J. Biosci., Vol. 18, Number 2, June 1993, pp 291–301. © Printed in India.

Induction and accumulation of phytoalexins in cowpea roots infected with a mycorrhizal fungus *Glomus fasciculatum* and their resistance to *Fusarium* wilt disease

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MS received 10 January 1992

Abstract. The interaction of a vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatum* with a wilt-causing soil borne pathogen, *Fusarium oxysporum,* was studied in cowpea (*Vigna unguiculata*). It was found that pre-establishment by vesicular-arbuscular mycorrhizal fungus reduced the colonization of the pathogen and the severity of the disease, as determined by reduction in vascular discolouration index. In mycorrhizal plants, the production of phytoalexin compounds was always higher than in the non-mycorrhizal plants. There appeared to be a direct correlation between the concentration of the phytoalexins and the degree of mycorrhizal association. Three different compounds with R_f values of 0·23 (I), 0·17 (II) and 0·11 (III) were obtained from mycorrhizal plants. Similar compounds were also found to be induced by an abiotic elicitor $CuSO_4$. The first compound was identified as an isoflavonoid, daidzein and the other two remain to be identified. These compounds were checked for their antifungal activity *in vitro.* The germination of conidial spores of *Fusarium oxysporum* was strongly inhibited by the compound III than the other two. It is argued that the production of phytoalexin compounds in mycorrhizal plant could be one of the mechanisms imparting tolerance of the plants to wilt disease.

Keywords. Phytoalexins; mycorrhizae; cowpea; *Glomus fasciculatum;* disease control.

1. Introduction

It has been widely accepted that vesicular-arbuscular mycorrhizal (VAM) fungi enhance plant mineral nutrition especially phosphorus (P) (Mosse 1973; Hayman 1986). Other than their influence on plant nutrition, their interaction with plant pathogens such as fungi, bacteria and nematodes may lead to either the reduction or increase in severity of disease (Schenck *et al* 1977; Schonbeck 1980; Schenck 1981; Schonbeck and Dehne 1981; Dehne 1982; Bagyaraj 1984). For instance, when tomato plants were inoculated with *Glomus mosseae,* the damage caused by *Fusarium oxysporum* f. sp. *lycopersici* was considerably reduced (Dehne and Schonbeck 1975). Such tolerance, due to mycorrhizal association may be imparted by one or more of the following mechanisms: alterations in the physiology of the host, improvement of the plant nutritional status, anatomical changes and production of phenolic compounds (Baltruschat and Schonbeck 1972; Ling-Lee *et al* 1977; Dehne *et al* 1978; Krishna and Bagyaraj 1983; Morandi *et al* 1984). Of these the production of phytoalexins is considered to be an important mechanism of

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disease resistance in plants (Morandi *et al* 1984). Only a few reports are available on phytoalexin production in VAM plants. Morandi *et al* (1984) observed accumulation of isoflavonoid compounds during VAM symbiosis. To understand the role of VAM fungi in disease resistance, we examined the interaction between VAM and a wilt causing fungus, *Fusarium oxysporum,* in cowpea. Further, the elicitation and accumulation of phytoalexin compounds in response to fungal infection and their antifungal activity were examined *in vitro.*

2. Materials and methods

2.1 *Plant material*

Seeds of cowpea (*Vigna unguiculata* (L) Walp.) were obtained from Pulses Research Centre, Pudukkottai, Tamil Nadu. They were planted in a phosphorous-deficient, black sandy loam soil (Sundaresan 1989) containing 4 mg/kg of extractable P ($NaHCO_3$-soluble P) and grown in greenhouse condition (approx. 26–31°C, rh 80%, light 16 h, 85 W m-2).

2.2 *Fungal cultures*

An isolate of *F. oxysporum* used in the present study was originally isolated from diseased cowpea roots and maintained on potato dextrose agar (PDA) at 26° C in dark. A VAM fungus, *Glomus fasciculatum* (Thaxter sensu Gerd.) Gerd, and Trappe (obtained from Prof. D J Bagyaraj, University of Agricultural Sciences, Bangalore) was maintained in pot cultures with *Panicum maximum* (Jacq.) (Bagyaraj and Manjunath 1980).

