Avirulent mutants of *Macrophomina phaseolina* and *Aspergillus fumigatus* initiate infection in *Phaseolus mungo* in the presence of phaseolinone; levamisole gives protection

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To evaluate the role of phaseolinone, a phytotoxin produced by *Macrophomina phaseolina*, in disease initiation, three nontoxigenic avirulent mutants of the fungus were generated by UV-mutagenesis. Two of them were able to initiate infection in germinating *Phaseolus mungo* seeds only in the presence of phaseolinone. The minimum dose of phaseolinone required for infection in 30% seedlings was 2·5 μg/ml. A human pathogen, *Aspergillus fumigatus* was also able to infect germinating seeds of *P. mungo* in the presence of 5 μg/ml concentration of phaseolinone. Phaseolinone seemed to facilitate infection by *A. fumigatus*, which is not normally phytopathogenic, by reducing the immunity of germinating seedlings in a nonspecific way. Levamisole, a non-specific immunopotentiator gave protection against infection induced by *A. fumigatus* at an optimum dose of 50 μg/ml. Sodium malonate prevented the effects of levamisole.

1. Introduction

Macrophomina phaseolina (Tassi) Goid, a fungal phytopathogen, infects about 500 plant species world wide (Sinclair 1982). Tropical crop plants are seriously affected by this pathogen (Malaguti 1990). *M. phaseolina* elaborates a number of phytotoxins, namely, asperlin, isoasperlin, phomalactone, phaseolinic acid, phomenon and phaseolinone (Dhar *et al* 1982; Mahato *et al* 1987; Bhattacharya *et al* 1992a). Phaseolinone appears to be most important of these and it induces disease symptoms in plants similar to those produced by the pathogen (Mathur 1968; Dhingra and Sinclair 1974; Siddiqui *et al* 1979; Bhattacharya *et al* 1992b). The direct role of toxins in pathogenesis induced by *M. phaseolina* has been questioned by Chan and Sackston (1969, 1973) and supported by the important role of cell wall dissolving enzymes in disease initiation and progression. However, several groups had reported that the virulence of *M. phaseolina* isolates is related to their ability to produce toxins (Mathur 1968; Rai and Singh 1973; Dhingra and Sinclair 1974; Bhattacharya *et al* 1992b). The role of toxin(s) produced by *M. phaseolina* in disease initiation has not yet been reported. A large number of fungal phytopathogens produce disease related phytotoxins; but the way these toxins interact with the cell machinery has been established only for very few of them (Ballio 1991).

In this study it has been shown that avirulent, nontoxigenic mutants of *M. phaseolina* and *Aspergillus fumigatus* which is not normally phytopathogenic, are capable of initiating infection in the germinating seeds of *Phaseolus mungo* in the presence of phaseolinone. Levamisole, a nonspecific immunomodulator in animals, can prevent such infection.

2. Materials and methods

2.1 *Materials*

M. phaseolina was isolated from infected black-gram (*P. mungo* L.) seeds (Siddiqui *et al* 1979). The culture was maintained in potato dextrose agar (PDA) and/or Czapek Dox (CD) agar media (Booth 1971). *A. fumigatus* a human pathogen was obtained from the School of Tropical Medicine, Calcutta. This strain was maintained in Sabouraud's agar (Booth 1971).

Keywords. *Aspergillus*; immunopotentiator; infection; levamisole; *macrophomina*; phaseolinone; protection

Phaseolinone was isolated and purified from culture filtrate of *M. phaseolina* as reported earlier (Siddiqui *et al* 1979; Dhar *et al* 1982). The preparation of phaseolinone showed a single spot in thin layer chromatography (TLC) plate.

The antihelminthic as well as immunopotentiator drug, levamisole, was obtained from Sigma Chemical Co. (USA).

Sodium malonate was prepared by neutralizing malonic acid to pH 7·2 with sodium hydroxide.

