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Molecular Characterization of Native Trichodermaharzianum Sp. and Evaluation of Different Biocontrol Agents under in Vitro and in Vivo Conditions against Stem Rot and Wilt Disease of Carnation

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Abstract:
Different fungal antagonists viz., Trichodermaharzianum, T. hamatum, T. viride, T. polysporum,T. virens, Penicilliumsp., Aspergillussp., Rhizopus sp. and two bacterial antagonistic species namely Bacillus subtilis and Pseudomonas fluorescens were evaluated under in vitro and field conditions against stem rot of carnation and seven microorganisms representing five fungal viz., T. harzianum, T. viride, T. virens, Aspergillus sp. and Penicillium sp. and two bacteria viz., Bacillus sp. and bacterial isolate 1 were tested against Fusarium wilt of carnation during 2012 and 2013. Under in vitro conditions biocontrol agents significantly inhibited the growth and under field conditions they caused significant reduction in disease incidence and there is increase plant growth and quality parameters as compared to untreated control. The Trichodermaharzianum was found most effective and identity of T.harzianum were also established by PCR amplification and sequencing of either 18S rRNA or ITS gene sequences and it was submitted with an accession number KF924562.

1. Introduction

Among different ornamental crops, carnation (*Dianthus caryophyllus*L.) is one of the major cut flowers of the world. Carnation express love, fascination and distinctions. Carnation are native to Eurasia and historically, carnation are known to have been used for the first time by Greeks and Romans in garlands but commercial propagation started in 1954. Carnation is subjected to be attack by number of fungal, bacterial and viral pathogens which result in huge loss of planting material and quality flowers. Among fungal diseases Fusarium wilt (*Fusariumoxysporum* f. sp. *dianthi*), *Rhizoctonia* stem rot (*Rhizoctoniasolani*) are considered more devastating in nature.Owing to the huge losses caused by the disease and the importance of the flower crops in national and international market attempts were made to use a consortium of biological agents to get persistent control of plant pathogens.

2. Materials and Methods

2.1. Extraction of Genomic DNA

Isolate of *Trichodermaharzianum* were grown in 100 ml of potato dextrose broth (Difco, Detroit, MI, USA) at 26 °C on an orbital shaker (150 rpm) for 7 days. Mycelium was harvested by filtration through sterile Mira cloth, frozen with liquid nitrogen, and stored at –80 °C until use. Fungal genomic DNA was extracted according to a modified method of Saghai-Maroof*et al.*, (1984).Ground mycelium in liquid nitrogen was suspended in 500 µl of 200 mMTris-HCl (pH: 8.5), 25 mM of NaCl, 25 mM of EDTA, and 0.5% SDS. Samples were incubated for 30 min at 65 °C. After adding an equal volume of phenol-chloroform, the mixture was centrifuged at 13,000 ×g for 15 min. Then, 25 µl of RNAse-A was added and incubated for 30 min at 37 °C. The suspension was extracted once

with chloroformisoamyl alcohol and precipitated with 1 volume of isopropanol. The pellet was then rinsed with ethanol, suspended in TE buffer (pH 7.4) and stored at –20 °C.

2.2. PCR Amplification

PCR was performed in a total volume of 25 µl of reaction containing 10 mM of Tris-HCl (pH 8.8), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.32 µM of primer, 0.125 mM of dNTPs, and 0.6 U of Taq polymerase (MBI,Fermantase).Amplification was carried out in a thermal cycler (Biometra) programmed as follows: 40 cycles of 20 s at 94 °C, 1 min at 36 °C, 1 min at 72 °C, and a final cycle of 8 min at 72 °C. The identity of *Trichodermaharzianum* were also established by PCR amplification and sequencing of either 18S rRNA or ITS gene sequences and it was submitted with an accession number KF924562.

2.3. In vitro Evaluation of Antagonists against Stem rot and Wilt Disease of Carntaion

Different native fungal antagonist species of *T. harzianum, T. hamatum, T. viride, T. polysporum,T. virens*, *Aspergillus* sp., *Rhizopus*, Actinomycete, *Fusarium*,*Penicillium* sp.and two bacterial antagonistic species namely *Bacillus subtilisand Pseudomonas fluorescens*were procured from the Department of Plant Pathology and Department of Basic Science of Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.). Fungal antagonists (*Trichoderma*spp.) were tested for their antagonistic activitiesby dual culture technique as adopted by Huang and Hoes (1976). Culture discs (4 mm diameter) of each of antagonists and the pathogen were taken from margin of their vigorously growing culture and transferred aseptically to solidified PDA (Potato Dextrose Agar) contained in Petri plates (90 mm) on the opposite side facing each other at a distance of 1 cm from the margin of the plate. The Petri plates containing only culture of the pathogen served as control. The experiment was laid out in CRD and each treatment was replicated thrice and the Petri plates were incubated at 27±1°C in BOD incubator. The colony diameter of test fungus was recorded till the control plates achieved full growth of the test fungus.