2.3 *Preparation of conidial suspension of F. oxysporum*

F. oxysporum was cultured on PDA for 26 days at 26° C and conidia were collected in sterile Czapek-Dox liquid nutrient containing a trace amount of Tween-80. This suspension was sieved through 45 μm sieve to remove mycelial clumps. The conidial concentration was adjusted to 1×10^5 conidia/ml.

2.4 *Interaction of VAM with F. oxysporum*

Three cowpea plants were grown in each pot containing 5 kg of sterilized experimental soil in greenhouse conditions. Prior to sowing, the pots were either inoculated with 500 spores of *G. fasciculatum* or with 25 ml of conidial suspension of *F. oxysporum* (1×10^5/ml). *F. oxysporum* was inoculated to both mycorrhizal (MF) and non mycorrhizal (NMF) plants simultaneously or at 10 days intervals. Control, mycorrhizal (M) and non-mycorrhizal (NM) plants were also maintained. There were three replicates per treatment and the plants were harvested after 75 days of growth.

2.5 *Quantification of VAM infection and spore number*

The VAM colonization was quantified using a modified method of Phillips and Hayman (1970) using 0·5% lactoglycerol-trypan blue after the *G. fasciculatum* infected roots were cleared in 10% KOH (Kormanik *et al* 1980). The stained roots were observed under the microscope and the degree of colonization was calculated according to Read *et al* (1976). The number of VAM fungal spores in the soil was determined after sieving the soil samples (Gerdemann and Nicolson 1963) and observing the soil suspension under the microscope.

2.6 *Plant parameters*

The plants were harvested after 75 days and their weight was determined after drying the samples at 80° C for 24 h. The total P content of the plant was estimated using the method of Allen (1940).

2.7 *Calculation of vascular discolouration index*

Vascular discolouration index (VDI) was calculated as an index of the colonization of *F. oxysporum* using the following formula (Davis *et al* 1979).

$$\text{VDI} = \frac{\text{Sum of vascular discolouration rating (0–3)*}}{\text{(Number of plants/treatment)} \times 3} \times 100$$

2.8 *Phytoalexin production in cowpea roots*

Surface sterilized cowpea seeds were germinated in 1 kg of sterile experimental soil. Five seedlings per pot and three replicates for each treatment were maintained. Ten days old cowpea plants were provided with either 50 ml of $CuSO_4$ (10^{-3} M) solution (positive control) or with distilled water (negative control). After four days of treatment, the plants were harvested and phytoalexins were extracted in case of abiotic elicitor treated plants. Five hundred spores of G. *fasciculatum* was given as inoculum to develop mycorrhizal plants and for control plants no inoculum was added. VAM and non-VAM plants were harvested at 15, 30, 45 and 60 days intervals. The roots were collected and used for phytoalexin extraction.

2.9 *Extraction and characterization of phytoalexins*

One gram of roots from each treatment was macerated in 10 ml of 95% ethanol. The extract was dried and redissolved in chloroform (2 ml/g fresh weight) and applied to a column (1×10 cm) of silica gel (60–120 mesh, Glaxo, India) equilibrated with chloroform. The column was first eluted with 40 ml of chloroform and later with 20 ml of ethyl acetate : chloroform (1:1 v/v). The fractions were concentrated and applied to a thin-layer chromatography (TLC) plate (0·25 mm

*Based on vascular discoloration scale of 0 = No discolouration to 3= 100% discolouration of the xylem tissues in each root.

thickness) and was developed with a mixture of hexane: diethyl ether (1:3 v/v) Three spots were scraped from the plate and were eluted with 10 ml of ethanol Compounds were further purified by repeated TLC. They were redissolved in ethanol and the maximum absorbance for each compound was measured in spectrophotometer (Hitachi U 2000).

2.10 *Quantitative analysis of phytoalexins*

Since compound I was identified as daidzein by co-chromatographing with authentic sample daidzein, the extracted phytoalexin was quantified and expressed as μg/g fresh weight of roots. Compounds II and III were not identified and they were quantified with their relative absorbance at their respective absorption maximum.

