BIOLOGIA (LAHORE)
December, 2019, 65 (II), 329-334

PKISSN 0006 – 3096 (Print)
ISSN 2313 – 206X (On-Line)

# Evaluation of biocontrol strategies and its synergistic interaction permitting the chickpea plant to trigger the appropriate defense responses against *Sclerotium rolfsii*

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**ARTICLE INFORMAION**

Received: 06-09-2019
Received in revised form: 06-01-2020
Accepted: 10-01-2020

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**Original Research Article**

**ABSTRACT**

Brassicaceae member i.e. *Rhaphinus sativus* L. and three species of *Trichoderma* (*T. harzianum*, *T. viride* and *T. hamatum*) were evaluated for their antifungal effect against *Sclerotium rolfsii*, the causal agent of collar rot disease in chickpea. *In vitro*, methanolic leaves extract of *R. sativus* significantly decreased pathogen biomass by 6-96% and three *Trichoderma* species also caused significant inhibition in pathogen growth variably between 30-100%. In potted soil, application of separate or combined application of myco- and phyto-fungicides proved highly efficient in managing collar rot disease. However, combined effect of *T. harzianum* or *T. viride* + leaves biomass of *R. sativus* found more promising in managing disease through significantly improving growth attributes and activities of antioxidant enzymes. It was concluded that disease suppression is directly linked with increase in activities of antioxidant enzyme (peroxidase, catalase, polyphenol oxidase and phenylalanine ammonia lyase) thus provides the basis for resistant in chickpea.

**Keywords**: Catalas, Peroxidase, Phenylalanine ammonia lyase, Polyphenol oxidase

## INTRODUCTION

*Sclerotium rolfsii* is one of the most destructive and aggressive soil inhabiting fungal pathogens belongs to phylum basidiomycota which generally prevails in warm humid climate and can infect over 500 plant species (Rafi *et al*., 2017). The pathogen is responsible for causing 55-95% seedling mortality through causing collar rot disease in chickpeas (*Cicer arietinum* L.) (Gurha and Dubey, 1982) and the disease has been documented all over the world wherever chickpea is grown (Nene *et al*., 1984). In Pakistan, chickpea is mainly grown in rainfed areas, where climatic conditions favors development of collar rot disease development, therefore huge losses to crop could be expected (Javaid and Khan, 2015).

Versatility of *S. rolfsii* to thrive in harsh environmental conditions through production of oxalic acids, vigorous mycelia growth and melaninized sclerotia make it difficult pathogen to manage through available cultural and chemical methods. Besides, the sclerotia has ability to persist in the soil for several years, serve as the principal over-wintering structure and primary inoculum for disease (Rafi *et al*., 2017). Antifungal biocontrol agents are powerful, safer and sustainable alternative for profitable agricultural productivity. Biocontrol agents hold potential to produce toxins, antibiotics, enzymes against invading pathogen and these can also compete pathogen for available resources, induce resistance in plant and hyper parasitize pathogen (Shoaib *et al*., 2019). Different species of *Trichoderma* are well known as potential antagonists against plant pathogens and these antagonistic fungi are found extensively in almost all agricultural soils (Tian *et al*., 2018). More than 50% of the *Trichoderma* species have been investigated for their potential to combat against fungal pathogen during 4-decades (Karuppiah *et al*., 2019). The antifungal properties of *Trichoderma* spp. are endorsed to their ability to produce siderophores, phosphate-solubilizing enzymes, antibiotics and phytohormones besides promoting

**Author's Contribution: A.S. & A.J**., Designed experiments, applied statistical analysis, compiled and wrote manuscript; **A.S., S.A. & Z.A.A**.; Conducted laboratory and pot experiments and collected data

plant growth and yield (Karuppiah *et al.*, 2019). Amongst many species, *T. harzianum, T. viride* and *T. hamatum* always gain attraction of scientists because of diverse physiological traits and important chitinolytic system (Shoaib *et al*., 2018).

Use of different types of plant extract and biomass is also another way of managing fungal pathogen. Recently Brassicaceae family has gained attention of scientists due to its antifungal properties against numerous strains of fungi (Javaid *et al*., 2017). The anti-pathogenic, allelopathic and anti-herbivore properties of Brassicaceae are due to production of glucosinolate (Wang *et al*., 2017). This family also comprises of many secondary metabolites and anti-microbial compounds. Some members of Brassicaceae like radish and mustard have been well-known for presence of antimicrobial agents in them (Jahangir *et al*., 2009). *Raphanus sativus* L. (radish) is a short season widely cultivated vegetable in Pakistan and has high therapeutic value (Pervez *et al.,* 2003). *R. sativus* leaves have pungent flavor and contain 'raphanin' which has an ability to possess antifungal and antibacterial potential against many plant pathogens (Singh and Singh, 2013; Singh Duy *et al*., 2019). Its extracts have many bioactive molecules and secondary metabolites including flavonoids, alkaloids and tannins that inhibit the fungal, bacterial and nematode growth by stimulating antimicrobial activity (Wang *et al*., 2017).