2.2 *Infection of seeds*

Seeds of *P. mungo* were surface sterilized by 10% bleach for 1 min and sterility was confirmed by placing seeds crushed in sterile pestle and mortar on CD plates. The batches of seeds that did not show microbial growth up to three days were considered healthy. Healthy seeds were allowed to germinate in Petri dishes over sterile moist filter paper along with different colony forming unit (c.f.u.) (10^2–10^6) of *M. phaseolina*, obtained by homogenizing 4-day old cultures grown in CD broth. The homogenate was washed twice with sterile distilled water by centrifugation at 10,000 *g* for 10 min.

Seeds were also infected by placing them over fine pieces of *M. phaseolina* culture grown on CD agar for assured contact with seeds. Spore suspensions of *A. fumigatus* were prepared in sterile distilled water from cultures grown in Sabouraud's agar. For infection study 10^4 spores per plate was used. However, only 50% spores of *A. fumigatus* were germinated in water containing seeds within 24 h. A total of 3 ml liquid was present at the beginning of germination in Petri dishes. Seeds were incubated at 30°C for two to three days in dark. Visible lesions, either black or brown, on the plant surface were considered as signs of infection. Different concentrations (5·0, 2·5 and 1·25 μg/ml) of phaseolinone were included in the plates to determine its role in disease initiation. Beside pure phaseolinone, autoclaved crude culture filtrate of *M. phaseolina* was also used as source of phaseolinone to judge whether the presence of other substances interferes or accelerates infection process. The concentration of phaseolinone in culture filtrate was determined either by ELISA (Bhattacharya *et al* 1992b) or by determining percentage of inhibition of seed germination (Bhattacharya *et al* 1994). The minimum concentration required for 100% inhibition of seed germination was 25 μg/ml. To inhibit 30% seedling growth generally 1 : 4 or 1 : 8 dilution of culture filtrate was required depending upon the phaseolinone produced in the culture.

Effect of cystine, cysteine, sodium malonate and levamisole on seed germination in the presence and in absence of phaseolinone was determined by including them in the germination medium. Ten independent experiments were performed on infection and protection assay and data presented as mean values with standard error of three experiments.

2.3 *UV-mutagenesis*

Mycelium from 4-day old culture of *M. phaseolina* in CD broth was homogenized in a pestle and mortar, diluted with sterile water, filtered through a thin pad of sterile cotton and the filtrate was centrifuged at 700 *g* for 7 min. The pellet was washed with distilled water twice and resuspended in water to obtain approximately 10^4 c.f.u./ml. This suspension was irradiated at 254 nm with Mineralight model UV2-58 lamp from a distance of 5 cm. The suspension was constantly stirred by a magnetic stirrer during UV-irradiation. Samples (1 ml) were withdrawn at 5 min intervals then kept in the dark for 30 min, diluted and plated on CD agar. Colonies isolated after 3 days of growth were transferred to fresh plates and maintained in both PDA and CD until characterized. Two hundred colonies were screened for infectivity and phaseolinone production in culture. Colony morphology and sclerotia formation were also studied.

3. Results

Colonies of *M. phaseolina* turned deep black in colour after 3 days of growth due to pigment formation. The cells were multinucleate. As induction of stable mutation in multinucleate cells is difficult, high dose of UV-irradiation was given to induce stable mutation. For easy scoring of mutants, albinism was considered as a sign of genetic change, although there is no report that virulence of *M. phaseolina* is related with pigmentation. White colonies were easily distinguished from black colonies after 3 days of growth.

3.1 *Isolation of avirulent mutant*

In initial plating 20% albino-mutants were detected. Albino mutants were obtained from 5 to 30 min UV exposure but no time dependent enhancement in the number of white colonies was observed.

One brown colony and 40 white colonies were selected out of 200 after primary screening and analysed further for infectivity, phaseolinone production and sclerotia formation. Among them, ten colonies were stable with respect to growth pattern, colony colour, infectivity and phaseolinone production. Ten consecutive subcultures were made on CD from the growing margins of mycelium from 4-day agar cultures to determine the stability. Seven albino colonies out of the above ten were moderately virulent compared to wild type strain. They were sclerotia forming and produced phaseolinone in culture. A brown variant, designated Muv 21, and two other albino mutants, designated Muv 19 and Muv 20, were avirulent and produced no phaseolinone in culture. Mutants Muv 20 and

Table 1. Properties of mutants of *M. phaseolina*.

