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Rhizosphere fungi of tomato : their effect on seed germination, seedling growth and the causal agent of wilt of tomoto

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Rhizosphere of tomato is colonized by several different types of microorganisms that influence its seed germination, seedling growth as well as health. Number, type and distribution of rhizosphere microorganisms change at different growth stages of the plants. In the present investigation, twenty four species of fungi were isolated from the rhizosphere of tomato plants at different growth stages. These included *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., *Curvularia lunata, Cladosporium cladosporioides, Helminthosporium* sp., *Mucor plumbeus, Alternaria alternata, Cephalosporium acremonium, Trichoderma* spp. and a white sterile fungus. The metabolites of *Trichoderma* spp., *Aspergillus* spp., *Cladosporium cladosporioides* and the white sterile fungus were found to enhance the seed germination as well as the seedling growth of tomato. These fungi showed antagonistic activity against tomato wilt causing pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), when tested by colony interaction method. The metabolites of these fungi exhibited differential degrees of growth inhibition of Fol

Key words: *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), metabolites, rhizosphere fungi, tomato wilt.

INTRODUCTION

Rhizosphere of tomato is colonized by several species of fungi. Species of *Fusarium, Penicillium, Trichoderma, Aureobasidium* and *Talaromyces* are the typical colonizers of soil environment of many crop plants including tomato (Kurzawinska and Pacyna, 2000; Jamiolkowska and Wagner, 2005). Species of *Trichoderma* were isolated more frequently at early phase of tomato growth particularly during the month of August, probably due to higher soil temperature (Kredics *et al.*, 2002). The ability of *Trichoderma* spp. to produce antibiotics increases with rise in temperature and decreases with increase in soil pH (Howell, 1998). However, it has been found that the frequency of wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* was significantly lower in the rhizosphere of tomato grown in rye mulch. This might be due to the residues of rye that provide nutrients to many soil microorganisms and subsequently increase their population (Huber and Watson, 1970; Pieta *et al.*, 1999). Some of these microorganisms are antagonistic to *Fusarium oxysporum* f. sp. *lycopersici* and, therefore, their presence in the tomato rhizosphere can reduce the population of this pathogen both by competition and antibiosis (Kurzawinska and Pacyna, 2000; Jamiolkowska and Wagner, 2003).

Metabolites of *Trichoderma virens, Trichoderma* spp., *Cladosporium cladosporioides* and a non pathogenic form of *Fusarium moniliforme* have been reported to promote seed germination and seedling emergence of various host plants (Hoflich *et al.*, 2001). The stimulation of seed germination and seedling growth might be due to the presence of growth promoting substances in the metabolites of these fungi (Hoflich *et al.*, 2001; Egamberdieva, 2007). A number of rhizosphere fungi cause inhibition of seed germination and seedling growth of a number of plants which may be due to the presence of some growth of a number of plants which may be due to the presence of some growth inhibitory substances in the metabolites of such fungi or due to unfavorable acidic nature of such

metabolites (Dennis and Webster, 1971b). Metabolites of *Aspergillus niger, Fusarium culmorium, Penicillium* sp. and *Rhizoctonia solani* have been reported to reduce the seed germination and seedling growth of soybean. (Haikal, 2008).

Many rhizosphere fungi have been reported to suppress the growth of pathogenic microorganisms (Hyakumachi *et al.*, 1994; Elad, 2000). The antagonistic rhizosphere fungi inhibit the growth of several plant pathogens by competing at the active sites (Biswas and Sen, 2000) and subsequently, reduce the intensity of disease development (Hyakumachi *et al.*, 1994). Antagonistic nature of *Trichoderma virens, Trichoderma* sp. and *Aspergillus* against *Phytophtyora capsici* causing foot root disease of black pepper has been reported (Noveriza *et al.*, 2004). *T. harzianum* and *T. reesei* have been found to inhibit the mycelial growth of *Sclerotium rolfsii* causing damping-off disease of tropical pine (Widyastuti *et al.*, 2003). Metabolites of *T. harzianum. T. viride* and *T. virens* have been found to inhibit the mycelial growth of *Fusarium oxysporum* f. sp. *ciceri* causing wilt disease of chick pea (Dubey *et al.*, 2007). Non-volatile metabolites of *Trichoderma* spp. inhibited the mycelial growth of *Fusarium oxysporum* (Dennis and Webster, 1971a). *Aspergillus niger, Aspergillus nidulans, Trichoderma* sp., *Trichoderma viride* and *Penicillium citrinum* were found to be antagonistic against *Fusarium moniliforme* (Vam and Saravanamathu, 1994).

MATERIALS AND METHODS

The following methodologies were followed for various experiments conducted during the study.

Isolation of Fusarium oxysporum f. sp. lycopersici (Fol) from tomato Plants

The wilted plants of tomato were collected for the study from a tomato field of Agricultural Farm, Banaras Hindu University (BHU), Varanasi (India) and a farmer's field of Karaudi, situated adjacent to the BHU campus, in sterilized polyethylene bags and brought to the Laboratory of Mycopathology and Microbial Technology, Department of Botany, Banaras Hindu University (BHU). Roots of the plants were washed separately with tap water to separate the adhering soil particles. The roots of various diameters were aseptically cut into small pieces of two centimeters each (Harley and Waid, 1955). The root pieces were surface sterilized with 0.1% aqueous solution of NaOCl for one minute and then washed with sterilized distilled water for 5-6 times to remove traces of NaOCl. Five such sterilized root pieces were transferred on to 20 ml of cooled patato dextrose agar (PDA) medium in Petri plates with the help of sterilized forceps. The plates were then incubated at 25 ± 2°C for seven days. The wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* (FOL) was isolated and its pure culture was maintained on PDA slants for further studies.

