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In vitro evaluation of induced systemic resistance (ISR) elicitors against collar rot of chilli caused by *Sclerotium rolfsii* Sacc.

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Abstract
Management of collar rot of chilli disease mainly depends on fungicide treatments. However, the hazardous effect of these chemicals or their degradation products on the environment and human health strongly requires the search for new, harmless means of disease control. There must be some natural phenomenon of induced resistance to protect plants from disease. Elicitors are compounds, which activate chemical defense in plants. Various biosynthetic pathways are activated in treated plants depending on the compound used. Induction of resistance in plants to overcome pathogen infection is a promising approach for controlling plant diseases. Seed treatments with Chitosan along with its spraying recorded significantly highest seed germination (90.00%), while maximum reduction in mean seedling mortality was recorded with chitosan (ST) (10.89%) followed by Chitosan (ST) + its spraying (14.17%) as compared to control.

Keywords: Chilli, collar rot, *Sclerotium rolfsii*, chitosan, salicylic acid

1. Introduction
Chilli (*Capsicum annuum* L.) is mainly cultivated for green fruits as table purpose and dry chilli as spice. Chilli crop suffers from a number of fungal, bacterial, nematode and many viral diseases. Recently, the collar rot caused by *Sclerotium rolfsii* Sacc.is becoming sever disease of chilli in India. Crop losses up to 16-80 per cent due to collar rot disease have been reported by many researchers in this crop (Wangikar *et al*., 1988; Singh and Dhancholia, 1991; Mathur and Gurjar, 2001) [22, 17, 12]. *S. rolfsii* is a soil borne facultative pathogen, which has wide host range of more than 500 species of cultivated and wild plants in tropical and sub-tropical regions (Punjab, 1985; Xu, 2009) [14, 23].
Management of collar rot disease mainly depends on fungicide treatments. However, intensive application of fungicides causes hazards to human health and environmental degradation and is not always satisfactory. Therefore, alternative approaches for the control of plant diseases should be emphasized (Mandal *et al*., 2009) [11]. Many plants respond to local attack by pathogens with a *de novo* production of compounds reducing or inhibiting further attack by, or performance of, their enemies. Responses occur both in the plant organ originally attacked (local response) and in distant, yet unaffected, parts (systemic response). One of these responses is Induced Systemic Resistance (ISR). (Hunt *et al*., 1996, Schneider *et al*., 1996, Sticher *et al*., 1997; Hammerschmidt, 1999) [9, 16, 19, 7]. Elicitors are compounds that are able to trigger defense mechanisms like hypersensitive response, production of reactive oxygen species, activation of defense-related genes as well as phytoalexin synthesis (Thakur and Sohal, 2013) [20]. Higher plants have the ability to initiate various defense reactions such as hypersensitive responses, production of phytoalexins and anti-microbial proteins, and reinforcement of cell walls when they are infected by various pathogens (Dangl and Jones, 2001) [3].
Induction of resistance in plants to overcome pathogen infection is a promising approach for controlling plant diseases. Exogenous or endogenous factors could substantially affect host physiology, lead to rapid and coordinated activation of defense-gene in plants normally expressing susceptibility to pathogen infection (Schneider *et al*., 1996; Smith, 1996) [16, 18]. This induced resistance to pathogens can be achieved by the application of various abiotic agents (elicitors/chemical inducers) such as Salicylic acid, β-amino butyric acid, Jasmonic acid and Chitosan (Heil and Bostock, 2002; Yigal, 2002; El-Khallal, 2007; Abdel-Monaim, 2010; Thakur and Sohal, 2013) [8, 24, 4, 1, 20].
The present study was conducted to investigate the *in vitro* evaluation of ISR elicitors against collar rot of chilli caused by *S. rolfsii* under greenhouse condition.

2. Material and methods

Isolation of pathogen: Chilli plants showing the typical symptoms of collar rot were collected and then washed with tap water and rinsed with sterile distilled water. Diseased collar regions were cut into small pieces and then surface sterilized in 0.1% mercuric chloride for 30 seconds and repeatedly washed with sterilized distilled water to remove traces of mercury and then transferred to Potato Dextrose Agar Medium and incubated at 27 ± 1 ºC. Fungal mycelium developed from the infected tissue on Potato Dextrose Agar Medium was finally transferred to PDA slants and incubated at 27 ± 1 ºC to obtain pure culture of *Sclerotium rolfsii*.