Strains	Colony colour	Infectivity	Phaseolinone production in culture (μg/ml)	Sclerotia formation	Infection initiation by phaseolinone
Wild type	Deep black	Virulent	10	Positive	Not performed
Mutant Muv 21	Brown	Avirulent	0	Positive	+
Mutant Muv 20	White	Avirulent	0	Positive	+
Mutant Muv 19	White	Avirulent	0	Negative	–

Muv 21 were sclerotia forming, but Muv 19 was not. Some of the properties of the mutants are listed in table 1.

3.2 *Infection of seeds*

Different dilutions of mycelial homogenate of avirulent mutants were used to infect *P. mungo* seeds. None of the avirulent mutants (Muv 19, Muv 20, Muv 21) infected seeds at a concentration up to 10^4 c.f.u./plate (figure 1C). The wild type strains were able to infect 100% seeds with 10^3 c.f.u./plate (figure 1E). To evaluate the effect of phaseolinone in disease initiation, different concentrations of phaseolinone (5, 2·5, 1·25 μg/ml) were included along with avirulent mutant in plates during germination. The mutants, Muv 20 and Muv 21 were able to initiate infection, and infective lesions were visible within two days. The minimum concentration required for infection in 30% seedlings was 2·5 μg/ml; at 5 μg/ml concentration about 70% seedlings were infected within 60 h (figure 1D). The disease was progressive once initiated but the rate of progression was slow compared to the wild type. At the lower concentration phaseolinone had no effect on seed germination, but the seedling length was reduced by 30 to 32% of control at 5 μg/ml after 72 h (figure 3). No infection was observed with the strain Muv 19 under similar conditions.

3.3 *Specificity*

To check the specificity of phaseolinone-induced disease initiation, *A. fumigatus*, a human pathogen, which normally does not infect *P. mungo* plant was chosen for study. Surprisingly, it was observed that *A. fumigatus* also induced infective lesions on the cotyledons in 50% of germinating seedlings in the presence of phaseolinone at a concentration of 5 μg/ml (figure 2b, table 2). The lesions developed were not just a superficial discolouration; mycelium entered deep into the tissue (figure 2d). However, on the fourth day, the signs of recovery were prominent when seedlings were kept in light after covering the roots with sterile sand. At low doses of phaseolinone or in its absence *A. fumigatus* produced no sign of infection, even the seeds had no unhealthy appearance (figure 2a).

3.4 *Protection by levamisole*

Levamisole is known to be an immunopotentiator in animals. We were interested in studying its effect on plant immunity, because infection initiated by *A. fumigatus* in the presence of phaseolinone appeared to us to be due to suppression of immunity of plantlets. When *P. mungo* seeds were allowed to germinate in the presence of levamisole a dose dependent inhibition of seedling growth was observed after 50 μg/ml concentrations (figure 3). *A. fumigatus* can initiate infection in the seedlings above 50 μg/ml concentration of levamisole alone and the percentage of infected seeds increased with dose (table 2, figure 1F). On the other hand, levamisole gave 100% protection against infection at optimum dose between 40 to 50 μg/ml concentration when *P. mungo* seeds were allowed to be infected by *A. fumigatus* in the presence of phaseolinone (table 2, figure 4). A dose dependent prevention of infection was observed up to 50 μg/ml, there after harmful effect was induced similar to levamisole alone (table 2, figure 3). The concentration dependent effects of levamisole on infection by *A. fumigatus* and its protecting activity in the presence of phaseolinone is shown in table 2. However, levamisole failed to give protection against virulent strain of *M. phaseolina*. It was observed that levamisole protected germinating *P. mungo* seeds from cytotoxic effects of phaseolinone when levamisole and phaseolinone were added together in the germinating medium. Levamisole prevented reduction in seedling length (figure 3). It was thought that a mercapto derivative of levamisole, L-2-oxo-3-(2-mercaptoethyl)-5-phenyl-imidazolidine (OMPI) (figure 5) may have been generated in the plantlets that may be responsible for detoxification, therefore cystine and cysteine were added along with phaseolinone during germination, but no change in seedling length was detected even at a concentration of 200 μg/ml. On the other hand sodium malonate, known to oppose the action of levamisole in animal cells (Klein *et al* 1996) was found to neutralize the action of levamisole in germinating seedlings too (figure 3). Concentration dependent seedling growth inhibition at high concentrations by levamisole was completely abolished by sodium malonate at 10 mM concentration. It even inhibited infection induced by *A. fumigatus* at 100 μg/ml concentration of levamisole and the protecting ability of levamisole was also lost when infection was induced by phaseolinone. The effect of levamisole was completely reversed by sodium