2.11 *Large scale extraction of phytoalexins*

One kg of VAM roots of cowpea was air dried and repeatedly extracted with hot ethanol. The extracts were combined, made into a slurry by adsorbing it over silica gel (60–120 mesh) and packed in a column (3×40 cm). This was eluted successively with benzene and increasing amounts of acetone. Fractions of 100 ml were collected each time and the solvent was distilled. The residues obtained were subjected to TLC separation using different solvent systems. The residue of fractions 1 to 21 (fraction A; eluted with 5, 10, 15 and 20% of acetone in benzene) had the properties of wax and therefore was discarded. The residues of fractions 22 to 30 (fraction B; eluted with 25 and 30 % of acetone in benzene), fractions 31 to 43 (fraction C; eluted with 35, 40, 45 and 50 % of acetone in benzene) and fractions 44 to 55 (fraction D; eluted with 55 and 60% of acetone in benzene) had a single compound each with minor impurities. However, these three compounds were found to be different from each other (R_f values were 0·11, 0·17 and 0·23 for the fractions B, C and D respectively). They were recrystallized from methanol, dissolved in ethanol and their UV spectrum recorded. As compounds obtained from fractions B and C were in small quantity, the melting points for them could not be determined. However, fraction D yielded sufficient amount to determine the melting point.

2.12 *Antifungal activity of phytoalexin compounds in vitro*

The antifungal activity of the phytoalexins separated from the large scale purification was checked *in vitro*. Stock solutions (0·2%) of each phytoalexin in ethanol were prepared and 10, 25, 75 and 100 μg were dispersed into multiwell assay trays. The volume of each well was made up to 50 μl by the addition of ethanol and then 950 μl of a conidial suspension containing fungal conidia (1×10^5) in Czapek-Dox liquid nutrient was added to each well. There were three replications for each treatment. Controls, without phytoalexin were kept in all assays. The assay trays were incubated for three days at 26° C in dark. The lowest concentration at which no mycelial development occurred was taken as the minimum inhibitory concentration (MIC).

3. Results

3.1 *Tolerance of mycorrhizal plants to F. oxysporum infection*

Plants inoculated with *G. fasciculatum* (M) showed a significant increase in plant biomass, whereas the *F. oxysporum* inoculated non-mycorrhizal plants (NMF) showed the lowest biomass (3·90 g/plant) and recorded maximum VDI (74·1%) (table 1). However, when *F. oxysporum* was inoculated simultaneously with VAM, the biomass of the plants was more than the *F. oxysporum* inoculated plants and the VDI was 51·6%. When *F. oxysporum* was inoculated after the establishment of VAM fungus in the roots, the VDI was much less. For example, *F. oxysporum* inoculated after 30 days of VAM establishment showed 14·8% VDI and about 19·2% reduction in the severity of disease. The disease tolerance of the mycorrhizal plants was not correlated to their P status. Mycorrhizal plants showed a marked increase (2·6 times) in P content over the non-VAM plants. *F. oxysporum* treated non-mycorrhizal plants (NMF) showed a significant reduction in the P level. On the contrary, plants simultaneously inoculated with *F. oxysporum* and *G. fasciculatum* (MF) showed higher P content. The reduction in P status of mycorrhizal plant was found to be less if *F. oxysporum* was inoculated to established mycorrhizal plants.

Table 1. Interaction between *G. fasciculatum* and *F. oxysporum* after inoculating them at various intervals.

Treatment	Total dry wt (g/plant)	Total P content (mg/plant)	Infection (%)	VDI	Spore No./50 ml soil
NM	$6{\cdot}80^{b}$	$7{\cdot}4^{u}$	—	—	—
M	$12{\cdot}15^{a}$	$19{\cdot}2^{t}$	84^{a}	—	402^{a}
NMF	$3{\cdot}90^{g}$	$3{\cdot}2^{z}$	—	$74{\cdot}1^{a}$	—
NMF10	$4{\cdot}08^{g}$	$3{\cdot}7^{z}$	—	$57{\cdot}1^{b}$	—
NMF20	$5{\cdot}13^{f}$	$5{\cdot}9^{y}$	—	$41{\cdot}0^{d}$	—
NMF30	$5{\cdot}87^{f}$	$6{\cdot}4^{y}$	—	$34{\cdot}0^{e}$	—
MF	$4{\cdot}43^{g}$	$6{\cdot}5^{y}$	32^{c}	$51{\cdot}6^{c}$	118^{e}
MF10	$6{\cdot}97^{b}$	$8{\cdot}2^{x}$	57^{d}	$40{\cdot}7^{d}$	172^{d}
MF20	$7{\cdot}30^{d}$	$10{\cdot}3^{w}$	63^{c}	$37{\cdot}0^{e}$	206^{c}
MF30	$9{\cdot}13^{c}$	$15{\cdot}2^{v}$	69^{b}	$14{\cdot}8^{f}$	317^{b}

Values are mean of 3 replicates. Values not followed by the same latter are significantly different (P=0.05, Duncan's new multiple range test).