Disease management options tend to alleviate the disease stress by inducting resistance in plants and altering activities of defense related enzymes. Several authors have demonstrated a distinct correlation between the degree of plant resistance and peroxidase (POX), catalase (CAT), phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) (Awan *et al*., 2018). The activities of these inducible defense enzymes may alter to variable extent to repair or resist damage. However, this requires comprehensive studies and understanding of the regulatory mechanisms and responses in chickpea. Considering the importance of crop and highly destructive nature of *S. rolfsii*, the current research work was designed to assess the antifungal potential of *T. harzianum, T*. *hamatum, T. viride* and *R. sativus* against *S. rolfsii* both *in vitro* and *in vivo* condition. Quantitative estimations of antioxidant enzymes (POX, PPO, PAL and CAT) were carried out to check their role in induction of systemic resistance in chickpea by soil amendment with leaves biomass of *R. sativus* and *Trichoderma* spp. against collar rot disease.

## MATERIALS AND METHODS

### In vitro bioassay

The antagonistic activity of cultural filtrate in broth of *T. viride* (FCBP # 644), *T. hamatum* (FCBP # 907) and *T. harzanium* (FCBP # 1277) against *S. rolfsii* (FCBP 1409) was determined following protocols of Derbalah *et al*. (2012). In brief, different concentrations of 5, 10, 15, 20, 25, 30, 35 and 40% of the filtrate of *Trichoderma* spp. were prepared in 2% ME (malt extract) medium and treatments were inoculated with the pathogen. Broth flasks having an inoculum of pathogen but without culture filtrate were served as control. After incubation of 7 days, oven dried biomass of fungus at 60 °C was recorded

Alongside, plating with agar (2% MEA) medium was also amended with different concentrations of filtrate (5, 10, 15, 20, 25, 30, 35 and 40%) and inoculated with *S. rolfsii* inoculum disc (5 mm). Control treatments comprised of MEA medium only were also inoculated with *S. rolfsii*. After 7 days of incubation, radial growth inhibition (%) by metabolites of different *Trichoderma* spp. was noticed (Shoaib *et al*., 2018).

Antifungal assays with methanolic leaf extract of *R. sativus* were carried out following the method of Shoaib *et al*. (2018). Briefly described, methanolic leaf extract (14.7 g) of *R. sativus* was mixed in 6 mL dimethyl sulphoxide (DMSO) and diluted with 12 mL of distilled water to prepare stock. DMSO (6 mL) was homogenized with 12 mL of distilled water for control solution. Different concentrations (1, 2, 3, 4, 5, 6, 7 and 8%) were prepared by mixing measured amount of stock solution in control solution, where 0% was control treatment. Then 20 mL each concentration was taken in 100 mL flask and all flasks were inoculated with of *S. rolfsii* (5 mm mycelial disc). After 7 days of incubation at 27 °C, the fungal biomass was dried at 60 °C and measured.

### *In vivo* bioassays

Two perspective strains *T. harzianum* and *T. viride* were selected for *in vivo* pot trials and fumigated soil, a method described by Awan *et al*. (2018), was filled in earthen pot (10" × 8" length and width). Furthermore, each potted soil (4 kg $pot^{-1}$) was inoculated with mycelial plug (5 mm $pot^{-1}$) of *S. rolfsii*. A week later, cultural suspension (conidia 4 × $10^6$, 15 mL $pot^{-1}$) of each of two *Trichoderma* spp. was drenched in treated and untreated pots. After 96 hours interval, three doses (0.5, 1 and 1.5%) of