The antagonistic activity of *Bacillus subtilis* and *Pseudomonas fluorescens against the stem rot pathogen was observed by* streak plate method (Utkhede and Rahe, 1983). The Petri plates containing sterilized PDA were streaked at the centre with 48 hours old colonies of bacteria with the help of bacterial loop. Mycelial bit (4 mm diameter) of the test pathogen was placed on opposite sides of the streak at a distance of 1 cm from the margin of the plate. Petri plates without bacterial streak served as control for comparison. Each treatment was replicated thrice under Completely Randomized Design (CRD) and incubated at 27±1°C in BOD incubator. Percent mycelial inhibition in the growth of test pathogen was calculated as per Vincent (1947).

2.4. Field Testing of Antagonists on Incidence of Stem Rot and Wilt Disease of Carnation

All the effective fungal bio-control agents and bacterial bio-control agents *Bacillus subtilis* and *P. fluorescens* were applied before planting at the rate of 10percent i.e. by mixing 10 g of the formulation in 1Kg FYM per bed of 1m × 1m size. Fungal antagonists were applied in solid form by mixing properly in soil before planting of cutting, whereas, bacterium antagonist *Bacillus subtilis* and *P. fluorescens* was used as broth culture at the rate of 1 percent by mixing 10 ml of the broth culture in 1 litre of sterilized distilled water and applied by dip method. The rooted cuttings of carnation variety 'Rubesco' were dipped in this culture for 30 minutes prior to planting.

After treatment with antagonists, the carnation cuttings of variety 'Rubesco' were planted at a distance of 20 × 20 cm in 1m × 1m bed with 25 cuttings per bed. The beds without application of any bio-control agents were kept as control. Each treatment was replicated thrice in (RBD) Randomized Block Design. The application of antagonists was repeated at monthly intervals maximum to three times till the bud formation of the crop was achieved with same concentration. The data pertaining to stem rot incidence (%), average plant growth and flower parameters viz., plant height (cm), number of flowers per plant, stem length (cm), day of 1st flowering and flower size (cm) were recorded at 10 days intervals as specified earlier and analysed statistically.

3. Results and Discussion

3.1. In Vitro Evaluation of Microorganisms against Stem Rot of Carnation

All the microbial antagonists evaluated under *in vitro* conditions, inhibited the growth of the stem rot pathogen ranging from 51.63 to 65.08 percent (Table 1). Out of five native species of fungal antagonists *Trichodermaharzianum*was found most effective and showed significant superiority among all the antagonists tested that resulted in 65.08 percent inhibition of the stem rot pathogen followed by *T. viride with 63.70 percent inhibition.*

*Out of two bacterial antagonists, Bacillus subtilis*was found better than *Pseudomonas fluorescens* which inhibited the mycelial growth upto *55.05 percent*. While, *T. virens and P. fluorescens* were found least effective among all treatments with 51.63 and 52.26 percent inhibition. Out of these antagonists, four fungal *(T. viride, T. hamatum, T. harzianum, T. polysporum)* and one bacterial *(B. subtilis)* antagonist were further tested under field conditions.

3.2. Field Testing of Antagonists on Incidence of Stem Rot and Plant Health

Among fungal antagonists *Trichodermaharzianum* and *T. viride* were found most effective among all the treatments which reduced the incidence of carnation stem rot to 17.33 and 20.00 percent in comparison to 40.33 percent in control as both the treatments were found statistically at par with each other. However, *Bacillus subtilis* antagonists resulted in 28.00 percent reduction in the incidence of

stem rot followed by *T. polysporum* (37.33%). A least effect was registered in *T. polysporum* with maximum disease incidence (37.33%) of stem rot pathogen.

Among fungal antagonists *T. harzianum* was found most effective and also resulted in giving maximum average plant height (71.80 cm), stem length (65.57 cm), number of flowers per plant (3.53), flower size (6.24 cm) and required least number of days (133.20) for first flowering compared to control where the plant had on an average significantly shorter plant height (51.55)

Antagonists	**Percent inhibition in mycelial growth**
Trichoderma harzianum	65.08
T. hamatum	56.32
T. viride	63.70
T. polysporum	53.18
T.virens	51.63
Bacillus subtilis	55.05
Pseudomonas fluorescens	52.26
$CD_{(0.05)}$	**0.38**

Table 1: In vitro efficacy of antagonists against the stem rot pathogen (Rhizoctoniasolani)