Preparation of mass culture of the wilt pathogen (Fol)

The mass culture of the test pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) was prepared on barley grains (Shivanna *et al.*, 1994). Clean and intact barley grains were taken for this purpose. The grains were pre-wetted by boiling them in water for 20-30 minutes so as to raise the moisture content of the grains up to 40-50% and to make them soft enough for the profuse growth of the fungus. After boiling, the grains were spread on wire mesh so as to drain the excess of water. The grains were then mixed with 2% Gypsum (calcium sulphate) and 0.5% chalk powder (calcium carbonate) on dry weight basis. These would help to check the pH of the medium and prevent them from sticking with each other. Clean glucose bottles were filled with 100g (in each case) of such barley grains and the mouth of such bottles were plugged with non-absorbent cotton, which were then steam sterilized in autoclave at 22 p.s.i. for 1-2 hours. The bottles were then allowed to cool at room temperature and were inoculated with five agar blocks (5 mm diameter each) cut from the margin of the actively growing culture of the test pathogen. The bottles were incubated at 25 ± 2°C for ten days. The bottles were shaken once or twice daily for rapid and uniform colonization of the fungus. The barley grains colonized by the pathogen were air dried and aseptically stored at 4°C for further use.

Seeds of the susceptible variety of tomato H-24 were first surface sterilized with 1% NaOCl for one minute and then washed with sterilized distilled water for five to six times to remove traces of NaOCl. Ten such sterilized seeds were sown in each infested and control pots in triplicate. The pots were kept under greenhouse conditions and the plants were regularly observed for disease symptoms. The moisture content was maintained at 15-20% by watering with sterilized distilled water at regular intervals. The pathogen was reisolated from the diseased plants and compared with the original

ones to confirm its pathogenicity as per Koch's postulates (Koch, 1876).

Isolation of Fungi from Rhizosphere of Tomato

For the study of rhizosphere fungi both healthy and wilted tomato plants from the above captioned fields were uprooted at regular growth intervals i.e. seedling, vegetative, flowering and fruiting stages. Care was taken to dig out, as far as possible, the whole root system with a sterilized spatula. The root systems were then brought to the laboratory in separate polyethylene bags. The roots were given gentle taping to loosen-off the lightly adhering soil, in order to have just the rhizosphere soil attached to the root system. Precautions were taken to keep the roots of healthy and diseased plants separately. Small pieces of roots (2 cm in length) of different diameters were cut with sterilized scissors under aseptic condition and twenty five such root pieces for each one were transferred into two separate 250 ml Erlenmeyer flasks (one for healthy and the other for diseased root) containing 100 ml of sterilized distilled water. The flasks were shaken vigorously with the help of a shaker to get a homogenous suspension of the rhizosphere soil. Taking this as the stock solution, conventional soil dilution plate method (Warcup, 1950) was followed for isolation of the rhizosphere fungi. Dilutions as 1 : 100, 1 : 1000 and 1 : 10000 were prepared in separate sterilized conical flasks for the study. Three replicates of sterilized Petri plates were inoculated with one ml aliquots from all the diluted suspensions. To this was added 20 ml melted and cooled (40°C) potato dextrose agar medium and the plates were rotated slowly in clock-wise and anti-clock wise directions to disperse the soil solution uniformly in the culture medium. All the inoculated plates were then incubated at 25 ± 2°C. The plates were examined regularly and the colonies of fungi appearing on the medium were transferred into fresh sterilized Petri plates containing PDA medium were transferred into fresh sterilized Petri plates containing PDA medium to avoid over-running by the fast growing forms. The pure cultures of the fungi thus isolated were preserved on Potato Dextrose Agar slants at 4°C.

The per cent occurence of the fungi appearing on culture plates was calculated by the formulae given below.

Per cent occurrence

$$= \frac{\textit{Average number of total colonies of a species per plate} \times 100}{\textit{Average number of total colonies of all species per plate}} \times 100$$

The frequency was recorded from the fungal population of ten Petri plates. Each Petri plate was considered as a unit of study just like a quadrat in phyto-sociological study of higher plants (Saksena, 1955).

Per cent Frequency

$$= \frac{\text{Number of replicates in which species occurred}}{\text{Total number of replicates studied}} \times 100$$

Isolation of metabolites from the rhizosphere fungi

Five equal size blocks (5 mm each) of individual fungi, cut from the actively growing margins of five day old cultures, were inoculated separately into 250 ml conical flasks each containing 100 ml liquid Czapek-Dox medium. After ten days of incubation at 25 ± 2°C the static cultures were filtered firstly through Whatman filter paper no. 44 and finally through Seitz filter of porosity grade G–5 by vacuum filtration to obtain cell free culture filtrates. These cell free culture filtrates were stored at 4°C for further studies.

In vitro screening of the rhizosphere fungi for enhanced seed germination and seedling growth of tomato through production of their metabolites

A susceptible variety of tomato (var. H-24) was selected for the present study. Certified seeds of this variety were obtained from Indian Vegetable research Institute (IVRI) Adalhat, Varanasi.

