PHYTOALEXIN RESPONSE IN *EUCALYPTUS GLOBULUS* LABILL.

CHAPTER - V

PHYTOALEXIN RESPONSE IN EUCALYPTUS GLOBULUS LABILL.

Eucalyptus is an Australian genus consisting of about 600 sp. It was first introduced in India in 1782 at Nandi hills (Karnataka). Extensive plantations of Eucalyptus sp. have been and are being raised in Gujarat, Punjab and Haryana under plantation schemes. Eucalyptus is an excellent tree for production of wood, since it grows faster and produces more wood than any other tree.

Eucalyptus globulus Labill is a large tree attaining a height of 100 m or more with a clean straight bole under forest conditions, but often tending to branch freely when grown in the open. Bark is deciduous in long thin strips or sheets. Leaves on juvenille shoots, opposite, sessile, cordate-ovate, covered with a bluish white bloom, adult leaves alternate, lanceolate, 15-30 cm long and 3-5 cm broad. Stem of seedlings and coppice shoots are quadrangular.

A cool, moist, equitable climate and deep fertile soil which is not calcareous or saline, are favourable for the growth of E. globulus. It is propagated by seeds.

The timber of E. globulus is utilised for a variety of purposes. Immunity to insect attack and durability under water are the main considerations which render the timber useful for

ship building. It is fire resistant and is used for the construction of godowns, sheds and platforms. It is also used for fence posts, rough carpentry and fuel.

The wood has been tested for its suitability as raw material for paper pulp. It yields 72 % of a brownish mechanical pulp useful in the manufacture of wrapping paper and cardboard. White pulp of good quality has been obtained from the wood by sulphite and soda processes. Mixed with spruce pulps, E. globulus pulp may be used for producing high grade paper.

The bark contains tannic acid. The oil obtained from the flowers has a composition similar to that of the leaf oil.

The leaves of E.globulus yield an essential oil containing 1,8-cineole, pinene, sesquiterpene alcohols, lower boiling alcohols, aldehydes, ketones, acids, cuminaldehyde, pinocarveol and phenols. Gell et al., (1958) reported 7-OMe 1,4 dihydroflavonol in leaves of some spp. of Eucalyptus.

E.glubulus is the principal source of medicinal eucalyptus oil. In India it is the only species from which oil is distilled for commercial purposes. Eucalpytus oil is largely used as a mosquito and vermin repellant and as an ingredient of germicidal and disinfecting preparations. It is used locally as an antiseptic especially in the treatment

of infections of the upper respiratory tract and mixed with an equal amount of olive oil in certain skin diseases. It is useful as a rubefacient for rheumatism and also used as a stimulating expectorant in chronic bronchitis and asthma. Dried leaves of E. globulus are used in the form of tincture in asthma and chronic bronchitis.

Casualities due to the fungus Ganoderma lucidum (Leyss) Karst. have been reported from some Eucalyptus plantations.

DaCosta and Rudman (1958) have reported the presence of antifungal compounds in Eucalyptus. They found that the outer heartwood of E.microcorys was extremely resistant to decay causing organisms such as, Coniophora carebella, Coriolus versicolor and Fomes durus. But methanol extracted outer heartwood was promptly decayed by these fungi and when the extract was added to the heartwood of E. regnans, susceptible to the fungus, it conferred protection against decay. Inhibition of wood rotting fungi by stilbenes and other polyphenols in E. sideroxylon has also been reported (Hart and Hillis, 1974). Haerdtl (1962) has shown that Eucalyptus oil exhibited antifungal activity against Aspergillus niger.

Antimicrobial compounds have also been reported from E. globulus (Osborne & Thrower, 1964) and several other spp. such as E. citriodora (Reis, 1973; Singh, 1971), E.marginatus

(Rudman, 1963b) and E.trifolora (Egawa et al.,1977).

In the present work healthy and infected leaves of E. globulus were screened for post-infectional compounds and fresh leaves of the same tree were induced with spore suspension of a pathogen and a non-pathogen to elicit phytoalexin responses.

MATERIALS AND METHODS

Healthy and infected leaves of Eucalyptus globulus were collected from cultivated trees in Baroda, Gujarat State.

The procedures followed for the isolation and culture of the fungus, the pathogenicity tests, the extraction, isolation and identification of compounds, drop diffusate technique and the facilitated diffusion technique have been described in chapter II.

