop diffusate technique also showed the same pattern in both the plants as the case of in vivo and this confirms the view that chemical changes occur in such a way that the new compounds produced after infection offer better resistance to infection.

The highly hydroxylated polyphenols such as quercetin and kaempferol, being more reactive, are more toxic to fungi and therefore the plants resorted to the production of these compounds in place of the less hydroxylated (i.e. methylated) derivatives when they were infected. There is evidence that quercetin inhibits the growth of fungi such as Daedalea quercina and Fomes annosus (Walchli and Scheck, 1976; Alcubillamartin, 1970). The antifungal activity of quercetin and kaempferol are reported earlier (Sporoston, 1957; Dixit et al., 1978). These compounds were found to be absent in plants like Populus maximowiczii, P. laurifolia and other hybrids which are susceptible to Dothichiza populea, while they were present in the resistant varieties such as P.nigra var. Italica and its hybrids (Pukacka, 1975). Furthermore the antifungal activity exerted by quercetin on A.niger and C. gleosporoides by inhibition of mycelial growth, spore

germination and germ tube elongation also suggests that the plants resorted to the production of the compounds to equip itself against infection. The demethylation leading to the production of these hydroxylated compounds at the sites of infection also helps to inhibit further growth of the pathogenic fungus or prevent secondary infection, therefore the antifungal compounds, quercetin and kaempferol could be considered as phytoalexins (Joy and Daniel, 1988). The increase in the concentration of flavonoids in the infected leaves also suggests their higher rate of production as a response to microbial attack.

Since the reactive group in phenols is the hydroxyl group and the toxic action exerted by them is attributed to this group, the production of hydroxylated compounds in place of methoxylated compounds (substituting OH group in place of methyl group) seem to be the logical step to combat the invader.

The production of o-coumaric acid in place of p-coumaric acid in C.fistula is another improved mode of resistance. Though p-coumaric acid is also known to be antifungal (Clauss, 1961; Trappe et al., 1973), its conversion to o-coumaric acid has an added significance in that o-coumaric acid on lactonisation produces coumarin (Fig. 22B) which is a more potent antimicrobial agent (King et al.,1954; Martin et al., 1966; Berkenkemp, 1971 Rudman, 1963 a). Moreover, o-coumaric acid has been reported to be a systemic fungicide (Gangulee and kar, 1985), and this explains well the biosynthesis of this phenolic acid in place of its p-substituted relative in infected leaves of C.fistula.

Table - V : Distribution of proanthocyanidins, iridoids alkaloids, saponins, and tannins in healthy and fungal infected leaves of Cassia fistula and Morinda tomentosa.

Name of the plant	1	2	3	4	5
Cassia fistula					
a. Healthy leaves	+	.	+	.	+
b. Infected leaves	+	.	+	.	+
Morinda tomentosa					
c. Healthy leaves	.	+	+	.	.
d. Infected leaves	.	+	+	.	.

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

Table - VI. Pre-infectional and post-infectional compounds of Cassia fistula.

Pre-infectional compounds	Post-infectional compounds	Drop diffusate technique		Type of post-infectional compounds	Mode of formation of post-infectional compounds
		Control	treated with the pathogen		
Quercetin-4'-OMe	Quercetin	Quercetin-4'-OMe	Quercetin	Flavonols	Demethylation of existing flavonols
Kaempferol 4'-OMe	Kaempferol	Kaempferol-4'-OMe	Kaempferol		
Quercetin 7,8'4'-triOMe					
Syringic acid					
p-coumaric acid	o-coumaric				
Quinones	Quinones				

Table - VII. Pre-infectional and post-infectional compounds of Morinda tomentosa.

Pre-infectional compounds	Post-infectional compounds	Drop diffusate technique		Type of post-infectional compounds	Mode of formation of post-infectional compounds
		Control	Treated (pathogen)		
Kaempferol-4'-OMe	Kaempferol	Kaempferol-4'-OMe	Kaempferol	Flavonols	Demethylation of existing flavonols
Quercetin 3',4'-diOMe	Quercetin	Quercetin 3',4' diOMe	Quercetin		
Syringic acid	Syringic acid				
Ferulic acid	Ferulic acid				
Vanillic acid	Vanillic acid				
Gentisic acid	Gentisic acid				