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Induced Systemic Resistance in Oilseed Rape by Some Bio-Elicitors Agents Against Rot Roots Diseases Caused by *Rhizoctonia solani*

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ABSTRACT

Rot roots disease in oilseed rape crop caused by phytopathogen Rhizoctonia solani. Four different bio-elicitors, Pseudomonas fluorescens PF83, Bacillus subtilis BS87, Trichoderma harzianum TH12 and T. viride TH10 tested to inhibition of R. solani, which reached 100%, 94.20%, 100% and 84.44% respectively. Their culture filtrates (CF) also were tested for growth inhibition of the pathogen in potato dextrose agar (PDA), which reached 66.07%, 55.72%, 63.90% and 54.00% respectively.

The degree of infection and disease severity of R. solani were tested for their efficacy in oilseed rape Brassica napus in greenhouse conditions. Bio-elicitors and their CF demonstrated the ability to cause induced systemic resistance (ISR) in oilseed rape against rot root disease. Furthermore, a high ability to reduce the degree of infection in B. napus with the biotic elicitors P. fluorescens, B. subtilis, T. hazanim and T. viride was observed, with numbers reaching 5.67%, 10.33%, 7.00% and 14.67%, respectively. When CF was used, reached 15.00%, 25.33%, 19.67% and 33.00%, respectively, while for disease severity, with numbers reaching 4.33%, 7.00%, 6.67% and 10.33%, respectively. When CF was used, reached 12.33%, 20.00%, 17.67% and 28.33%.

These results show that biotic elicitor treatments can increase the fresh and dry weights of both roots and shoots as well as plant height compared with controls. Results suggest that bio-elicitors and their CF tested in this study can be used to control rot roots diseases of oilseed rape crop.

Key words: *Oilseed rape, Pseudomonas fluorescens, Bacillus subtilis, Trichoderma harzianum and Trichoderma viride.*

INTRODUCTION

Brassica napus L. is one of the major oilseed crops of the world providing 13% of the world's supply, seeds of this crop contain about 40% oil and produce meals with 35 to 40% protein[30]. This crop suffer from various pathogens that reduce the quantity and quality of production and some of which infect the crop in its early stages of growth.

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Rhizoctonia solani Kühn (teleomorph = *Thanatephorus cucumeris* Donk) is one of a ubiquitous soil-borne pathogenic fungus which causes pre-and post-emergence damping off and root rots disease in oilseed rape[34]. Root plant diseases are estimated to cause yield losses ranging from 10-15% per year in the world[5].

Plant Growth Promoting Rhizobacteria (PGPR) and Plant Growth Promoting Fungi (PGPF) are heterogeneous group can protect plants from various pathogens by Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR) as *Pseudomonas fluorescens*[6], *Bacillus subtilis*[21] and *Trichoderma harzianum* and *Trichoderma viride*[3] are bio-elicitors agent to ISR or SAR. The ability of culture filtrate of elicitors agents to play a role of bio protestants via induced systemic resistance has been demonstrated, and their ability to provide protection from pathogens in plants[3,6].

The defense mechanisms triggered by general elicitor's leads to plant are innate resistance. Elicitors as signal compounds at low concentrations, supplying information to the host to trigger defense, discriminate between elicitors and toxins, which may effect on the plant detrimentally at higher concentrations without active plant metabolism, inside cells of a host, some of the elicitors possess antimicrobial properties, generally lead to the production of phytoalexin biosynthesis, active oxygen species (AOS), reinforcement of plant cell wall related with phenylpropanoid compounds, synthesis of defense enzymes, deposition of callose and the accumulation of pathogenesis-related (PR) proteins effective against diverse pathogens, including fungi, bacteria and viruses[31]. The ultimate purpose of this study was determine the ability and effect of the biotic elicitors *P. fluorescens*, *B. subtilis*, *T. harzianum* and *T. viride* as well as cell-free culture filtrate (CF) to *R. solani* in a Petridisha and to induce systemic resistance in oilseed rape crop in the greenhouse against rot roots diseases.

MATERIALS AND METHODS

Plant materials, pathogen and biotic elicitors

The genotype of *Brassica napus* and isolates of the fungus *R. solani* and the biotic elicitors *P. fluorescens* PF83, *B. subtilis* BS87, *T. harzianum* TH12 and *T. viride* TV10 were obtained from the College of Agriculture, Wasit University, Wasit, Iraq. The genotype of plant materials *B. napus*, *R. solani* and biotic elicitors were used for greenhouse experiments during the entire period of investigation. The *R. solani* fungus, *T. harzianum* and *T. viride* were maintained and cultured on Potato dextrose agar (P.D.A) medium (200 g peeled potato, 20 g dextrose, 15 g agar, and 1 liter distilled water) in the dark at 22 ± 1°C, and 5-mm-diameter mycelia agar plugs were punched from the growing margin after 4 days. Fluorescent *Pseudomonas* was grown in pour plate method on King's medium[20], and incubated at 30 °C. Nutrient agar medium for *B. subtilis* was added and incubated at 28± 2°C for two days. Culture filtrate of bio-elicitors was prepared as previously described[3,33] (Yoshida et al, 2001., Alkooranee et al, 2015). The cell concentration was then adjusted to 1.5×10^8 CFU/ml (PF83), 1×10^6 CFU/ml (BS87), 1.5×10^7 CFU/ml (TH12) and 1×10^7 CFU/ml (TV10).