BIOASSAY TESTS

Assay of mycelial growth: The antifungal activity of the diffusates, control (A) and treated (B) were found out by bioassay tests. The diffusates were made upto 100 ml (stock solutions, A and B). 2,5 and 10 ml of the stock solution (B) were added to three different PDA containing petriplates respectively. This three different dilutions of the stock (B) were considered as the test solutions. In the case of control, only one solution i.e. 10 ml of the stock (A) was added to one

petriplate containing the medium. Three replicates were maintained in each case of treatment and control. The centre of each petriplate was inoculated with an agar plug of the test fungus cut from the margin of a parent colony growing on PDA. The assay plates were incubated at 25° ± 2°C for a period of six days. Measurements of the mycelial growth was taken every 2 days. Mycelial growth was calculated by measuring the perpendicular diameter of each of the three replicate colonies and subtracting the diameter of the mycelial plugs used to inoculate the plates. The assay plates of the treated were compared with that of the control.

Assay for spore germination and germ tube growth:- The test solutions were prepared as mentioned above. The procedure followed for the bioassay of spore germination and germ tube elongation have been described in Chapter - II.

RESULTS

Alternaria alternata (Fr.) Keissler (IMI, 316666) was the fungus isolated from the infected leaves. Pathogenicity tests have confirmed that A. alternata is pathogenic on leaves of E.globulus (Fig. 23). The leaf spot disease of E.globulus is found to occur in the months of December and January.

The lesions due to the disease developed 4-5 days after inoculation. The symptoms were first visible as

Fig. 23. Diseased leaves of Eucalyptus globulus Labill.

Fig.24. Spores of Alternaria alternata(fr.) Keissler.

FIG. 23

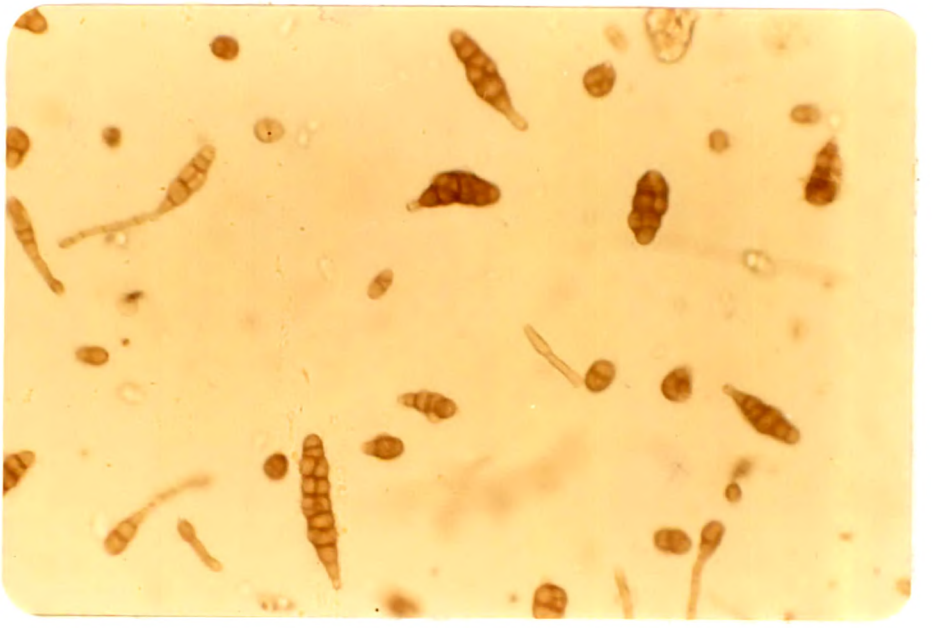

FIG. 24

small round black spots scattered on the leaf blade. The black spots were found restricted to the upper surface of the leaf.

The distribution of proanthocyanidins, iridoids and alkaloids in healthy and infected leaves of E. globulus is presented in Table VIII. Proanthocyanidins, iridoids and alkaloids were located in both healthy and infected leaves. Tannins and saponins were absent from all the plant specimens.

The distribution of pre-infectional and post-infectional phenols of E. globulus is presented in Table IX. The infected leaves of E. globulus contained the same flavonol 4'-OMe kaempferol and 3'-OMe quercetin as in the case of healthy leaves. Vanillic, syringic and p-hydroxybenzoic acids were the phenolic acids found in both the healthy and infected leaves.