malonate.

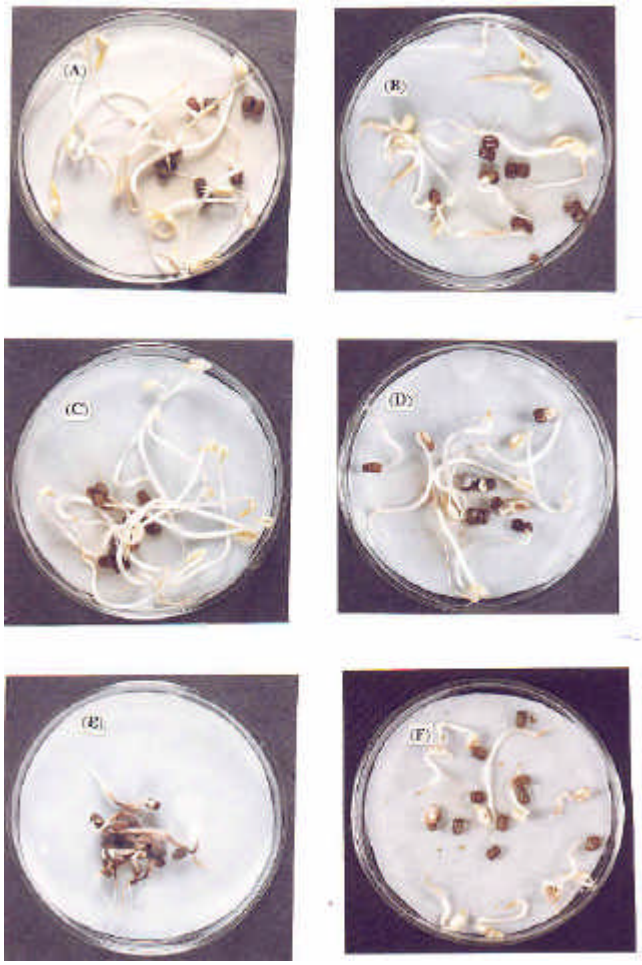

Figure 1.

4. Discussion

The importance of phaseolinone, the major phytotoxic substance produced by *M. phaseolina* in disease initiation has been evaluated using nontoxigenic, avirulent mutants and another fungus, *A. fumigatus*, which infects human and animal tissues but not plants. Initiation of infection by nontoxigenic, avirulent mutants of *M. phaseolina* (figure 1) and *A. fumigatus* (figure 2) in the presence of phaseolinone clearly indicated that phaseolinone somehow made *P. mungo* seedlings susceptible to infection. There are reports that some toxins can act as suppressors of induced resis-