Mass multiplication of the pathogen's inoculum: In order to get mass multiplication of inoculum, sand-maize medium was used. Sand: maize medium (2 part partially broken maize grains + 1part sand + distilled water to moisten the medium) was prepared, filled into the polypropylene bags (9x12 cm) and autoclaved at 121 ^{0}C (20 psi pressure) for 30 min, for two consecutive days. After cooling at room temperature, the sterilized sand: maize medium in bags was inoculated with 8-10 mycelial discs (5 mm diameter) of the test pathogen separately obtained from a week old culture and incubated at 27±1^{0}C for 12 days. This mass multiplied inoculum was used for inoculating soil in the pots to create sick soil.

Preparation of sick soil for pot culture: For preparation of sick soil, polythene bags disinfected with 5 per cent copper sulphate solution were filled with sterilized potting mixture of soil: sand: FYM (2:1:1) was used. and inoculated (@ 50 g/kg soil) separately with mass multiplied (sand: maize) culture of *S. rolfsii* to the upper 4-5 cm layer, mixed thoroughly, watered adequately and incubated in greenhouse for two weeks to proliferate the test pathogens and create sick soil.

Greenhouse (pot culture) experiment: The efficacy of four ISR elicitors *i.e.* Salicylic acid, β-amino butyric acid, Jasmonic acid and Chitosan (alone and in combination) used at different concentrations, was tested against collar rot of chilli by sick soil method in pot culture under greenhouse conditions. These chemicals were applied as seed treatment, followed by foliar spray application 35 days after sowing, applied as detailed follows:

T. No.	Name of chemical/elicitor	Concentration (per kg of seed or l of water)
T_1	Salicylic acid (ST)	1.5 µM
T_2	Salicylic acid (ST) + its spraying	1.5 µM
T_3	β-amino butyric acid (ST)	30 µM
T_4	β-amino butyric acid (ST) + its spraying	30 µM
T_5	Jasmonic acid (ST)	1.5 µM
T_6	Jasmonic acid (ST) + its spraying	1.5 µM
T_7	Chitosan (ST)	20 µM
T_8	Chitosan (ST) + its spraying	20 µM
T_9	Salicylic acid (ST) + Tebuconazole (spraying)	1.5 µM+0.1%
T_{10}	Jasmonic acid + Tebuconazole (spraying)	1.5 µM+0.1%
T_{11}	Control (Carbendazim (ST))	0.1%
T_{12}	Control (Untreated)	---

ST= Seed treatment

Polyethene bags (20 cm diameter) were filled with soil artificially inoculated with *Sclerotium rolfsii* pathogens. The test ISR chemicals/elicitors were applied (alone and in combination) as seed treatment to the healthy seeds of chilli cv. Pusa Jwala and sown (10 seeds/bag) in the polythene bags containing *S. rolfsii* sick soil. Two bags /treatment/replication were maintained. The polythene bags containing *S. rolfsii* sick soil sown with surface sterilized seeds of chilli and without application of any ISR chemicals/elicitors and with application of carbendazim (fungicide) were maintained as untreated and treated control, respectively. One spray of treatments was undertaken at 30 days after sowing of the crop. Soil moisture was maintained at 80% by spraying with sterile water. Each treatment was replicated three times.

Mortality percentage (pre-emergence rot (PREM) and post-emergence seedling mortality (POSM)) was recorded after 10 and 35 days of sowing respectively by the following formula:

$$\text{PREM\%} = \frac{\text{No. of seeds ungerminated}}{\text{Total No. of seeds sown}} \text{ x } 100$$

$$\text{POSM\%} = \frac{\text{No. of emerged seedlings died}}{\text{Total No. of seedlings}} \text{ x } 100$$

The data recorded were analysed statistically wherever necessary. The variations of the different parameters revealed by different treatments were tested for the significance using completely randomized design.

3. Results and Discussion

Effect on seed germination: Results (Table 1) revealed that all the treatments exhibited improved seed germination, over untreated control and it was ranged from 65.00 to 90.00 per cent, as against 36.67 per cent in untreated control. However, significantly highest seed germination was recorded with the treatment Chitosan (ST) + its spraying (90.00%) followed by Salicylic acid (ST) + its spraying (86.67%), Chitosan (ST) (86.67%), Salicylic acid (ST) (83.33%), β-amino butyric acid (ST) + its spraying (80.00%), Salicylic acid (ST) + Tebuconazole (spraying) (78.33%), β-amino butyric acid (ST) (76.67%), Jasmonic acid (ST) + its spraying (75.00%), Jasmonic acid + Tebuconazole (spraying) (71.67%) and Jasmonic acid (ST) (73.33%).