dried powdered leaf material of *R. sativus* was added in treated and untreated pots seeds of chickpea were sown. Positive control was treated with *S. rolfsii* only, whereas negative control were not given in any treatment. Following were the treatment of pot trials in a completely randomized form with three replicates and three plants per pot. $T_1$: Negative control; $T_2$: Positive control [*S. rolfsii* (SR)]; $T_3$: 0.5% leaves biomass of *R. sativus* + SR; $T_4$: 1% leaves biomass of *R. sativus* + SR; $T_5$: 1.5% leaves biomass of *R. sativus* + SR; $T_6$: *T. harzianum* + SR; $T_7$: *T. viride* + SR; $T_8$: 0.5% leaves biomass of *R. sativus* + *T. harzianum* + SF; $T_9$: 1% leaves biomass of *R. sativus* + *T. harzianum* + SR; $T_{10}$: 1.5% leaves biomass of *R. sativus* + *T. harzianum* + SR; $T_{11}$: 0.5% leaves biomass of *R. sativus* + *T. viride* + SR; $T_{12}$: 1% leaves biomass of *R. sativus* + *T. viride* + SR; $T_{13}$: 1.5% leaves biomass of *R. sativus* + *T. viride* + SR.

Variations in antioxidant enzymes (POX, CAT, PPO and PAL) in different treatments were assessed at 20th days after inoculation following protocol described by Awan *et al*. (2018). The disease incidence (Shoaib *et al*., 2018) and the disease severity (Horsfall and Barratt, 1945) was checked at 35th post pathogen inoculation.

**Table I: Disease rating scale**

| Disease rating | Description | Disease reaction |
|---|---|---|
| **0** | Healthy plant, no wilting | Resistant |
| **1** | Yellowing appeared on 25% plant parts. | Mild |
| **2** | About 50% plant diseased with yellowing and browning | Moderate |
| **3** | More than 50% plants wilted and died | Severe wilt |

Data concerning different plant growth attributes (plant height, weight) were also recorded. Triplicate data of all treatments were subjected to analysis of variance (ANOVA) followed by least significant difference (LSD) test by using software Statistix 8.1.

## RESULTS AND DISCUSSION

Antimitotic potential of three *Trichoderma* spp. (*T. harzianum*, *T. viride* and *T. hamatum*) and *R. sativus* was tested through *in vitro* and *in vivo* bioassays against pathogen of collar rot disease of chickpea (*S. rolfsii*). Increasing concentrations (5-40%) of fungal metabolites showed significant reduction (20-100%) in the growth of pathogen in both broth as well as in agar medium. The biomass of *S. rolfsii* was significantly reduced from 40-90% due to metabolites of *T. harzianum*, followed by parallel reduction of 30-80% due to *T. viride* and *T. hamatum* (Table II). In solid medium, metabolites significantly reduced fungal growth by 27-100%, 19-100% and 24-100% due to increase in metabolites concentrations (5-40%) of *T. harzianum*, *T. hamatum* and *T. viride*, respectively as compared to their respective control treatments (Table II). The inhibited growth of *S. rolfsii* might be result of secretion of metabolic enzymes (harzianic acids, tricholin, viridian, glisoprenins, massoilactone gliovir and heptelidic acid) by *Trichoderma* spp. in broth (Rini and Sulochana, 2007).

**Table II. Percentage reduction in *Sclerotium rolfsii* biomass due to effect of different concentrations of *Trichoderma* species filtrate.**

| Antagonistic fungal sp. | Percentage reduction in *S. rolfsii* biomass due to effect of metabolite of *Trichoderma* spp. in broth | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5% | 10% | 15% | 20% | 25% | 30% | 35% | 40% |
| *T. harzanium* | 38 ij | 41 h-j | 46 gh | 52 fg | 59 ef | 70 cd | 78 bc | 86 a |
| *T. hamatum* | 25 k | 38 ij | 42 hi | 44 gh | 55 f | 63 d-f | 68 d | 80 a-c |
| *T. viride* | 25 k | 44 gh | 49 f-h | 58 d-f | 60 ef | 69 cd | 73 b-d | 83 a-c |
| **Antagonistic fungal sp.** | **Percentage reduction in radial growth of *S. rolfsii* due to effect of metabolite of *Trichoderma* spp. on agar** | | | | | | | |
| | 5% | 10% | 15% | 20% | 25% | 30% | 35% | 40% |
| *T. harzanium* | 27 kl | 30 i-k | 46 fg | 58 e-g | 64 cd | 71 c | 81 b | 100 a |
| *T. hamatum* | 19 m | 27 jk | 41 h | 57 e-g | 60 c-e | 62 c-e | 82 b | 100 a |
| *T. viride* | 24 k-m | 30 i-k | 47 fg | 60 c-e | 64 cd | 67 c | 79 b | 100 a |

Values with different letters show significant difference (P≤0.05) as determined by LSD Test.

