Original Research Article

Evaluation of culture filtrates of endophytic microorganisms from tomato against *Ralstonia solanacearum*

ABSTRACT

A novel approach to biological control using endophytes has gained immense potential in disease management. The endophytes constitute a valuable source of secondary metabolites such as alkaloids, terpenoids, sterols and phenolic compounds which play a major role in plant defense mechanisms. Therefore an experiment was carried out to study the effect of secondary metabolites of endophytes from tomatoes against the bacterial wilt pathogen *Ralstonia solanacearum* both *in vitro* and *in vivo* conditions using their culture filtrates. *In vitro* evaluation of the culture filtrates of the endophytes was carried out by inoculating the pathogen on a five per cent culture filtrate amended Potato Dextrose Agar medium. Complete inhibition of the pathogen was observed in all endophytes indicating the inhibitory effect of their culture filtrates. In order to study the effect of secondary metabolites of endophytes on *R. solanacearum* under *in planta* conditions, culture filtrates along with culture suspensions for comparison were used. The tomato seedlings dipped first in culture filtrate/suspension then in bacterial ooze showed lowest wilt incidence and among the two, seedling dip in filtrate was the effective one. The minimum wilt incidence was noticed with seedlings dipped first in culture filtrate of endophyte *Trichoderma viride*-2 recording 10.92, 15.68 and 19.84 per cent followed by *T. harzianum*-1 and *Bacillus subtilis*, which showed 12.5, 16.67 and 22.22 per cent at 7, 10 and 14 days after inoculation respectively. Thus the above findings clearly indicated the inhibitory effect of secondary metabolites of the endophytes on bacterial wilt pathogen.

Keywords: *Ralstonia solanacearum*; *Trichoderma viride*; *Trichoderma harzianum*; *Bacillus subtilis*; culture filtrate; secondary metabolites

1. INTRODUCTION

The biological control method using endophytes has entered the arena of disease management with attempts to make the plant, defend itself from the pathogens. The beneficial effects that the endophytes can confer on plants have made their role highly significant in biological control of diseases in various crops. The biological control method using endophytes has entered the arena of disease management with attempts to make the plant, defend itself from pathogens. The beneficial effects that the endophytes can confer on plants have made their role highly significant in the biological control of diseases in various crops.

The term 'endophyte' is derived from two Greek words, 'endon' meaning 'within' and 'phyton' meaning 'plant'. Endophytes have been defined in several ways and the definitions have been modified as the research in this field advanced. The earliest definition was given by (2) as the microorganisms that colonize internal plant tissues. Perotti, R (10) reported the presence of non pathogenic microorganisms in plant tissues for the first time. According to (11), endophytes are the microorganisms that inhabit for at least one period of their life cycle inside plant tissues without causing any apparent harm to the hosts. They may originate from indigenous species that occur either naturally in soil or may be introduced through various agricultural practices. Endophytes also constitute a valuable source of secondary metabolites for the discovery of new potential therapeutic drugs (5). Secondary metabolites such as alkaloids, terpenoids, sterols and phenolic compounds are the constituents which play a major role in plant defense mechanisms (6). Some of the endophytic bacterial strains have been reported to produce metabolites which can play an important role in ISR against many plant diseases. Hence an attempt was made to study the significance of secondary metabolites produced by endophytes in defence mechanism.

2. MATERIALS AND METHODS

The effect of culture filtrates of the endophytes on *R. solanacearum* was evaluated under both *in vitro* and *in vivo* conditions.

2.1 Preparation of culture filtrate

The endophytic isolates were inoculated separately in 100 ml potato dextrose broth and incubated at 28 $\pm$ 2^0C for 21 days. The cell free culture filtrate of the isolates was prepared by removing the mycelium and other cells by filtering first through double layered filter paper and then by passing through a bacterial proof filter of pore size 0.22 µm.

2.2 *In vitro* evaluation of culture filtrate of endophytes against *R. solanacearum*

For *in vitro* evaluation, *R. solanacearum* was inoculated on a five per cent culture filtrate amended PDA medium. The medium without filtrate served as a control. Observations on the growth of the pathogen were recorded.

2.3 Evaluation of culture filtrate of the endophytes against *R. solanacearum* under *in planta* condition

In order to study the effect of secondary metabolites of endophytes on *R. solanacearum* under *in planta* condition, culture filtrates along with culture suspensions for comparison were used. Both undiluted and 30 per cent diluted culture filtrates were employed for the study.

2.3.1 Preparation of culture suspension

The endophytic bacteria, fungi, and actinomycetes were inoculated separately in 100 ml PDB and incubated for 2, 7 and 14 days respectively. These culture suspensions were diluted to the concentrations of 10^6 spores ml^{-1} for fungi, 10^8 cfu ml^{-1} for bacteria and 10^5 cfu ml^{-1} for actinomycete.

2.3.2 Preparation of inoculum

Wilted plants were collected, washed thoroughly in running tap water and the root portion was removed. 100 g of stem portion cut into smaller bits were suspended in 100 ml sterile water for 30 min and the concentration was adjusted to OD_{600} = 0.3.