Effect on pre and post-emergence mortalities: Results (Table 2) revealed that all the treatments significantly influenced the both pre-emergence seed rot (PREM) and post-emergence seedling mortality (POSM), caused by *S. rolfsii* in chilli. The pre-emergence seed rot recorded with all the treatments was ranged from 10.00 to 35.00 per cent, as against 63.33 per cent in untreated control. However, the treatment found most effective with significant least PREM was treatment Chitosan (ST) + its spraying (10.00%) followed by Salicylic acid (ST) + its spraying (13.33%), Chitosan (ST) (13.33%), Salicylic acid (ST) (16.67%), β-amino butyric acid (ST) + its spraying (20.00%), Salicylic acid (ST) + Tebuconazole (spraying) (21.67%), β-amino butyric acid (ST)

(23.33%), Jasmonic acid (ST) + its spraying (25.00%), Jasmonic acid + Tebuconazole (spraying) (26.67%) and Jasmonic acid (ST) (28.33%).

Similar trend with increased post-emergence seedling mortality was also observed and it was ranged from 11.78 to 36.35 per cent, as against 89.26 per cent in untreated control. However, significantly least POSM was recorded with the treatment Chitosan (ST) + its spraying (11.78%) followed by Salicylic acid (ST) + its spraying (15.00%), Chitosan (ST) (16.67%), Salicylic acid (ST) (19.98%), β-amino butyric acid (ST) + its spraying (23.47%), Salicylic acid (ST) + Tebuconazole (spraying) (31.11%), β-amino butyric acid (ST) (31.25%), Jasmonic acid (ST) + its spraying (32.54%), Jasmonic acid + Tebuconazole (spraying) (34.72%) and Jasmonic acid (ST) (35.90%).

The mean mortality recorded with all the treatments was ranged from 10.89 to 35.67 per cent, as against 76.30 per cent in untreated control. However, significantly least POSM was recorded with the treatment Chitosan (ST) + its spraying (10.89%) followed by Salicylic acid (ST) + its spraying (14.17%), Chitosan (ST) (15.00%), Salicylic acid (ST) (18.33%), β-amino butyric acid (ST) + its spraying (21.74%), Salicylic acid (ST) + Tebuconazole (spraying) (26.39%), β-amino butyric acid (ST) (27.29%), Jasmonic acid (ST) + its spraying (28.77%), Jasmonic acid + Tebuconazole (spraying) (30.70%) and Jasmonic acid (ST) (32.12%).

Reduction in mortality: All the treatments were found to reduce both pre-emergence seed rot and post-emergence seedling mortality, over untreated control (Table 2). The reductions in both PREM and POSM were ranged from 44.73 to 84.21 per cent and 59.28 to 86.80 per cent, respectively. However, highest reductions in PREM and POSM were recorded with the treatment Chitosan (ST) + its spraying 84.21, 86.80 per cent respectively with a mean of 85.51 per cent; followed by Salicylic acid (ST) + its spraying 78.95, 83.20 per cent respectively with a mean of 81.07 per cent; Chitosan (ST) 78.95, 81.32 per cent respectively with a mean of 80.14 per cent; Salicylic acid (ST) 73.68, 77.62 per cent respectively with a mean of 75.65 per cent; β-amino butyric acid (ST) + its spraying 68.42, 73.70 per cent respectively with a mean of 71.06 per cent; Salicylic acid (ST) + Tebuconazole (spraying) 65.79, 65.15 per cent respectively with a mean of 65.46 per cent; β-amino butyric acid (ST) 63.16, 64.99 per cent respectively with a mean of 64.08 per cent; Jasmonic acid (ST) + its spraying 60.52, 63.55 per cent respectively with a mean of 62.03 per cent; Jasmonic acid + Tebuconazole (spraying) 57.89, 61.10 per cent respectively with a mean of 59.49 per cent; and Jasmonic acid (ST) 55.26, 59.78 per cent respectively with a mean of 57.52 per cent.