Two ml of the metabolite of each rhizosphere fungus was poured into separate sterilized Petri plates containing three layers of blotting paper. Plates with the same amount of potato dextrose broth medium and sterilized distilled water in place of the culture filtrates, served as control. Seeds of the susceptible variety of tomato were surface sterilized with 1% NaOCl for one min. and then washed thoroughly with sterilized distilled water for several times to remove traces of NaOCl. Ten such surface sterilized seeds were then kept on top of the moistened blotting paper and incubated for seven days at 25 ± 2°C in NUV light for twelve-hour cycle of light and darkness. On seventh day, the observation was made for the seed germination and seedling growth by measuring the length of radicles and plumules. Each test was carried out with three replicates. The metabolites giving higher percentage of seed germination and greater seedling growth, in comparison to control, were considered to be due to the PGPF. The metabolites of ten PGPF mentioned

below showed significant growth promoting effect in comparison to control. Therefore, they were selected for further studies. The ten PGPF were *Trichoderma harzianum, Trichoderma koningii, Trichoderma viride, Trichoderma virens, Tricholderma sp., Aspergillus niger, Aspergillus flavus, Cladosporium cladosporioides, Penicillium citrinum* and a white sterile fungus.

Antagonistic properties of the selected rhizosphere fungi against the wilt causing pathogen in vitro

The selected fungi isolated from the rhizosphere of the tomato plants were tested for their antagonistic activity against the wilt causing pathogen by colony interaction method and through the effect of their culture filtrates as described below.

(a) Colony interaction between the screened PGPF and the test pathogen (Fol)

Equal sized blocks (5 mm in diameter) of the individual PGPF and the test pathogen cut from the actively growing margins of five day old cultures were placed over solid potato dextrose agar (PDA) medium (in triplicate Petri plates) approximately 3 cm apart in paired combinations. The inoculated plates were incubated at 25 ± 2°C and the observations were made after 7 days of incubation. Interactions were assessed with the help of colony interaction models of Skidmore and Dickinson (1976). The colony growth of both the type of microorganisms was measured at both sides i.e. towards and opposing each other from their central loci. The parameters used for the assessment of colony interaction were the breadth of inhibition zone, intermingled zone and per cent inhibition of radial growth which was calculated with the help of formula, $(r_1 - r_2/r_1) \times 100$ (Fokkema, 1976), where r_1 denotes diameter of the radial colony growth of the test pathogen towards opposite side and r_2 denotes its growth towards the opponent PGPF.

(b) Effect of volatile metabolites of PGPF on the radial colony growth of the test pathogen

The method of Denis and webster (1971b) was followed to determine the effect of volatile metabolites of the PGPF on radial colony growth of the test pathogen. Five mm disc of each PGPF was inoculated centrally in sterilized Petri plates containing 20 ml cooled PDA. The lid of each Petri plate was replaced with the same sized bottom plate containing 20 ml PDA medium pre-inoculated centrally with an agar block (5 mm in diameter) cut from the margin of actively growing culture of the test pathogen (FOL). Both the plates were then taped together properly with the help of cello tape. Plates containing 20 ml cooled PDA only on the lower half and PDA pre-inoculated with an agar block (5 mm diameter) cut from the margins of actively growing culture of the test pathogen (FOL) on the upper half served as control. The plates were incubated at 25 ± 2°C and the colony diameter of the test pathogen was measured after 48 hours. The per cent inhibition of growth of the test pathogen was calculated by using the formula given below.

Per cent growth inhibition

$$= \frac{\text{Growth in control - Growth in treatment}}{\text{Growth in control}} \times 100$$

(c) Effect of culture filtrates (non-volatile metabolites) of PGPF on the radial colony growth of the test pathogen

With the help of sterilized cork-borer, equal sized (5 mm) wells were cut centrally in cooled PDA medium poured in Petri plates. Each well was filled with 0.5 ml of metabolites of the individual PGPF and then covered with 5 mm blocks cut from the actively growing margin of the test pathogen (FOL). The control consisted of sterilized distilled water placed in the wells which were also covered with same sized block of the test pathogen. The experiment was set in replicates of three. The plates were then incubated at 25 ± 2°C for seven days after which the per cent inhibition in growth of the test pathogen was calculated by using the formula given below.

Per cent growth inhibition

$$= \frac{\text{Growth in control - Growth in treatment}}{\text{Growth in control}} \times 100$$

RESULTS AND DISCUSSION

Rhizosphere fungi of tomato

Twenty four species of fungi were isolated from the rhizosphere of tomato plants during seedling, vegetative, flowering and fruiting stages (Ref. Table 1). The isolated fungi were found to be most common at vegetative stage. This was followed by the fungi recorded at flowering, seedling and fruiting stages respectively.

Aspergillus niger, Aspergillus terreus and *Aspergillus luchuensis* could be noted during all the stages of the plant growth. *Fusarium oxysporum, Trichoderma viride* and *Penicillium citrinum* were recorded at vegetative as well as the flowering

stages. Other fungal genera such as *Fusarium equiseti, Aspergillus clavatus, Aspergillus sydowii, Penicillium purpurogenum, Curvularia lunata, Cladosporium cladosporioides, Helminthosporium* sp. *Mucor plumbeus, Rhizopus* sp. *Cephalosporium acremonium, Trchoderma virens, Trichoderma harzianum, Trichoderma koningii, Trichoderma* sp. and the white sterile fungus were recorded at vegetative stage only. *Alternaria alternata* was recorded at flowering stage only. The species of *Trichoderma* were more commonly found during the vegetative stage. A white sterile fungus (which did not fructify) could be isolated at vegetative stage. In nut shell, out of all the 24 fungi which were isolated from the rhizosphere of tomato, the population of *Aspergillus* spp. was found to be dominant which gradually increased from seedling stage to vegetative stage. At this stage it attained a climax and then declined from flowering stage to the fruiting stage.