Antagonists	**Conc. (%)**	**Disease incidence (%)**	**Plant height (cm)**	**Stem length (cm)**	**No. of days taken for 1st flowering**	**No. of flowers /plant**	**Flower size (cm)**
Trichoderma harzianum	1%	17.33 (24.57)	71.80	65.57	133.20	3.53	6.24
T. hamatum	1%	26.67 (31.08)	67.20	64.27	133.20	3.47	6.21
T. viride	1%	20.00 (26.49)	69.40	60.60	137.60	3.27	5.82
T. polysporum	1%	37.33 (37.66)	62.80	56.46	143.05	2.87	5.65
Bacillus subtilis	1%	28.00 (31.91)	66.88	59.28	137.80	3.20	5.76
Control		40.33 (40.01)	51.55	50.28	149.67	2.35	5.37
$CD_{(0.05)}$		(3.70)	5.27	5.21	10.79	0.72	0.37

Table 2: Effect of antagonists on incidence of stem rot, plant growth and flower parameters of carnation

Figures in parentheses are arc sine transformed values

cm), shorter stem length (50.28 cm), less number of flowers per plant (2.35), shorter flower size (5.37 cm) with maximum number of days (146.67) for first flowering.

3.3. In Vivo Evaluation of Microorganisms against Stem Rot of Carnation

The result of field experiment during 2012-13of different potential antagonists on disease incidence and different plant growth and flower parameters are presented in Table 2. It is evident from the data that all the treatments significantly reduced the stem rot incidence and also resulted in improvement of the different growth and flower parameters of the carnation in comparison to control. Among fungal antagonists *Trichodermaharzianum* and *T. viride* were found most effective among all the treatments which reduced the incidence of carnation stem rot to 17.33 and 20.00 percent in comparison to 40.33 percent in control as both the treatments were found statistically at par with each other. However, *Bacillus subtilis* antagonists resulted in 28.00 percent reduction in the incidence of stem rot followed by *T. polysporum* (37.33%). A least effect was registered in *T. polysporum* with maximum disease incidence (37.33%) of stem rot pathogen. Among fungal antagonists *T. harzianum* was found most effective and also resulted in giving maximum average plant height (71.80 cm), stem length (65.57 cm), number of flowers per plant (3.53), flower size (6.24 cm) and required least number of days (133.20) for first flowering compared to control where the plant had on an average significantly shorter plant height (51.55 cm), shorter stem length (50.28 cm), less number of flowers per plant (2.35), shorter flower size (5.37 cm) with

maximum number of days (146.67) for first flowering. The antagonist, *T. viride* was found next best to *T.harzianum*in efficacy and both were found statistically at par except for flower size whereas, *T. polysporum*was found least effective in efficacy as the average plants in the treatment were found with minimum plant height (62.80 cm), stem length (56.46), flowers per plant (2.87), flower size (5.65 cm) and 143.05 days taken for first flowering. It is also observed that none of the treatments including control had any adverse effect on calyx splitting. Elad*et al*. (1981) tested wheat bran culture of *T. harzianum* for the control of *R. solani* in carnation field pretreated with methyl bromide that could get 70 percent reduction in disease incidence @150 g (dry weight) per square meter.

3.4. In Vitroand in Vivo Evaluation of Microorganisms against Fusarium Wilt of Carnation

The maximum inhibition of the mycelia growth was observed by *Trichodermaharzianum* (76.54 %) with minmum radial growth (19.00 mm) which was superior over other treatments. Other microorganisms viz. *T. viride* (70.06%), *T. virens* (59.57 %) and *Aspergillus* sp. (58.02%) also gave good inhibition of mycelia growth. Minimum inhibition was given by *Rhizopus* sp. (28.70 %) which is statistically at par with Pseudomonas sp. (29.94 %)

Amongst the bacterial isolate 1 was reported as more effective which inhibited the mycelia growth upto 47.84 percent and was found statistically at par with *Penicillium* sp. and *Bacillus* sp. whereas Bacterial isolate 11 gave 41.36 percent inhibition of mycelia growth and was found statistically at par with Actinomycete sp.

seven microorganisms representing five fungal viz., *Trichodermaharzianum, T. viride, T. virens, Aspergillus* sp. and *Penicillium* sp. and two bacteria viz., Bacillus sp. and bacteria isolate 1 were tested against Fusarium wilt of carnation.

Micro-organisms	Radial growth	Inhibition of mycelia growth
Trichodermaharzianum	19.00	76.54 (61.06)
T. viride	24.25	70.06 (57.70)
T. hamatum	43.50	46.30 (42.85)
T. virens	32.75	59.75 (50.54)
*Aspergillus*sp.	34.00	58.02(49.72)
Bacillus sp.	40.75	49.69 (44.85)
*Penicillium*sp.	42.00	48.14 (43.96)
*Fusarium*sp.	45.00	44.44 (41.83)
Pseudomonas sp.	56.75	29.94 (32.88)
Rhizopus sp.	57.75	28.70 (31.75)
Actinomyces sp.	46.75	42.28 (40.50)
Bacterial isolate 1*	42.25	47.84 (43.75)
Bacterial isolate 11*	47.50	41.36 (40.04)
Control	81.00	-
$CD_{0.05}$		1.17