These results are in conformity with the findings of those reported earlier by several workers against *S. rolfsii* and other soil borne diseases. Jabnoun-Khiareddine *et al.* (2015) [10] reported antifungal activity of chitosan and salicylic acid (SA) against ten tomato phytopathogenic fungi and found that Chitosan-and SA-based treatments resulted in 42.1-73.68, 60.86-78.26 and 45-50 per cent reductions in wilt severity. Sathiyanarayanan and Muthukrishnan (2014) [15] reported chitosan for its potential to induce resistance against *Pythium aphanidermatum*. Its application induces defense enzymes such as chitinases and chitosanases which played a role in restricting the development of disease symptoms. The eliciting properties of chitosan make chitosan as potential antifungal agent against rot. Similar results are reported earlier by several workers (EL-Mougy *et al*., 2004; EL-Mougy and Nehal, 2004; Matny and Al-Jarrh, 2014 and Amini, 2015) [6, 5, 13, 2].

Table 1: Effect of ISR elicitors seed treatment on seed germination (Pot Culture)

S. No.	Treatments	Conc. (per kg of seed or l of water)	Germination (%)	Per cent increase over control
T_1	Salicylic acid (ST)	1.5 µM	83.33 (65.93)*	55.99
T_2	Salicylic acid (ST) + its spraying	1.5 µM	86.67 (68.64)	57.69
T_3	β-amino butyric acid (ST)	30 µM	76.67 (61.12)	52.17
T_4	β-amino butyric acid (ST) + its spraying	30 µM	80.00 (63.41)	54.16
T_5	Jasmonic acid (ST)	1.5 µM	71.67 (57.84)	48.83
T_6	Jasmonic acid (ST) + its spraying	1.5 µM	75.00 (59.98)	51.11
T_7	Chitosan (ST)	20 µM	86.67 (68.64)	57.69
T_8	Chitosan (ST) + its spraying	20 µM	90.00 (71.54)	59.26
T_9	Salicylic acid (ST) + Tebuconazole (spraying)	1.5 µM+0.1%	78.33 (62.27)	53.19
T_{10}	Jasmonic acid + Tebuconazole (spraying)	1.5 µM+0.1%	73.33 (58.91)	49.99
T_{11}	Control (Carbendazim (ST))	0.1%	65.00 (53.71)	43.58
T_{12}	Control (Untreated)	-	36.67 (37.24)	0.00
	S.E. m ±		0.89	
	C.D. (P = 0.05)		2.62	
* Figures in parenthesis are angular transformed values				

Table 2: Effect of ISR elicitors on mortality of seedlings (Pot Culture)

S. No.	Treatments	Conc. (per kg of seed or l of water)	Per cent Incidence			Per cent reduction over control		
			PREM	POSM	Mean	PREM	POSM	Mean
T_1	Salicylic acid (ST)	1.5 µM	16.67 (24.04)	19.98 (26.50)	18.32	73.68	77.62	75.65
T_2	Salicylic acid (ST) + its spraying	1.5 µM	13.33 (21.33)	15.00 (22.78)	14.17	78.95	83.20	81.07
T_3	β-amino butyric acid (ST)	30 µM	23.33 (28.84)	31.25 (33.97)	27.29	63.16	64.99	64.08
T_4	β-amino butyric acid (ST) + its spraying	30 µM	20.00 (26.55)	23.47 (28.91)	21.74	68.42	73.70	71.06
T_5	Jasmonic acid (ST)	1.5 µM	28.33 (32.13)	35.90 (36.77)	32.12	55.26	59.78	57.52
T_6	Jasmonic acid (ST) + its spraying	1.5 µM	25.00 (29.99)	32.54 (34.74)	28.77	60.52	63.55	62.03
T_7	Chitosan (ST)	20 µM	13.33 (21.33)	16.67 (24.04)	15.00	78.95	81.32	80.14
T_8	Chitosan (ST) + its spraying	20 µM	10.00 (18.43)	11.78 (20.06)	10.89	84.21	86.80	85.51
T_9	Salicylic acid (ST) + Tebuconazole (spraying)	1.5 µM+0.1%	21.67 (27.70)	31.11 (33.86)	26.39	65.79	65.15	65.46
T_{10}	Jasmonic acid + Tebuconazole (spraying)	1.5 µM+0.1%	26.67 (31.06)	34.72 (36.08)	30.69	57.89	61.10	59.49

T_{11}	Control (Carbendazim (ST))	0.1%	35.00 (36.26)	36.35 (37.05)	35.67	44.73	59.28	52.01
T_{12}	Control (Untreated)	-	63.33 (52.72)	89.26 (68.78)	76.30	0.00	0.00	0.00
	S.E. m ±		0.49	0.46				
	C.D. (P = 0.05)		1.43	1.34				
* Figures in parenthesis are angular transformed values								

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