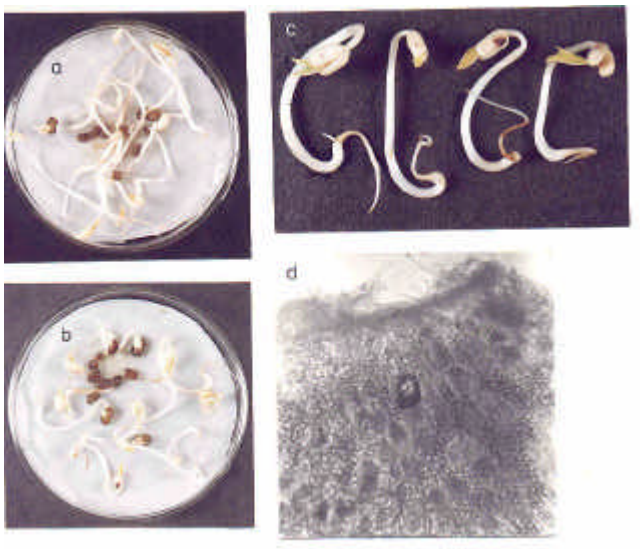

Figure 2. Infection of germinating *P. mungo* seeds by *A. fumigatus* in the presence of phaseolinone. (**a**) Seed germination in the presence of *A. fumigatus*. (**b**) Infection by *A. fumigatus* in the presence of 5 μg/ml concentration of phaseolinone (arrow indicating infection). (**c**) Close-up view of infective lesions (taken from **b**). (**d**) TS of infected cotyledon (taken from **b**).

Figure 1. Infection of germinating *P. mungo* seeds by virulent and avirulent mutant of *M. phaseolina* in the presence and in absence of phaseolinone and infection by *A. fumigatus* in the presence of levamisole. (**A**) Control seed germination. (**B**) Seed germination in the presence of 5 μg/ml phaseolinone. (**C**) Seed germination in the presence of avirulent mutant Muv 21. (**D**) Infection of seedlings by Muv 21 in the presence of 5 μg/ml concentration of phaseolinone. (**E**) Infection by wild type virulent *M. phaseolina*. (**F**) Infection by *A. fumigatus* in the presence of 100 μg/ml of levamisole (magnified).

Table 2. The effects of levamisole on infection of germinating *P. mungo* seeds in the absence or in presence of phaseolinone alone or in combination with malonate.

Concentration of levamisole (μg/ml)	Seed infected by *A. fumigatus* (%)*		
	Control (no phaseolinone)	Phaseolinone (5 μg/ml)	5 μg/ml phaseolinone + 1·5 mg/ml Na-malonate
0	0	50·0 ± 5·7	50·0 ± 5·7
1	0	43·3 ± 3·3	36·6 ± 3·3
10	0	36·6 ± 3·3	40·0 ± 0
20	0	26·6 ± 3·3	50·0 ± 5·7
30	0	13·3 ± 3·3	46·6 ± 8·8
40	0	0·0 ± 0	43·3 ± 6·6
50	0	0·0 ± 0	50·0 ± 5·7
60	6·6 ± 3·3	13·3 ± 3·33	36·6 ± 3·3
70	20·0 ± 5·7	23·0 ± 3·3	43·3 ± 7·3
80	43·3 ± 3·3	43·3 ± 3·3	43·3 ± 3·3
90	66·3 ± 8·8	70·3 ± 3·3	56·6 ± 13·3
100	80·0 ± 5·7	86·6 ± 3·3	50·0 ± 15