NM and M denote non-mycorrhizal and mycorrhizal plants respectively. F10, F20 and F30 denote the day of *Fusarium* inoculation that followed the VAM inoculation.

3.2 *Development of VAM with Fusarium in roots*

The VAM colonization was considerably reduced when *F. oxysporum* was inoculated simultaneously with *G. fasciculatum*. However, this damage caused by the pathogen was reduced when the pathogen was inoculated after the establishment of VAM fungus in roots (table 1). Simultaneous inoculation of both the organisms reduced the VAM spore production in soil. However, when the

pathogen was inoculated in the later stages, the reduction in spore number was substantial. These results suggested that the VAM development control invasion of the pathogen not merely by increasing the P level of the plant. studied the production of phytoalexin compounds in the VAM plants and relevance to development of resistance of plants to the pathogen.

3.3 *Separation and identification of phytoalexins*

Treatment with both the elicitors increased the accumulation of phytoalexins in roots. From $CuSO_4$-treated and VAM colonized roots, three compounds with different R_f values (0·23, 0·17 and 0·11) were separated by TLC. The absorbance maxima of these compounds were found to be 248, 283 and 340 nm respectively. The first compound which had a R_f value of 0·23 and an absorbance maximum of 248 nm (figure 1) showed a melting point at 319° C and was identified as an isoflavonoid compound, daidzein. Figure 2 shows the absorbtion maxima of compounds II and III extracted from VAM plants.

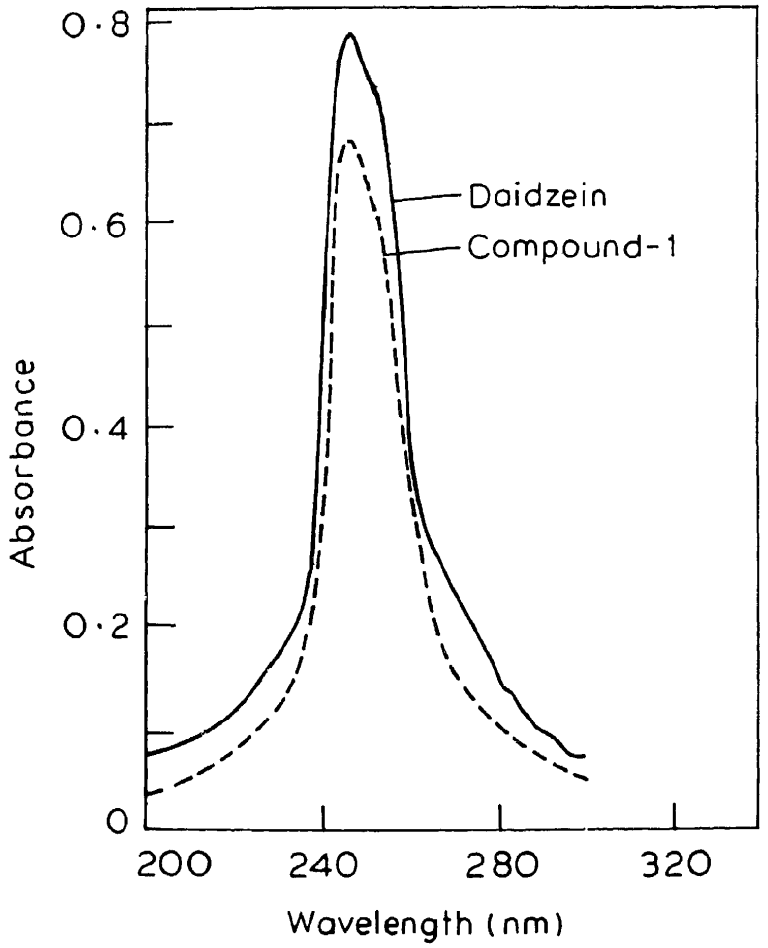

Figure 1. Maximal absorbance of daidzein pure and compound I.