Monoterpenes and steroids were similar in both healthy and infected leaves. Thin layer chromatograms of extracts from healthy and infected leaves showed the presence of three monoterpenes, (1) Rf-0.31 (2) Rf 0.42 (3) Rf-0.65) and two steroids ((1) Rf-0.23 (2) Rf-0.29).

When phytoalexin production was induced in the leaves of E.globulus with spores of the pathogenic fungus using the drop diffusate technique, a compound with blue fluorescence

under UV light (Rf-0.57 in Toluene: Ethyl formate : Acetic acid (5:4:1), $\lambda \frac{max}{MeOH}$ 276, 285, 296, 330, 341 nm) was detected in thin layer chromatograms along with other phenolic compounds (Fig. 25). The color reactions and absorption spectrum indicate that this compound could be a coumarin. This compound was not seen in the chromatograms of leaves used in control experiments. There was no qualitative or quantitative differences between the diffusates (control and treated) when the leaves were exposed to spores of the non pathogenic fungus, Fusarium solani (Mart.) Sacc. Facilitated diffusion technique employed with both the pathogen and the non-pathogen yielded no new compounds.

The diffusate from the treated leaves containing the phytoalexin inhibited the mycelial growth of the pathogen, A.laternata at 10 ml dilution (Fig.26). There was no inhibition at 5 ml and 2 ml dilution. The mycelial growth was more or less similar to that of control showing only a small per cent of inhibition. A maximum of 54 per cent inhibition of the mycelial growth over control was noted at 10 ml dilution of the diffusate (Fig.27). This diffusate inhibited the spore germination and germ tube elongation of A. alternata also (Fig.28). A maximum of 65 per cent inhibition of spore germination and 71 per cent inhibition of germ tube elongation was seen at 10 ml dilution.

Fig. 25. A thin layer chromatogram (under UV light) showing a coumarin from diffusates of Eucalyptus leaves treated with Alternaria alternata. Control (left) and treated (right)

Fig.26. Antifungal activity of the diffusate (treated with A.alternata) from leaves of Eucalyptus globulus. Control (left) and treatment: 10 ml dilution (right).