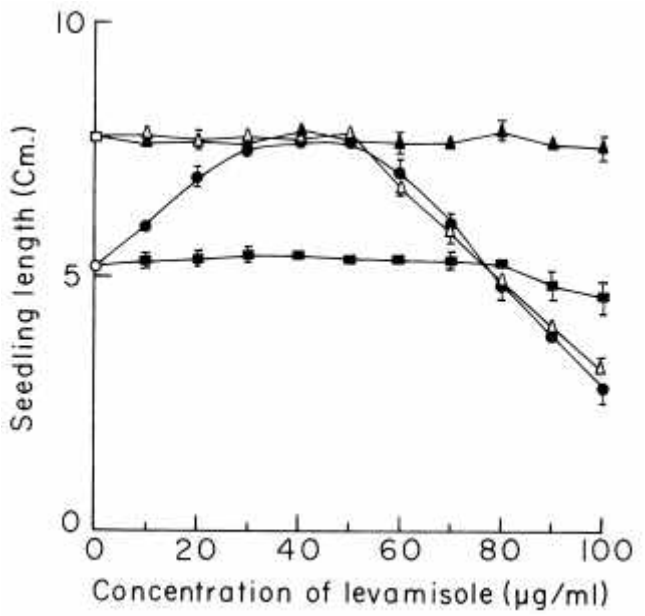

Figure 3. Seedling length of *P. mungo* after 72 h in the presence of different concentration of levamisole either alone or in combination with 5 mg/ml concentration of phaseolinone or 1·5 mg/ml sodium malonate. (□), Control seed germination; (Δ), levamisole; (▲), levamisole + sodium malonate; (○), phaseolinone; (●), levamisole + phaseolinone; (■), levamisole + phaseolinone + sodium malonate.

The error bars indicating standard error of three sets of experiment, error bars are obscured by symbols in some

tance (Hayami *et al* 1982; Kohmoto and Durbin 1989; Wada *et al* 1995) or can activate plant defense reaction (Bailey and O'Connel 1989). The concentration of phaseolinone at which avirulent mutants of *M. phaseolina* initiated infection in 30% of seedlings does not interfere with seed germination and seedling growth. Some specific defect or stress is induced by phaseolinone in the seedlings that render the plantlets susceptible to infection.

Protecting ability of phaseolinone-induced cytotoxicity of seedlings by levamisole was thought to be due to OMPI, a derivative of levamisole. Levamisole is metabolized in some animals to OMPI which contains a SH group (figure 5). If OMPI is generated in plants the SH group may react with the epoxide group of phaseolinone. However, a direct reaction with SH and epoxide group of phaseolinone does not appear to be responsible for detoxification because neither cystine nor cysteine had any effect under the condition used. It is reported that levamisole protects cancer patients and mouse from cytotoxic effects of 5-fluorouracil (Johnkoski *et al* 1996). Levamisole-mediated protection of animals from cytotoxicity induced by 5-fluorouracil and of germinating seedlings from phaseolinone indicates that some basic biochemical event is taking place both in plants and animals for detoxification which however does not depend upon the nature of the compound. Sodium malonate which has been reported to block the action of levamisole in animal cells (Klein *et al* 1996) was also found to block the effects of levamisole on seedlings in respect to growth and immunity against infection. The antagonistic effect of sodium malonate in animal cells has been explained as due to opposing effect in membrane potential. It may act on plants by similar mechanism.

Levamisole gave protection to seedlings against phaseolinone-induced infection initiated by *A. fumigatus* but failed to protect against virulent strain of *M. phaseolina*. This indicated that levamisole can restore loss of immunity against non-specific infection only. lmmunorestorative activity of levamisole in animal is documented by Turrizani *et*