High occurrence of the selective rhizosphere microflora at a particular growth stage of a plant might be because of the following two reasons:

(a) The rhizosphere of a plant is more favourable for only some microorganisms. These favourable microorganisms directly or indirectly suppress the other microflora and, (b) There is an increase in the quantity of root exudates upto flowering stage due to increase in vegetative growth of the plant and, thereafter, they get decreased upto the senescent stage. The root exudates show a selective action on the rhizosphere microflora. Hence, certain fungi such as *Trichoderma, Curvularia, Mucor, Helminthosporium* and *Rhizopus* sp. were confined to a particular growth stage only (Peterson, 1958; El-Amin *et al.*, 2007).

In the present study, the species of *Aspergillus* and *Trichoderma* (though at vegetative stage), as

Table 1 : Rhizosphere fungi of tomato at different growth stages.

Fungi	Seedling stage		Vegetative stage		Flowering stage		Fruiting stage	
	%Oc	%Fr	%Oc	%Fr	%Oc	%Fr	%Oc	%Fr
Fusarium oxysporum	—	—	5	12	4	15	—	—
Fusarium equiseti	—	—	1	4	—	—	—	—
Aspergillus niger	14	28	18	32	23	48	14	24
Aspergillus clavatus	—	—	5	10	—	—	—	—
Aspergillus fumigatus	1	6	2	8	—	—	—	—
Aspergillus terreus	15	26	13	30	11	27	17	28
Aspergillus flavus	—	—	11	17	19	23	11	17
Aspergillus luchuensis	3	8	4	8	7	11	1	2
Aspergillus sydowii	—	—	2	5	—	—	—	—
Penicillium citrinum	—	—	1	5	2	11	—	—
Penicillium purpurogenum	—	—	2	5	—	—	—	—
Curvularia lunata	—	—	2	10	—	—	—	—
Cladosporium cladosporioides	—	—	3	15	—	—	—	—
Helminthosporium sp.	—	—	9	17	—	—	—	—
Mucor plumbeus	—	—	2	4	—	—	—	—
Rhizopus sp.	—	—	7	11	—	—	—	—
Alternaria alternata	—	—	—	—	2	5	—	—
Cephalosporium acremonium	—	—	2	5	—	—	—	—
Trichoderma virens (Gliocladium virens)	—	—	4	10	—	—	—	—
Trichoderma harzianum	—	—	2	4	—	—	—	—
Trichoderma koningii	—	—	2	5	—	—	—	—
Trichoderma viride	—	—	2	5	3	8	—	—
Trichoderma sp.	—	—	3	9	—	—	—	—
White sterile fungus	—	—	1	3	—	—	—	—
Total No. of Species	4	4	23	23	8	8	4	4

Oc, Occurrence
Fr, Frequency
—, Absent

already mentioned above, were found to occur dominantly in the rhizoshphere of tomato plants. This might be due to the proportionate and selective nature of the root exudates (Wahid *et al.,* 1997). Also, the presence of *Trichoderma,* which has been reported by several workers to be antagonistic, might have suppressed the pathogenic fungi in the root region such as the wilt causing *Fusarium oxysporum* f. sp. *lycopersici* (Hyakumachi, 2000; Noveriza *et al.,* 2004; Brunner *et al.,* 2005).

The species of *Aspergillus* and *Trichoderma* have been reported to be common in tropical soils by several workers (Eapen *et al.,* 2005; Nkwelang *et al.,* 2008) that also constitute an important component of the rhizosphere. In the present case, more frequent occurrence of rhizosphere mycoflora at the vegetative stage might be attributed to the availability of sufficient nutrients from the root exudates of tomato (El-Amin *et al.* 2007). The gradual decline in the rhizosphere mycoflora from flowering to fruiting stage might be attributed to less exudation from the roots due to senescence and partly due to rise in soil temperature and loss of moisture after vegetative growth of the plant that prevails during the months of March and April in this part of the country.

In vitro screening of the rhizosphere fungi for enhanced seed germination and seedling growth of tomato through the production of their metabolites

In vitro screening of all the 24 fungi, which were isolated from the rhizosphere of tomato plants at different growth stages, was done for promoting seed germination and seedling growth of tomato through the production of their metabolites (Ref. Table 2).

It was noted that the metabolites of different fungi have differential effects on seed germination as well as seedling growth of tomato. Highest percentage of seed germination was recorded in case of the seeds placed on the blotting papers treated with the metabolites of *Trichoderma virens* which was followed by *Trichoderma harzianum.* The seed germination and the seedling growth was significantly higher as compared to control when the seeds were placed on the blotting papers treated with the culture filtrates of *Aspergillus niger, Apergillus flavus, Penicillium citrinum, Cladosporium cladosporioides, Trichoderma virens, Trichoderma* sp. and the white sterile fungus (Table 2). These fungi promoted the seed germination to the extent of more than 66%. In case of *Trichoderma virens* and *Trichoderma harzianum* it was even more than 90%. Minimum radicle growth was recorded with the treatment of the culture filtrates of *Aspergillus flavus* (2.16 cm) as compared to control where it was only 2.3 cm. The radicle growth was recorded to be 6.66 cm, 6.53cm and 6.43 cm in case of *Trichoderma harzianum, Trichoderma virens* and *Trichoderma* sp. treatments, respectively, which was approximately 3 times more than the control. It was almost 2-fold in case of *Trichoderma konigii* and *Trichoderma viride.*

Plumule growth was recorded to be maximum (6.13 cm) in case of *Trichoderma virens* treatment which is almost three times more than the control where it was only 2.0 cm.