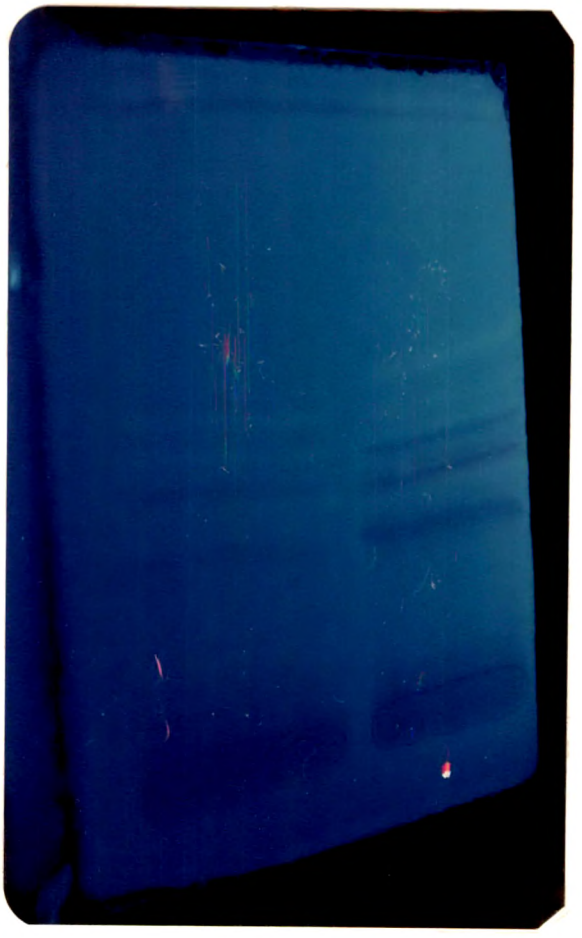

FIG. 25

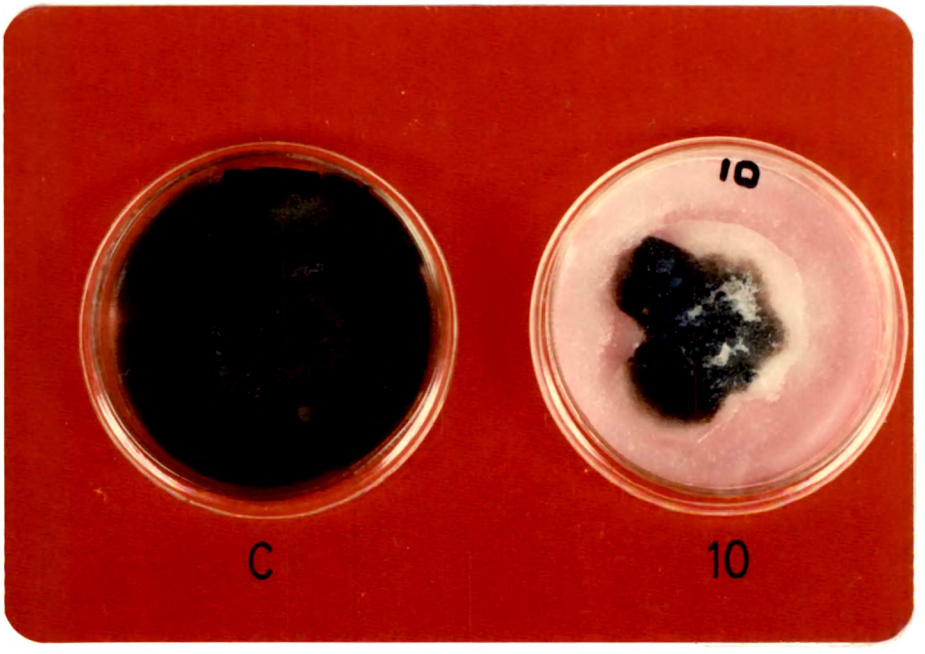

FIG. 26

Fig.27. The effect of the diffusate (treated) from leaves of Eucalyptus globulus on the mycelial growth of Alternaria alternata. Days after inoculation: 2 days (●----●), 4 days (X——X), 6 days (O——O).

Fig.28. The effect of the diffusate (treated) on spore germination (O---O) and germ tube length (●——●) of Alternaria alternata as observed after a incubation period of 24 hrs at 25° ± 2°C.