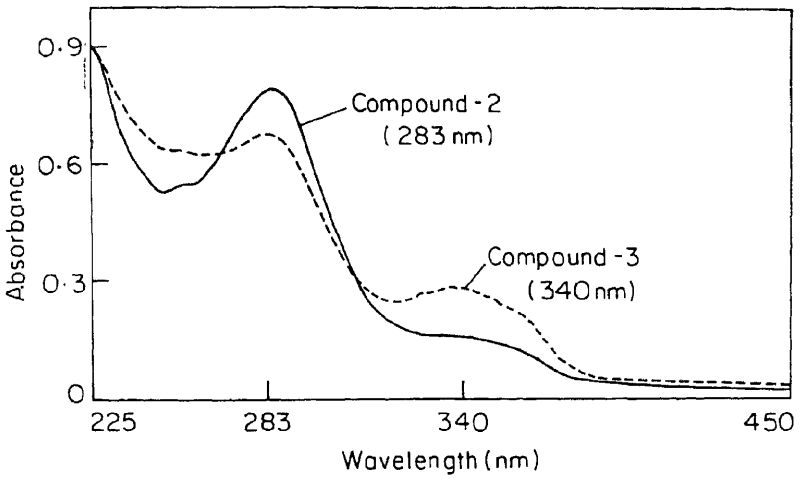

Figure 2. Maximal absorbance of compounds II and III.

3.4 *Accumulation of phytoalexins in roots*

When cowpea plants were treated with $CuSO_4$, phytoalexin production was enhanced with about 9·7-, 10·3- and 13-fold increase in the concentration of compounds I, II and III respectively over the untreated plants (table 2). VAM infection in plants greatly induced the synthesis of phytoalexin compounds; the extent of phytoalexin production was positively correlated with the degree of VAM colonization (table 3). At the final harvest, VAM plants had 3·0, 5·0 and 4·0 times more concentration of daidzein, compounds II and III respectively than the non-mycorrhizal plants.

Table 2. Synthesis of phytoalexin compounds in cowpea roots elicited with $CuSO_4$.

	Treatment	
	Control	$CuSO_4$
Daidzein*	0·9	8·7
Compound II**	0·006	0·06
Compound III**	0·003	0·04

Values are the means of three replicates.
*Expressed in µg/g fresh weight.
**The concentration of compounds II and III Were expressed in relative absorbance at 283 and 340 nm respectively since the compound were not identified. Values are the absorbance of compounds separated from 1 g of fresh roots

3.5 *Antifungal activity of phytoalexins in vitro*

Among the three compounds extracted from VAM roots, compound III showed a strong inhibition of fungal growth (table 4). The minimum inhibitory concentration

Table 3. Phytoalexin accumulation in cowpea roots in relation to the development of VAM infection.

Treatment	Days	VAM infection (%)	Daidzein*	Compound II**	Compound III**
Control	15	—	5·4	0·3	0·01
	30	—	9·7	0·9	0·03
	45	—	11·9	1·3	0·37
	60	—	12·7	1·6	0·78
VAM	15	18	17·8	2·6	0·11
	30	42	30·4	4·3	0·92
	45	64	33·5	5·9	1·91
	60	75	42·1	8·2	3·22

**The concentration of compounds II and III were expressed in relative absorbance at 283 and 340 nm respectively since the compounds were not identified. Values are the absorbance of compounds separate from 10 g of fresh roots.

Table 4. Inhibitory concentration of phytoalexins on germination of *F. oxysporum* spores.

	Minimum inhibitory concentration μg/ml
Daidzein	> 100
Compound II	50
Compound III	10

Values are the means of 3 replicates

of this compound for *F. oxysporum* spore germination was found to be 10 μg/ml, whereas for compound II, it was 50 μg/ml. The antifungal effect was very low with daidzein and the MIC was > 100 μg/ml.