This was followed by *Trichoderma harzianum* (5.5 cm), *Trichoderma* sp. (5.3 cm) *Trichoderma viride* (4.26 cm) and *Trichoderma koningii* (4.2 cm) treatments. These above listed ten species of fungi were screened as Plant Growth Promoting Fungi (PGPF) and they were further tested and confirmed in *in vivo* experiments. Apart from this, seed germination and seedling growth was noted to be reduced in case of some fungal species but in others it was inhibited in comparison to control. The seed germination was found to be reduced in case of *Curvularia lunata, Aspergillus terreus, Aspergillus clavatus, Fusarium equisetii, Aspergillus sydowii, Cephalosporium acremonium* and *Aspergillus fumigatus* treatments. Seed germination in case of *penicillium purpurogenum* was recorded to be 65.23% which was almost same as recorded in control where it was 64.5%. Some fungi had an inhibitory effect on seed germination in comparison to control. These included *Rhizopus* sp., *Alternaria alternata, Mucor plumbeus, Fusarium oxysporum* and *Helminthosporium* sp.

A few fungi showed retarding effect on the growth of radicle. These were *Alternaria alternata, Curvularia lunata* and *Aspergillus sydowii.* A similar effect was recorded in case of plumule growth by the treatments with the metabolites of *Aspergillus luchuensis, Aspergillus terreus* and *Penicillium purpurogenum.* Inhibition of plumule and radicle growth was caused by *Helminthosporium* sp., *Mucor plumbeus, Rhizopus* sp., *Fusarium equisetii* and *Fusarium oxysporum.* The culture filtrates of a few fungi namely *Penicillium purpurogenum* and

Aspergillus luchuensis had inhibitory effect on radicle growth but possessed retarding effect on plumule growth.

The stimulation of seed germination and seedling growth might be due to the presence of growth promoting substances in the metabolities of the PGPF (Hyakumachi *et al.,* 1992, 1994; Egamberdieva, 2007). The other reason could be the solubilization of nutrients by these fungi (Altmore, *et al.,* 1998, 1999; Rengel *el al.,* 2005; El-Azouni, 2008). It has been reported that the rhizosphere microorganisms stimulate the seedling growth of plants such as pepper and pine (Hoflich *et al.,* 2001).

A number of rhizosphere fungi cause inhibition of seed germination and seedling growth of a number of plants which may be due to the presence of some growth inhibitory substances in the metabolites of such fungi or due to unfavorable acidic nature of such metabolites (Dennis and Webster, 1971b). Singh (1982) reported inhibitory effect of certain fungal metabolites on the per cent seed germination and seedling growth of *Brassica campestris* and *Linum usitatissimum.* He believed it to be due to presence of some growth Inhibitory substances in such culture filtrates. Haikal (2008) reported that the culture filtrates of *Aspergillus niger, Fusarium culmorium, Penicillium* sp. and *Rhizoctonia solani* reduced the percentage of seed germination and inhibited the seedling growth of soybean.

Colony interaction between the screened PGPF and the test pathogen (FOL)

All the ten *in vitro* screened PGPF isolated from the rhizosphere of tomato plants were tested for their

Table 2 : *In vitro* screening of the rhizosphere fungi for enhanced seed germination and seedling growth of tomato through production of their metabolites.

Fungi	Seed germination (%)	Seedling growth (cm)	
		Radicle	Plumule
Fusarium oxysporum	$20.4^{b} \pm 0.8$	$0.87^{a} \pm 0.3$	$0.5^{a} \pm 0.1$
Fusarium equisetii	$53.26^{h} \pm 0.1$	$1.0^{ab} \pm 0.0$	$0.83^{ab} \pm 0.1$
Aspergillus niger	$75.73^{q} \pm 0.3$	$2.96^{fg} \pm 0.0$	$2.5^{g} \pm 0.1$
Aspergillus clavatus	$57.83^{f} \pm 0.4$	$2.2^{c} \pm 0.1$	$2.03^{efg} \pm 0.1$
Aspergillus fumigatus	$45.26^{e} \pm 0.3$	$2.3^{ef} \pm 0.2$	$2.2^{fg} \pm 0.1$
Aspergillus terreus	$60.26^{i} \pm 0.3$	$2.1^{de} \pm 0.2$	$1.86^{def} \pm 0.0$
Aspergillus flavus	$67.83^{m} \pm 0.4$	$2.16^{e} \pm 0.2$	$2.13^{efg} \pm 0.2$
Aspergillus luchuensis	$4.3^{e} \pm 0.1$	$1.33^{a} \pm 0.1$	$1.96^{efg} \pm 0.2$
Aspergillus sydowii	$51.2^{g} \pm 0.3$	$1.93^{ide} \pm 0.1$	$2.00^{fg} \pm 0.1$
Penicillium citrinum	$69.9^{o} \pm 0.2$	$2.5^{abcd} \pm 0.1$	$2.00^{efg} \pm 0.2$
Penicillium purpurogenum	$65.23^{l} \pm 0.1$	$1.36^{abc} \pm 0.2$	$1.56^{cde} \pm 0.2$
Curvularia lunata	$64.23^{kl} \pm 0.1$	$1.53^{bcd} \pm 0.2$	$1.0^{abc} \pm 0.0$
Cladosporium cladosporioides	$68.9^{n} \pm 0.2$	$2.36^{ef} \pm 0.2$	$2.30^{efg} \pm 0.1$
Helminthosporium sp.	$15.26^{a} \pm 0.1$	$1.43^{ab} \pm 0.2$	$0.76^{ab} \pm 0.1$
Mucor plumbeus	$24.9^{c} \pm 0.2$	$1.36^{abc} \pm 0.1$	$1.1^{bc} \pm 0.2$
Rhizopus sp.	$37.4^{d} \pm 0.3$	$1.36^{abc} \pm 0.2$	$1.33^{bcd} \pm 0.2$
Alternaria alternata	$25.4^{c} \pm 0.3$	$1.56^{bcd} \pm 0.2$	$1.1^{bc} \pm 0.2$
Trichoderma virens	$92.9^{u} \pm 0.2$	$6.53^{i} \pm 0.2$	$6.13^{k} \pm 0.2$
Cephalosporium acremonium	$47.86^{f} \pm 0.1$	$2.26^{e} \pm 0.1$	$2.13^{efg} \pm 0.2$
Trichoderma harzianum	$90.0^{t} \pm 0.5$	$6.66^{l} \pm 0.2$	$5.5^{j} \pm 0.2$
Trichoderma koningii	$70.0^{R} \pm 0.2$	$4.66^{h} \pm 0.2$	$4.2^{l} \pm 0.1$
Trichoderma viride	$82.6^{s} \pm 0.3$	$4.66^{h} \pm 0.2$	$4.26^{l} \pm 0.1$
Trichoderma sp.	$89.03^{st} \pm 0.5$	$6.43^{l} \pm 0.2$	$5.3^{i} \pm 0.2$
White sterile fungus	$66.16^{p} \pm 0.4$	$3.43^{f} \pm 0.2$	$3.13^{h} \pm 0.1$
PD solution only	$64.0^{k} \pm 0.0$	$2.5^{af} \pm 0.0$	$2.0^{efg} \pm 0.0$
Distilled water (control)	$64.5^{kl} \pm 0.0$	$2.3^{e} \pm 0.0$	$2.0^{efg} \pm 0.0$