2.3.3 Method of application

Healthy 30 day old seedlings were uprooted and the roots were washed thoroughly under tap water and then with sterile water. In the first experiment, the seedlings were given root dip in 30 per cent diluted culture filtrate of endophytic isolates for 2 h, then dipped in bacterial ooze suspension for 30 min and planted in polybags. Another set of seedlings was dipped in culture suspension of the endophytes for 2 h followed by dipping in ooze for 30 min before planting. In the second experiment, the seedlings were dipped first in ooze for 30 min and then in culture filtrate/suspension for 2 h before planting. Seedlings dipped in PDB and sterile water served as controls. The experiments were carried out in three replications with 12 plants in each treatment and wilt incidence was recorded at periodical intervals. The statistical design used was completely randomized design and arc – sine transformed values are used to compare the means.

3. RESULTS

The effect of secondary metabolites of the potential endophytes on the pathogen was studied both *in vitro* and *in vivo* using their culture filtrates.

3.1 *In vitro* evaluation of secondary metabolites of endophytes against *R. solanacearum*

Complete inhibition of the pathogen was observed on the medium amended with culture filtrate of the endophytes indicating the inhibitory effect of the secondary metabolites.

3.2 *In vivo* evaluation of secondary metabolites of the endophytes against the pathogen

The effect of secondary metabolites on *R. solanacearum* was studied under *in vivo* conditions using the diluted and undiluted culture filtrate of the endophytes along with culture suspensions as a comparison. Seedlings dipped in undiluted culture filtrate before planting showed wilting symptoms in 24 to 48 h indicating phytotoxicity.

Therefore, the diluted culture filtrate of 30 per cent concentration was used for the experimental purpose.

The data furnished in the Table 1 revealed that, seedlings dipped first in culture filtrate/suspension and then in bacterial ooze showed lowest wilt incidence and among the two, seedling dip in the filtrate was the most effective one. The minimum incidence was noticed with seedlings dipped first in culture filtrate of *T. viride*-2 recording 10.92, 15.68 and 19.84 per cent followed by *T. harzianum*-1 and *B. subtilis*, which showed 12.5, 16.67 and 22.22 per cent at 7, 10 and 14 days after inoculation respectively. The isolates, *T. viride*-1 and *Streptomyces thermodiastaticus* were least effective, but found superior to control. Overall performance of various isolates at 14 days after inoculation revealed that, *B. subtilis* was the most efficient one as it recorded lowest wilt incidence of 22.22, 34.92, 26.19 and 38.89 against cent per cent in control, in all the four types of treatments employed. However, not much significant difference was noticed among the treatments with different isolates and was found to be on par.

In the case of seedling dip first in suspension and then in bacterial ooze, *B. subtilis* was found effective showing minimum wilt incidence at all intervals of observation and the same trend was observed in seedling dip first in ooze followed by filtrate.

When the seedlings were treated first with ooze and then in culture suspension, the isolate *B. subtilis* and *T. viride*- 2 showed less incidence as compared to control. Thus the above findings clearly indicated the inhibitory effect of secondary metabolites of the endophytes on bacterial wilt pathogen.

4. DISCUSSION AND CONCLUSION

In the experiment, the study of the effect of secondary metabolites of endophytes was carried out against the pathogen in both *vitro* and *vivo* conditions.

The selected endophytes tested *in vitro* were found positive for the production of inhibitory substances recording cent percent inhibition of the pathogen. It has been reported that culture filtrates of endophytic fungi showing variable antifungal activities

against the phytopathogenic fungi, *Botrytis cinerea* and *Rhizoctonia solani* (4). According to (12), *in vitro* antagonistic evaluation study of endophytic *Colletotrichum gloeosporioides* from *Camellia sinensis* carried out against the fungal pathogen viz., *Pestalotiopsis theae* showed 64% antagonistic activity. of *Camellia sinensis* .

The tested endophytes found effective under *in vitro* evaluation were tested *in vivo*, using culture filtrate along with culture suspension for comparison. Among the different treatments, dipping the seedlings either in culture filtrate or suspension prior to bacterial ooze treatment was more effective as it recorded lowest wilt incidence compared to dipping after bacterial inoculation. It may indicate the preventive or protective action by the way of antibiosis and defense mechanism of the endophytes. Among these two, dipping first in culture filtrate was found to be the best treatment with minimum incidence noted in the filtrate of *T. viride*-2 (19.84 %), which may be due to quick and direct action of secondary metabolites in the culture filtrate on the pathogen. It is also noted that, seedlings treated first in ooze and later in suspension/culture filtrate also showed lesser disease incidence as compared to control which indicates curative or suppressive action of filtrate/suspension after entry of the pathogen. It is also noted that, culture filtrate of *T. viride*-2 (a root endophyte from Mannuthy) and suspension of *B. subtilis* (a stem endophyte from Vellanikkara) recorded minimum incidence of 19.84 and 26.19 per cent respectively which may be due to the high amount of secondary metabolites in the fungal filtrate and the faster multiplication of bacterial cells in case of *B. subtilis*. Thus this study indicated the role of secondary metabolites present in the culture filtrates of the endophytes in enhancing the defense mechanism in tomato. A search through the literature did not give relevant information about the aforesaid aspects. (7) reported that, tomato seedlings dipped in culture filtrate of *T. harzianum* showed maximum control of Fusarium wilt after inoculation. Later, (3) studied and demonstrated the inhibitory activity of the filtrates or extracts of the fungal endophytes, *Drechslera biseptata* and *Epicoccum nigrum* from *Lupinus luteus* against the pathogen *Phytophthora cinnamomi* both under *in vitro* and *in planta* conditions. The report by (8) also demonstrated the antagonistic activity of four endophytic fungal isolates from *Solanum tuberosum* against *Rhizoctonia solani* and