Table 3: In vitro screening of micro-organisms against Fusariumoxysporumf.sp. dianthi

Figures in parenthesis are arc sine transformed values.
*Colonies creamy white
**Colonies yellowish in colour

The maximum percent disease control of 69.35 and 65.77 percent was recorded in *T. harzianum* and *T. viride*, respectively. This was followed by *T. virens* (53.55%), *Aspergillus* sp. (48.04%) and *Bacillus* sp. (46.83%).Though, *Aspergillus* sp. and *Bacillus* sp. were found equally at par in minimizing the disease. The maximum percent disease incidence (85.21%) was found in control plants.

The data also reveal that root dip and soil drenching methods do not differ statistically from each other. However, the disease incidence (50.29%) was more after 60 days of planting as compared to percent disease incidence (42.66%) after 35 days, their values differ with each other.

The interaction studies amongst antagonist, application method and days reveal that the minimum disease incidence was observed after 35 days of planting of carnation when rooted cuttings of carnation were dipped in spore suspension of *Trichodermaharzianum.*

4. Results and discussion

Chakraborty and Chatterjee (2008) reported 86.44 percent growth inhibition of *Fusariumsolani* causing wilt of brinjal by *T. harzianum* under *in vitro* condition. Johnson *et al.* (2008) reported *in vitro* inhibition of *F. oxysporum* by *T. viride, T. hamatum, T. harzianum and T. koenigi.* Gupta and Bansal (2006) used *T. viride* against chickpea wilt complex and found significant reduction in wilt and enhanced yield under field conditions. Rini and Sulochana (2007) found usefulness of *Trichoderma* and *Pseudomonas* against *Rhizoctoniasolani* and *Fusariumoxysporum* infecting tomato and reported that *Pseudomonas fluorescens* isolates P28 and P51 showed the greatest inhibition against the pathogen while *Trichodermaviride* isolates TR19 and TR22 found effective against *F. oxysporum*.

Antagonist	Percent disease incidence				Mean	Percent disease control
	Root dip		Soil drenching			
	After 35 days	After 60 days	After 35 days	After 60 days		
Trichodermaharzianum	22.83 (28.54)	25.00 (29.86)	25.00 (29.86)	31.54 (33.96)	26.12 (30.56)	69.35
T. viride	22.92 (28.54)	31.54 (33.96)	27.08 (31.33)	35.41 (36.52)	29.16 (32.56)	65.77
T. virens	33.33 (35.28)	43.75 (41.42)	35.42 (36.52)	45.83 (42.62)	39.58 (35.28)	53.55
*Aspergillus*sp.	39.58 (38.99)	47.92 (43.82)	41.67 (40.23)	47.92 (43.82)	44.27 (38.99)	48.04
Bacillus sp.	41.69 (40.20)	45.83 (42.62)	45.83 (42.62)	47.92 (43.82)	45.31 (40.20)	46.83
*Penicillium*sp.	43.75 (41.42)	56.25 (48.62)	45.83 (42.62)	54.16 (47.42)	49.99 (41.42)	41.33
Bacterial isolate 1	47.92 (43.82)	56.25 (48.62)	52.08 (46.22)	56.25 (48.62)	53.12 (43.83)	37.55
Control	81.25 (64.46)	91.67 (73.26)	76.25 (64.46)	91.67 (73.26)	85.21 (64.46)	_
Mean	41.63 (40.23)	49.74 (45.26)	43.64 (41.73)	50.85 (45.92)	_	_

Table 4: Evaluation of antagonists and application methods against Fusariumoxysporumf.sp.dianthi under pot culture conditions

Figures in paranthesis are arc sine transformed values.

Overall Mean Root Dip : 45.76 (42.73)
Drenching : 47.25 (43.30)
35 days : 42.66 (40.96)
60 days : 50.29 (45.50)

Rehman and Lawrence (2010) used *Trichodermaviride* and *T. harzianum* as seed treatment and soil drench against damping off in cabbage. Trillas *et al*., (2006) used composts from agricultural waste and the *Trichodermaasperellum* strain T-34 to suppress *Rhizoctoniasolani* in cucumber seedlings while Ngullie and Daiho (2013) recorded reduced incidence of seedling rot in both greenhouse and field condition and high test per plant yield from combination of *T. viride* + *P. fluorescens* followed by *T. viride.*Karimi *et al.,* (2007) found strain E121 of *Bacillussubtilis* and strain E130 of *Pseudomonas fluorescens* as most effective in inhibiting the mycelial growth of *F.oxysporum*f. sp.*dianthi*the cause of wilt of carnation by production of non volatile and volatile metabolites under laboratory conditions.

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