Furthermore, different concentrations (1-8%) of methanolic leaf extract of *R. sativus* significantly reduced the growth of *S. rolfsii* by 10-98% (Table III). Antifungal potential against *S. rolfsii* could be ascribed to involvement of many secondary metabolites including flavonoids, alkaloids and tannins as well as presence of glucosinolate (Wang *et al*., 2017).

**Table III. Percentage reduction in *Sclerotium rolfsii* biomass due to effect of different concentrations of methanolic extract of *Raphanus sativus*.**

| Methanolic extract concentration (%) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| 6 **e** | 10 **e** | 28 **d** | 34 **d** | 54 **c** | 72 **b** | 95 **a** | 96 **a** |

Values with different letters show significant difference (P≤0.05) as determined by LSD Test

During pot assays, the highest disease incidence (70%), severity (rating scale: 4) and significant reduction (~60%) in plant growth attributes were observed in positive control. Soil amendment with 0.5-1.5 g of leaves biomass reduced the disease incidence from 49 to 39% (rating scale: 3) leading to plant growth improvement by 50-150%. Individual effect of *T. harzianum* or *T. viride* significantly reduced the disease up to 40% (rating scale: 3) and improved the plant growth attributes by 100-200% over control. Synergistic effect of 1.5 g of leaves dry biomass + biocontrol agent showed more promising effect in disease management and growth improvement. When *T. harzianum* was applied with leaves biomass of *R. sativus*, the disease was significantly reduced from 37 to 17% (rating scale: 2 to 1) resulted in 100-215% enhancement in plant growth attributes. The results obtained due to individual effect of *T. viride* were statistically similar to *T. harzianum* in terms of disease reduction and growth attributes. So far, the disease incidence was considerably reduced to threshold level (7%) due to combined application of *T. viride* + 1.5 g leaves biomass (Table IV). Disease suppression by *Trichoderma* spp. might be attributed to their potential of better utilization of the resources through their rapid growth rate as compared to the pathogen (Vinale *et al*., 2008). Under combined effect of soil amendments with leaves biomass and *Trichoderma* spp., it seems that the pathogen was not only in competition for resources with *Trichoderma* but was also under stress due to antifungal effect of applied agents. Therefore, *Trichoderma* likely to get a chance to establish better as compared to pathogen, which ultimately reduced the disease and improved the host growth.

*S. rolfsii* inoculation significantly decreased POX, CAT, PPO and PAL activities in chickpea leaves by 20-50% over negative control (6.13, 1.33, 0.028 and 1.10 units/min per mg of protein) as the susceptible host does not have the ability to detect the threat pose by pathogen (Awan *et al*., 2018).

Various biofungicides alleviated the disease stress by improving the POX, PPO and PAL activities to variable extents. The enzymes activities were increased to maximum levels due to soil amendment with leaves biomass (0.5-1.5 g) as compared to rest of treatments. The improvement in the enzyme activities was gradually decreased with rising in leaves biomass doses either alone or combined with biocontrol agent over positive control. However, the minimum improvement in the activities of enzymes was observed due to separate and combined effect of *T. harzianum* or *T. viride* with 1.5% leaves biomass of *R. sativus* (Table V). A directly proportional relationship was observed between enzyme production and disease suppression after application of different management agents. It would be due to the fact that when plant is under stress it produces more enzymes to overcome stress. As the plant gets rid of the stress, the enzyme production was also reduced. Change in POX activity due to effect of various agents might ascribed its link with suberization and lignifications of host plant cells (Quiroga *et al*., 2000) and altered PAL activity may indicate regulation of defense related phenolics compounds. CAT is required to lower the concentration of $H_2O_2$ and variation in its activity after organic or bio-fungicides treatments therefore might be ascribed with resistance in plants. Alteration in PPO may signify its contribution in defensive host response by creating toxic environment for pathogen through oxidation of phenolic compounds into quinones (Araji *et al*., 2014).

## CONCLUSION

*T. harzianum* or *T. viride* with leaf biomass of *R. sativus* found effective in suppressing disease through improving intercellular communications of defense enzymes to a desired level to boost up plant immune system. These potential biocides would play a key role in management of collar rot disease in chickpea in environment friendly way and would serve as cost effective inputs for the farmers.