their ability to suppress black scurf disease of potato tubers. Endophytic bacterial strains also have been reported to produce many kinds of metabolites which can play an important role in ISR against many plant diseases. According to (9), *Pseudomonas fluorescens* EBS 20 produced secondary metabolites including salicylic acid, siderophore and hydrogen cyanide, and its culture filtrate at a 15% concentration totally inhibited mycelial growth of *Pythium aphanidermatum*. The culture filtrate effect of endophytic *Bacillus* sp. was studied against different plant pathogens of rice and observed that, *Bacillus* strains produced several cyclic peptides, amino-polyols and amino-glycosides, which are having significant effect on ISR development (13). Moreover, endophytes represent an inexhaustible reservoir of pharmacologically important compounds and the endophytic fungi could be exploited for the sustainable production of bioactive "plant metabolites" in the future. (1) The exact mechanism by which endophytic microorganisms induce protection in the host plants remains unclear, although production of secondary metabolites has been suggested as a possible mechanism. Intensive research is necessary to find out the facts and factors involved in the exact mechanism, which was beyond the scope of the present investigation.

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Table 1. Effect of culture filtrates of endophytes on bacterial wilt pathogen under *in planta* condition

Endophyte	* Per cent wilt incidence											
	7 DAI				10 DAI				14 DAI			
	Filtrate ↓ Ooze	Ooze ↓ Filtrate	Suspension ↓ Ooze	Ooze ↓ Suspension	Filtrate ↓ Ooze	Ooze ↓ Filtrate	Suspension ↓ Ooze	Ooze ↓ Suspension	Filtrate ↓ Ooze	Ooze ↓ filtrate	Suspension ↓ Ooze	Ooze ↓ Suspension
T. harzianum	12.50^{b} (0.35)	27.78^{bcd} (0.55)	27.78^{bc} (0.55)	44.44^{b} (0.733)	16.67^{b} (0.42)	38.89^{bc} (0.68)	33.33^{b} (0.61)	55.56^{bc} (0.85)	22.22^{b} (0.49)	44.44^{bc} (0.73)	44.44^{b} (0.73)	61.11^{bc} (0.90)
T. viride-1	22.22^{b} (0.49)	34.92^{b} (0.63)	33.33^{b} (0.61)	47.62^{b} (0.76)	33.33^{b} (0.61)	45.24^{bc} (0.74)	38.89^{b} (0.68)	57.94^{bc} (0.87)	38.89^{b} (0.67)	50.00^{bc} (0.79)	50.00^{b} (0.79)	63.49^{b} (0.93)
T. viride-2	10.92^{b} (0.33)	19.84^{cd} (0.46)	20.64^{bc} (0.47)	27.78^{bc} (0.55)	15.68^{b} (0.39)	29.37^{c} (0.56)	25.40^{bc} (0.52)	38.89^{cd} (0.67)	19.84^{c} (0.46)	36.51^{c} (0.65)	34.92^{bc} (0.63)	44.44^{cd} (0.73)
B. subtilis	12.50^{b} (0.35)	15.08^{d} (0.40)	17.26^{c} (0.41)	16.67^{c} (0.42)	16.67^{b} (0.42)	25.40^{c} (0.52)	17.26^{c} (0.41)	27.78^{d} (0.55)	22.22^{b} (0.49)	34.92^{c} (0.63)	26.19^{c} (0.53)	38.89^{d} (0.68)
S. thermodiastaticus	20.64^{bc} (0.47)	38.89^{b} (0.68)	22.22^{bc} (0.49)	44.44^{b} (0.73)	27.78^{b} (0.55)	50.00^{b} (0.79)	30.16^{bc} (0.58)	66.67^{b} (0.97)	38.89^{b} (0.67)	61.11^{b} 0.90)	40.48^{bc} (0.69)	77.78^{b} (1.09)
Control-1 (Medium →ooze)	83.33^{a} (1.15)	83.33^{a} (1.15)	83.33^{a} (1.15)	83.33^{a} (1.15)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)
Control-2 (Ooze → medium)	88.89^{a} (1.22)	88.89^{a} (1.22)	88.89^{a} (1.22)	88.89^{a} (1.22)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)

* Mean of three replications DAI – Days after inoculation

Treatment means with same alphabets in superscript do not differ significantly. Figures in parenthesis are arc-sine transformed values.