4. Discussion

There is increasing evidence on the interaction of VAM fungi and plant pathogenic organisms (Schenck and Kellam 1978; Schonbeck 1980; Schenck 1981; Schonbeck and Dehne 1981). Since VAM are established in the roots of host plants, it can primarily reduce the diseases caused by soil-borne pathogens (Dehne 1982). In the study, the interaction of *G. fasciculatum* with a wilt causing pathogen *F. oxysporum* and the production of phytoalexins were investigated. *F. oxysporum* infection lowered the plant growth and correspondingly increased the vascular discolouration index. However, simultaneous inoculation with VAM fungus *G. fasciculatum* reduced the severity of disease caused by the pathogen. Similar observations have been reported for other host-VAM fungus-pathogen combinations (Baltruschat and Schonbeck 1972; Dehne and Schonbeck 1975; Schenck *et al* 1975; Davis and Menge 1981; Chakravarty and Mishra 1986). In a few instances VAM fungus inoculation were also reported to increase or have no effect on the development of fungal root pathogen (Ross 1972; Davis *et al* 1978; Davis 1980). Disease incidence was substantially reduced when pathogens were inoculated to roots already colonized by the VAM fungus. In fact a clear negative association between the extent of VAM

colonization and severity of disease caused by the pathogen has been found. Similar observation with *F. oxysporum* f. sp. *lycopersici* (Dehne and Schonbeck 1975) and *Phytophthora parasitica* (Schenck *et al* 1977) have been reported.

Increased resistance of mycorrhizal plants to disease have been attributed to several mechanisms. One of the possible mechanisms of resistance of VAM plants to the pathogen could be because of the improved mineral nutrition, particularly P. VAM inoculated plants had an increased P level and exhibited decreased VDI. Krishna and Bagyaraj (1983) suggested the possible role of P in disease tolerance in a *G. fasciculatum-Sclerotium rolfsii*-peanut interaction system. Alternatively because mycorrhizal roots are more lignified especially in the stelar tissue they may restrict the entry of pathogen into the root cortex (Dehne 1982). Increased level of phenols especially ortho-dihydroxy phenols in the mycorrhizal plants (Ling-Lee *et al* 1977; Krishna and Bagyaraj 1984) has also been argued to impart disease resistance (Goodman *et al* 1967; Krishna and Bagyaraj 1983). Dehne *et al* (1978) suggested that the production of chitinase like hydrolytic enzymes in mycorrhizal plants may also act on the pathogens. The increased resistance of mycorrhizal roots to pathogen may be due to the alteration of host physiology by the accumulation of specific amino acids such as arginine which was found to decrease the sporulation of *Thielaviopsis basicola* (Baltruschat and Schonbeck 1972; Schonbeck and Dehne 1977; Dehne *et al* 1978). Finally, the resistance of mycorrhizal plants could also be due to phytoalexins (Bailey 1982; Mansfield 1982).

Our results showed that mycorrhizal infection increased the production of phytoalexins in cowpea. Further, the extent of accumulation of phytoalexin compounds was positively associated with colonization of *G. fasciculatum.* The production of phytoalexins was also found to be elicited by $CuSO_4$. Earlier, Morandi *et al* (1984) reported an increase in the accumulation of phytoalexin Compounds in soybean with increase in infection by *G. mosse* and *G. fasciculatum*; they also reported elicitation of phytoalexins by $CuSO_4$.

In our study, three compounds with different absorption maxima were separated and one of them was identified as daidzein. Synthesis of daidzein, an intermediate in the biosynthetic pathway of isoflavonoids such as coumestrol and glyceollin, has also been found in soybean-Glomus symbiosis (Morandi *et al* 1984). Compound III having an absorption maxima at 340 nm strongly inhibited *Fusarium* spore germination. Daidzein was found to be less active against the pathogen. No report is available on the toxic effect of daidzein on either fungi or nematodes (Morandi *et al* 1984). Based on our results, we suggest that mycorrhizal association in plants may restrict the development of pathogen by the production of phytoalexins. Thus, inoculation of the VAM fungus. *G. fasciculatum,* to cowpea not only improves plant growth and development, but offers increasing resistance to soil-borne pathogens such as *F. oxysporum.*

Acknowledgements

Authors acknowledge the Indian Council of Agricultural Research, New Delhi, for the financial assistance. Dr M Ramaiah and Dr H R Krishnan, Department of Natural Products, School of Chemistry, Madurai Kamaraj University, Madurai, for their help in the large scale extraction of phytoalexins and Prof. M Lakshmanan for his help during the project.

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