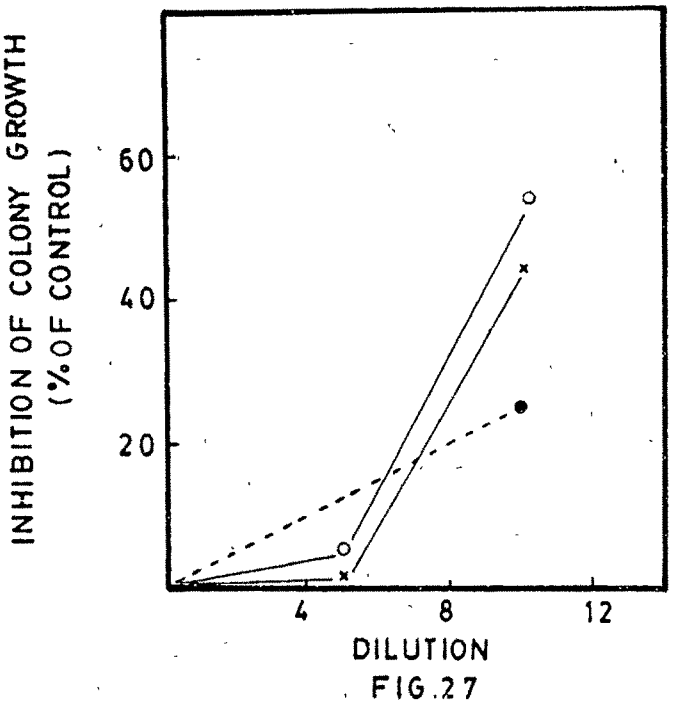
INHIBITION OF COLONY GROWTH
(% OF CONTROL)
60
40
20
4
8
12
DILUTION

FIG.27

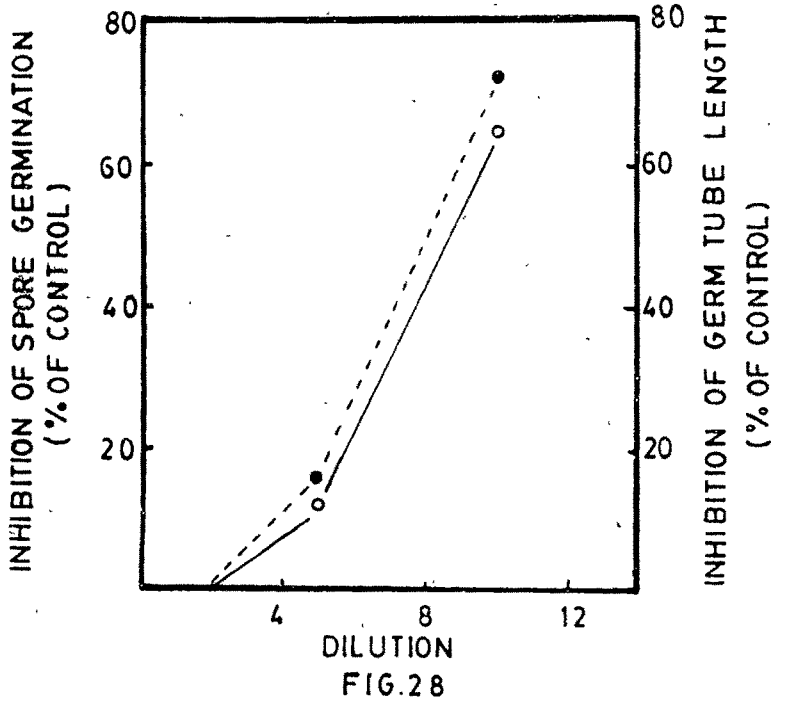
INHIBITION OF SPORE GERMINATION
(% OF CONTROL)
80
60
40
20
INHIBITION OF GERM TUBE LENGTH
(% OF CONTROL)
80
60
40
20
4
8
12
DILUTION

FIG.28

DISCUSSION

The absence of a new or modified compound in the infected leaves of *E.globulus* indicates that there is no qualitative change as a result of infection.

The blue fluorescent compound produced in response, when leaves of *E.globulus* was exposed to spores of *A.alternata*, in all probability is a coumarin. The absence of the same compound in the diffusate containing sterile distilled water suggests that it could be a product of the interaction between the fungal spores and the leaves of the plant. The inhibition of mycelial growth, spore germination and germ tube elongation by the diffusate confirms the antifungal activity of the coumarin phytoalexin. However, the absence of the coumarin in the diffusate of the leaves treated with the non-pathogen indicates that the production of the coumarin was more of a host-pathogen interaction.

Coumarins are lactones of o-hydroxycinnamic acids and the lactones in general are highly active. Coumarin phytoalexins such as ayapin from *Carthamus tinctorius* (Tal and Robeson,1986), Xanthotoxin from *Pastinaca sativa* (Johnson *et al.*, 1973), 6,methoxymellein from *Daucus carota* (Fumiya *et al.*,1984,1985) have also been reported. Accumulation of coumarin phytoalexins in potato (Clarke, 1973; Clarke and Baines, 1976; Malmberg and Theander, 1980) and tobacco (Sequeira, 1969; Fritig *et al.*,1972)

in response to various infections have also been reported. However not all coumarins are antifungal. Chakraborty et al., (1957, 1961) has found only four out of seventeen natural coumarins to inhibit the growth of Aspergillus niger. Vichkanova et al., (1973) has recorded that, out of 33 coumarins isolated from plants only 14 of them inhibited fungi.

Table - VIII. Distribution of proanthocyanidins, iridoids, alkaloids, tannins and saponins in healthy and infected leaves of *Eucalyptus globulus*.

Leaves	1	2	3	4	5
Healthy	+	+	+	.	.
Infected	+	+	+	.	.

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

Table -IX : Distribution of pre-infectional and post-infectional phenols of Eucalyptus globulus.

Pre-infectional compounds	Post-infectional compounds	Drop-diffusate technique	
		Control	Treated
4'-OMe kaempferol	4'OMe kaempferol		
3'-OMe quercetin	3'-OMe quercetin		
Vanillic acid	Vanillic acid		
Syringic acid	Syringic acid		
p-hydroxybenzoic acid	p-hydroxybenzoic acid	-	Coumarin