**Table IV. Effect of *Sclerotium rolfsii* (SR), soil amendment (*Raphanus sativus*) and *Trichoderma* spp. on disease, growth and dry weight of *Cicer arietinum* plant.**

| Treatments | Disease incidence (%) | Disease severity | Shoot length (cm) | Shoot biomass (mg) | Root length (cm) | Root biomass (mg) |
|---|---|---|---|---|---|---|
| **$T_1$**: Negative control (without any inoculation or amendment) | 0.0 | 0 | 34bc | 550f | 4.00b-e | 6.03ab |
| **$T_2$:** Positive control [(inoculated with *Sclerotium rolfsii* (SR) only)] | 70a | 4 | 18f | 223h | 1.76g | 2.43e |
| **$T_3$:** SR + 1% *R. sativus* | 48b | 3 | 24e | 390g | 3.33f | 3.93d |
| **$T_4$:** SR + 2% *R. sativus* | 44bc | 3 | 29de | 426g | 3.56d-f | 4.10d |
| **$T_5$:** SR + 3% *R. sativus* | 39cd | 3 | 31cd | 556ef | 4.40b | 4.66c |
| **$T_6$:** SR + *T. harzianum* | 36d | 3 | 38ab | 676a-c | 4.36b | 5.76b |
| **$T_7$:** SR + *T. harzianum* + 0.5% *R. sativus* | 37d | 3 | 32bc | 586d-f | 3.47ef | 4.93c |
| **$T_8$:** SR + *T. harzianum* + 1% *R. sativus* | 25f | 2 | 34bc | 639b-d | 4.23bc | 5.73b |
| **$T_9$:** SR + *T. harzianum* + 1.5% *R. sativus* | 17g | 1 | 35ab | 703a | 5.12a | 6.10a |
| **$T_{10}$:** SR + *T. viride* | 40cd | 3 | 33b-d | 576d-f | 4.06b-d | 5.83b |
| **$T_{11}$:** SR + *T. viride* + 0.5% *R. sativus* | 33e | 3 | 30c-e | 617cd | 3.76c-f | 5.10c |
| **$T_{12}$:** SR + *T. viride* + 1% *R. sativus* | 22fg | 2 | 33b-d | 660a-c | 4.33b | 6.03ab |
| **$T_{13}$:** SR + *T. viride* + 1.5% *R. sativus* | 7h | 1 | 39ab | 693ab | 5.23a | 6.27a |

Values with different letters in column show significant difference (P≤0.05) as determined by LSD Test. Values represent mean of three replicates.

**Table V. Effect of *Sclerotium rolfsii* (SR), soil amendment (*Raphanus sativus*) and *Trichoderma* spp. on biochemical traits (U/min/mg of protein) of *Cicer arietinum* leaf.**

| Treatments | CAT | POX | PPO | PAL |
|---|---|---|---|---|
| **$T_1$**: Negative control (without any inoculation or amendment) | 4.2be | 6.02d | 0.028de | 1.10g |
| **$T_2$:** Positive control [(inoculated with *Sclerotium rolfsii* (SR) only)] | 2.36e | 4.30g-i | 0.022e | 0.59h |
| **$T_3$:** SR + 1% *R. sativus* | 5.66a | 8.8a | 0.058a | 2.03a |
| **$T_4$:** SR + 2% *R. sativus* | 5.23a | 7.83b | 0.039bc | 1.80ab |
| **$T_5$:** SR + 3% *R. sativus* | 4.24be | 7.13c | 0.036b-d | 1.70bc |
| **$T_6$:** SR + *T. harzianum* | 3.31d | 5.33e | 0.034b-d | 1.04g |
| **$T_7$:** SR + *T. harzianum* + 1% *R. sativus* | 4.44bc | 5.00ef | 0.039bc | 1.53a-e |
| **$T_8$:** SR + *T. harzianum* + 2% *R. sativus* | 4.10bc | 4.83e-g | 0.035b-d | 1.48c-f |
| **$T_9$:** SR + *T. harzianum* + 3% *R. sativus* | 4.47b | 3.96hi | 0.033cd | 1.26e-g |
| **$T_{10}$:** SR + *T. viride* | 3.87cd | 4.62fh | 0.042b | 1.24fg |
| **$T_{11}$:** SR + *T. viride* + 1% *R. sativus* | 4.50b | 5.10ef | 0.040bc | 1.64b-d |
| **$T_{12}$:** SR + *T. viride* + 2% *R. sativus* | 4.36bc | 3.73i | 0.034cd | 1.30e-g |
| **$T_{13}$:** SR + *T. viride* + 3% *R. sativus* | 4.00bc | 3.93i | 0.029de | 1.39d-f |

Values with different letters in column show significant difference (P≤0.05) as determined by LSD Test. Values represent mean of three replicates.

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