±, Standard error of mean (SEM). It implies to all other tables where ever it has been used. PD, potato dextrose.

a, b, c, d, e, f, g, h, i, j, values in the column followed by same letter are not significantly different ($P < 0.05$)

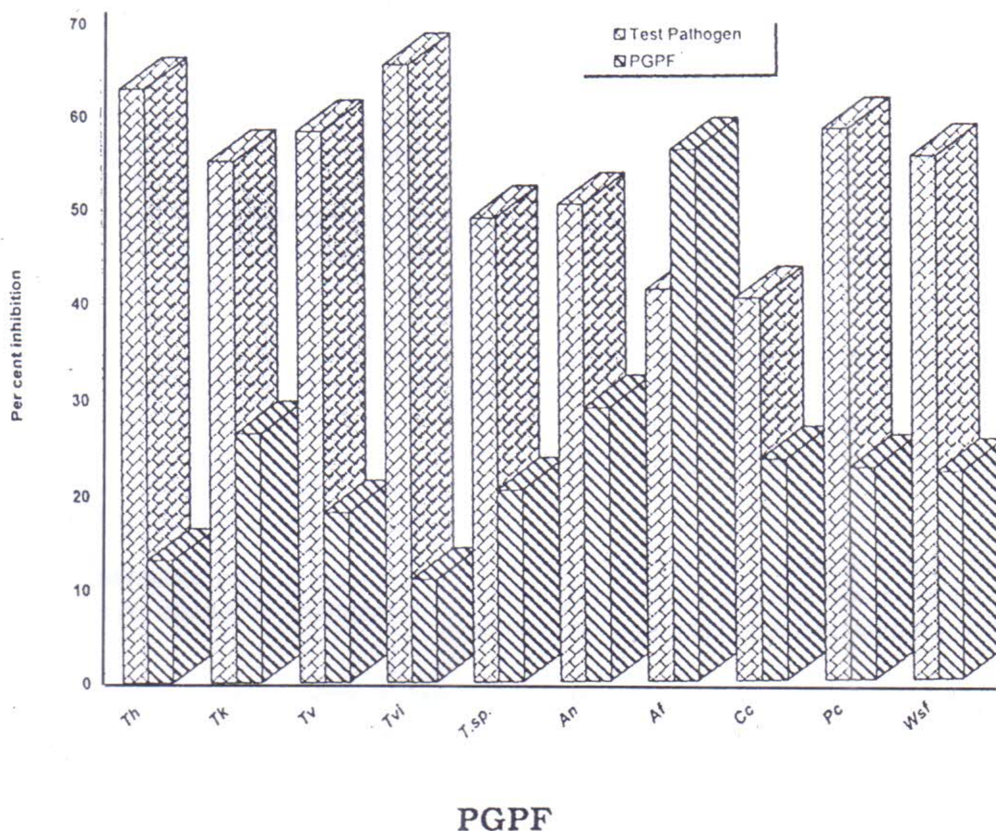

Fig. 1 : Colony interaction between the screened Plant Growth Promoting Fungi (PGPF) and the test pathogen*

*(Abbreviations : *Th, Trichoderma harzianum; Tk, Trichoderma koningii; Tv, Trichoderma virens; Tvi, Trichoderma viride; T.* sp., *Trichoderma* sp.; *An, Aspergillus nigher; Af, Aspergillus flavus; Cc, Cladosporium cladosporides; Pc, Penicillum citrinum; Wsf,* White sterile fungus). These abbreviations apply in other figures also.

antagonistic activity against the test pathogen *Fusarium oxysporum* f.sp. *lycopersici* (Fol) by colony interaction method (Fig. 1). Assessment of the colony interaction was made following the model of Skidmore and Dickinson (1976). The measurement of the colony growth of both the microorganisms was done on both sides i.e. towards and opposing each other from their central loci. The parameters used for assessment of the colony interaction were the width of inhibition zone and the intermingled zone. On the basis of the colony interaction between each PGPF and Fol, the below captioned grades were assigned.