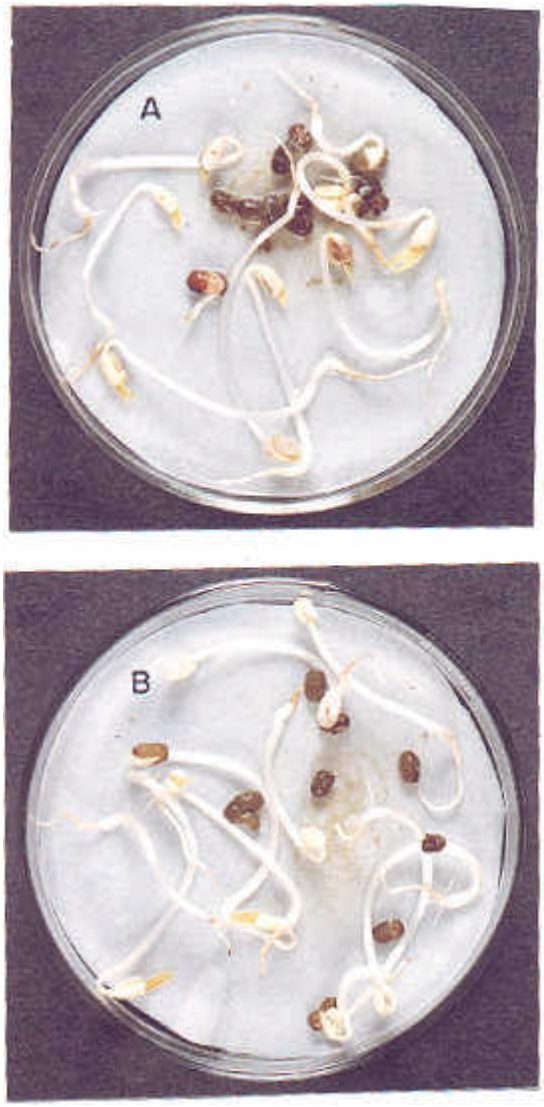

Figure 4. Protection of germinating seedlings from phaseolinone-induced infection by *A. fumigatus* in the presence of levamisole. (**A**) Infection of *P. mungo* by *A. fumigatus* in the presence of 5 µg/ml phaseolinone. (**B**) Protection of seedlings in the presence of 50 µg/ml levamisole.

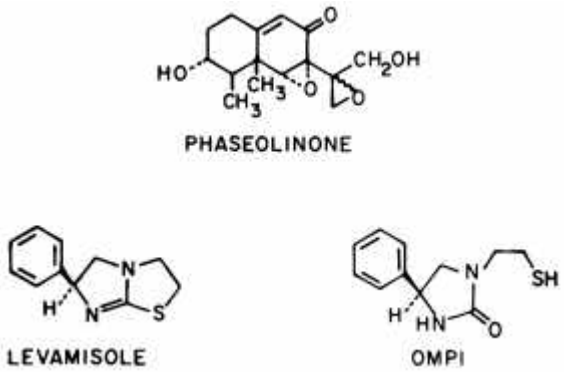

Figure 5. Chemical structure of phaseolinone, levamisole (L-2,3,5,6-tetrahydro-6-phenyl-imidazo[2,1-b]thiazole) and OMPI.

al (1991). They have shown that levamisole plays a significant role in adjuvant chemotherapy of colorectal cancer but it does not alleviate allograft response inhibition produced by high-dose dacarbazine or by a mouse RNA virus. Shiraishi *et al* (1983) have also shown that in tumour-bearing mice levamisole restores suppressed function to the immune system but does not enhance the functions to level above the normal.

As a non-specific irnmunomodulator in animals, levamisole in combination with chemotherapy is being increasingly used for the treatment of cancer and AIDS. The mode of action of levamisole in relation to immunity has not yet been fully elucidated. The mode of action of levamisole and its importance in chemoimmunotherapy has been reviewed by Wauwe and Janssen (1991) and Greenspan and Erlich (1991) respectively. Protection of *P. mungo* seedling by levamisole from phaseolinone-induced infection by *A. fumigatus* is difficult to explain at this stage precisely, but it is most likely some component of defense is activated which was suppressed by phaseolinone. Susceptibility of *P. mungo* to *A. fumigatus* in the presence of high concentration of levamisole alone is important in developing an immunodepressed plant model. It is reported that, in animals, levamisole beyond an optimum level exhibits an adverse effect on immunity and it has been ascribed to over stimulation. Recently, Delledonne *et al* (1998) suggested the probable existence of functional parallels between innate immunity in animals and hyper sensitive response in plants. The similarity in the action of levamisole in plants and animals at biochemical as well as defense level points to possible effects on a common target site(s). Recent findings indicate that innate immunity of plants and animals show similarity in several respects. Baker *et al* (1997) in a review on signal transduction in plant–microbe interactions pointed out that innate immunity of plants and animals has originated from a common ancestor. Detailed work on the action of phaseolinone and levamisole on plants may certainly provide valuable information on plant immunity as well as on the mode of action of levamisole in relation to innate immunity in general.

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