Grade Bi. The test PGPF grew over the test pathogen either above or below or both and suppressed its growth. This type of intermingling growth was common in case of *T. harziamum, T. koningii, T. viride, T. virens, Trichoderma* sp., *A. niger, A. flavus, C. cladosporioides* and the white sterile fungus.

Grade C. The test PGPF as well as the test pathogen grew and approached each other until almost in contact, leaving a narrow demarcation line between the two colonies and resulting into a slight inhibition zone. This type of colony interaction was recorded in case of *Penicillium citrinum* only.

The maximum inhibition in radial colony growth of FOL was caused by the metabolites of *Trichoderma virens* (65.46%). This was followed by *T. harzianum* (62.96%), *T. viride* (58.3%), *P. citrinum* (58.26%), the white sterile fungus (55.26%), *T. koningii* (55.1%). *A. niger* (50.3%), *Trichoderma* sp. (48.93%), *A. flavus* (41.26%) and *C. cladosporioides* (40.26%). On the other hand, maximum inhibition in the radial colony growth of PGPF by Fol was recorded in case of *A. Flavus* (56.16%). This was followed by *A. niger* (28.93%), *T. koningii* (26.43%), *C. cladosporioides* (23.5%), *P. citrinum* (22.53%), the white sterile fungus (22.03%), *Trichoderma* sp. (20.43%), *T. viride* (18.16%), *T. harzianum* (13.16%) and *T. virens* (11.0%).

Overgrowth of the PGPF over the test pathogen might be due to the rapid growth rate of the former and their tolerance to the antibiotics, if any, produced by the later (Garrett, 1981). *Trichoderma* has been identified as a fast growing fungus which is antagonistic against many other pathogenic and non-pathogenic fungi (Papavizas, 1985). This might be the reason for suppression of the growth of Fol (Ayers and Adams, 1981). *Trichoderma* has also been found to be effective against *Fusarium udum* (Bhatnagar, 1992, 1996). Gachomo *et al.* (2008) demonstrated that the growth of peanut moulds *(Aspergillus niger, A. flavus, A parasiticus, A. ochraceous* and *Fusarium* sp.) was significantly inhibited when co-cultured with *Trichoderma* spp.

Howell and Stipanovic (1995) reported that *Trichoderma virens (Gliocladium virens)* produced an antibiotic diketopiperazine (gliovirin) that was highly inhibitory to *Pythium ultimum.* Naseby *et al.* (2000) also made similar observation. Antagonistic nature of *A. niger, A. nidulans, Trichoderma* sp. *Trichoderma viride* and *Penicillium citrinum* against *Fusarium moniliforme* has been reported (Vam and Saravanamathu, 1994).

Antagonistic effect of *Trichoderma* against any microorganism occurs due to several factors like production of secondary metabolites, growth rate of the interacting microbe, competition for nutrients and space, nature and composition of culture media and physical factors (Skidmore and Dickinson, 1976) or because of its ability to produce and/or resist metabolites that exhibit antibiosis (kill the cells), fungistasis (inhibit spore germination) or mycoparasitism (parasitise the test fungus), etc. (Benitez *et al.,* 2004). It has been reported that *Trichoderma harzianum* and *Trichoderma reesei* possessed mycoparasitic activity in direct confrontation assay and reduced the incidence of damping-off disease caused by *Sclerotium rolfsii* (Widyastuti *et al.,* 2003). Mycoparasitic activity of *Trichoderma* might be because of its ability to produce cell wall-degrading enzymes (Di-Pietro *et al.,* 1993; Schirmbock *et al.,* 1994). Noveriza *et al.* (2004) studied the antagonistic activity of rhizosphere mycoflora of black pepper by dual culture technique and suggested that the antagonistic mechanism of *Mucor, Trichoderma, Cunninghamella, Mortierella* and *Aspergillus* against *Phytophthora capsici,* the pathogen which causes foot-rot disease of black pepper, was based on competition for nutrients and space since they rapidly overgrew the pathogen.

In the present study, the interaction of the PGPF and the test pathogen and occurrence of inhibition zone between them on agar media might have happened as a result of the production of antibiotics, changes in pH, and competition for nutrients and space (Biswas and Sen, 2000). A mutual growth of fungi in dual cultures is possible when both the microorganisms show equal growth rate, equal competition and equal capacity of tolerance to antibiotics produced by each other. The overgrowth is achieved when one microorganism exhibits higher growth rate, hgher capacity of antibiotic production and more tolerance against the other.

Effect of volatile and non-Volatile metabolites of the PGPF on the radial colony growth of Fusarium oxysporum f. sp. lycopersici.

The volatile as well as non-volatile metabolits of PGPF exhibited different degrees of growth inhibition on *Fol* (Fig. 2). The effect of non-volatile metabolites of the PGPF was more pronounced as compared to the volatile ones. In case of volatile metabolites of the PGPF, maximum inhibition of radial colony growth of *Fol* was caused by the metabolites of *Trichoderma harzianum* (28.1%). This was followed by the metabolites of *T. virens* (27.22%), *T. viride* (26.53%), *Trichoderma* sp. (24.56%), *T. koningii* (24.4%). the white sterile fungus (23.86%), *P. citrinum* (22.53%), *A. niger* (20.03%), *C. cladosporioides* (16.9%) and *A. flavus* (15.36%). As far as the non-volatile metabolites of the PGPF are concerned, *Trichoderma virens* was found to be the most effective fungus with its metabolites causing maximum inhibition of the radial colony growth of *Fol* (63.16%). This is followed by the non-volatile metabolites of *T. harzianum* (62.26%). *A. niger* (60.16%), *A. flavus* (53.16%), *Trichoderma* sp. (50.16%), *T. koningii* (49.83%), the white sterile fungus (48.23%), *T. viride* (47.1%), *P. citrinum* (47.0%) and *C. cladosporioides* (42.3%).

The inhibition of the radial colony growth of FOL may be attributed to growth inhibitory substances present in the volatile metabolites of the PGPF (Dennis and Webster, 1971b). *T. virens* has been reported to produce volatile substances in its culture filtrates that are harmful to *Pythium aphanidermatum* (Howell and Stipanovic, 1983). John *et al.* (2004) studied the interaction between *T. harzianum* and

Eutypa lata, the pathogen which causes dieback of grapevine and reported that the metabolites produced by *T. harzianum* reduced the growth of this test pathogen *in vitro.* Eziashi *et al.* (2006) tested the metabolites of *Trichoderma* spp. against *Ceratocystis paradoxa* and found them to be growth inhibitory. The inhibition of growth of *C. paradoxa* was reported to vary from 2.0% to 64% and 0.0% to 74% due to volatile and non-volatile metabolites of *Trichoderma* spp., respectively. The varied degree of inhibition of growth of the test fungus might be due to varied concentrations of the metabolites and quality and quantity of the inhibitory substances present in such metabolites (Dennis and Webster, 1971a, b; Kumar and Dubey, 2001). The mode of action of volatile metabolites may be by the activation of enzymes, removal or neutralization of inhibitors, influence on nutrient uptake from the medium, and stimulation of some limiting factors in the intermediary metabolites (Fries, 1973).

Inhibition of mycelial growth of *Fusarium oxysporum, Heterobasidion annosum and Phytophthora* spp. by non-volatile metabolites of *Trichoderma* spp. has been reported (Etebarian *et al.,* 2000) and the reasons have been attributed to the production of substances such as antibiotics, toxins, etc. in the culture filtrates of the test microorganisms (Dhendhi *et al.,* 1990). It has been reported that *Trichoderma* spp. produce non-volatile substances such as Trichodermin which could be the cause of inhibition of the growth of FOL in the present study (Dennis and Webster, 1971a). The variability in per cent inhibition of the pathogen by the selected PGPF might be due to difference in nature, quality and quantity of the inhibitory substances produced by the individual PGPF (Eziashi *et al.* 2006). Vinale *et al.* (2006) reported that *T. harzianum* produced a metabolite identified as T22 azaphilone (83) that inhibited the growth of *Rhizoctonia solani, Pythium ultimum* and *Gaeumannomyces graminis var. tritici.*

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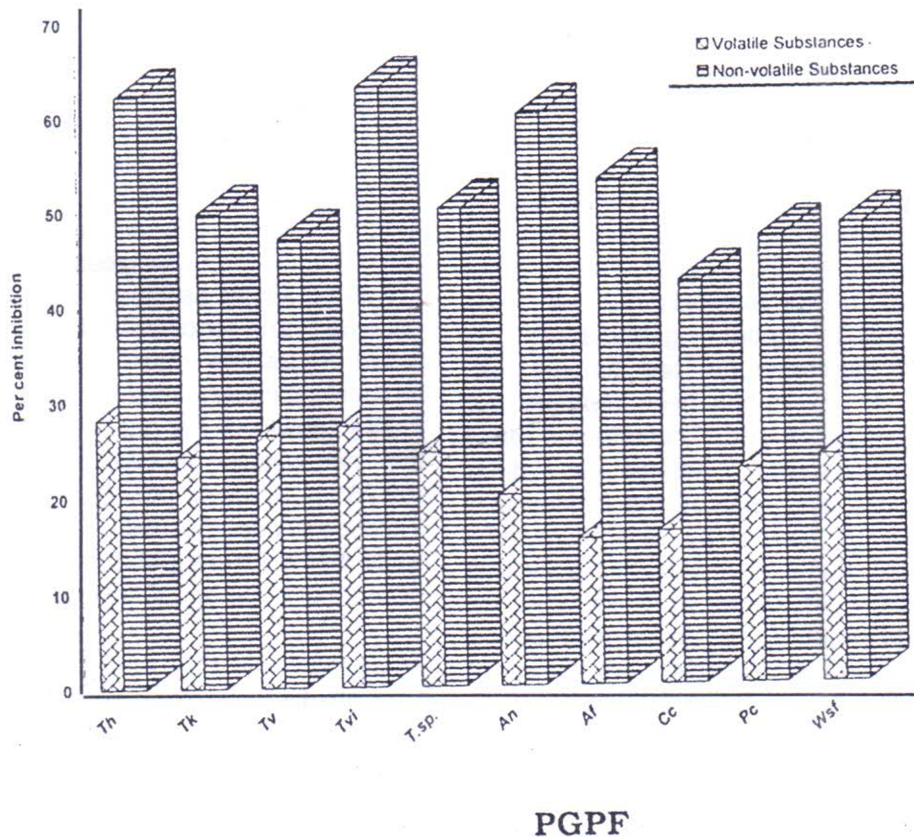

Fig. 2 : Effect of volatile and non-volatile metabilites of the PGPF on the radial colony growth of *Fusarium oxysporum* f. sp. *lycopersici.*

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