Production of culture filters of bio-elicitors

PF83 and BS87 were cultured in 150 ml flask with 100 ml king B and nutrient broth, respectively on a rotary shaker at 150 rpm for 24 h at 25°C. The cells were harvested by centrifugation at 6,000 rpm for 10 min and supernatants were filtered through Whatman membrane (2.4 μM) as previously described Katsumi Akutsu *et al*[18].

For TH12 and TV10, fifteen mycelial disks of each fungal bio-elicitor grown on PDA were separately inoculated into 150 ml flasks of PDB and incubated at 25 ± 2°C for 20 days. The cultures were then filtered as previously described Alkooranee *et al*[3]. Then culture filtrates of all bio-elicitors were used for inhibition of pathogen mycelia growth.

***Invitro* inhibition of mycelia growth**

To determine the effects of bio-elicitors on mycelia growth of the targeted pathogen *R. solani* in dual-culture techniques, PF83, BS87, TH12 and TV10 isolate suspensions and their culture filtrate (CF) were added to molten PDA media (40˚C ± 2˚C) to obtain a final concentration of 25% (v/v) each, which were mixed properly prior to plating. The media was poured in Petri dishes at 20 ml per plate. Plates were inoculated separately with 5-mm mycelia plugs of the pathogen *R. solani* placed in the centers of the plates. The inoculated plates were incubated at 25˚C ± 1˚C for 7 days. Percent mycelial growth inhibition of each pathogen was calculated using the formula:

Percent inhibition = C - T / C x 100

where C = control (radial growth of the pathogen) and T = treatment (radial growth of the pathogen after inhibition by the antagonist).

Plant cultivation under greenhouse conditions

Experiments were designed under greenhouse conditions using oilseed rape planted as five seeds per pot (10-cm-diamter) containing sand and peat moss autoclaved at a ratio of 3:1. All seeds were surface disinfected using 70% ethanol for 1 min, then 1% sodium hypochlorite solution for 5 min and rinsed three times in sterile distilled water prior to sowing. Plants were grown at 22 °C with a cycle of 12 h/12 h light/dark and a light intensity of 150 µE/m2 /s for 30 days. Irrigation was applied by drenching twice a week. After germination, only three plants were allowed to grow in each pot.

Preparation of pathogen inoculum

Rhizoctonia solani was cultured on potato dextrose agar medium for 7 days in the dark at 22°C. Five mycelia discs (8 mm in diameter) of this isolate collected from the edges of 5-day-old cultures were transferred into 100 ml of potato dextrose broth (Potato Dextrose Broth 24 g/L, Peptone 10 g/L, in water) in 300-ml Erlenmeyer flasks and incubated for 15 days at 20°C in a rotary shaker at 120 rpm. The resulting fungal suspension was adjusted to 1×10^3 fragments mL^{-1} and used as the inoculum source.

Screening of bio-elicitors based on ISR-eliciting potential and disease severity evaluation

To screen the bacterial and fungal biotic elicitors capable of eliciting ISR, 15-day-old oilseed rape seedlings, the leaves and stems of plants were treated by spread elicitors suspensions separately by 100 ml / pot water (as control) or inoculated 1.5×10^8 CFU/ml (PF83), 1×10^6 CFU/ml (BS87), 1.5×10^7 CFU/ml (TH12) and 1×10^7 CFU/ml (TV10) as well as their CF. The pathogen fungal *R. solani* was prepared in 2.4. propagules of each flask containing 100 ml media were blended and then mixed with the upper soil surface of each pot 1 day after application of the last biotic elicitor. Each treatment consisted of three replicates.

Observations were recorded after 15 days of inoculation. The degree of infection over control was calculated by applying the following formula:

Number of infected plants / Total number of plants assessed × 100

Root disease severity on oilseed rape was determined according to the development of severity scale as the infected area of the plant and multiplied by 100 given below: 0-4 (0= no infection, 1=25%, 2=50%, 3=75%, 4=100% of infection)[23]. Fresh and dry (70˚C for 48 h) weights of shoots and roots as well as plant height were determined.

Statistical analysis

The experimental design of the *invitro* and greenhouse experiments was completely randomized, with three replicates for all treatments. Data of the results were subjected to analysis of variance using GenStat software, and the means ($P < 0.05$) were compared between treatments of oilseed rape bio-elicitor treatments and SSR disease using least significant difference tests[16].

RESULTS AND DISCUSSION

Detection ability of antagonists of bio-elicitors *invitro*

All the bio-elicitors were tested for their ability to reduce mycelia growth fungi *R. solani* using the dual culture technique (Table 1). However, the degree of inhibition varied, which facilitated the election of biotic

elicitors. On the basis of percentage inhibition of the radial growth of the test pathogen four bio-elicitors namely *P. fluorescen* PF 83 (1.5× 10^8 CFU), *B. subtilis* BS 87 (1× 10^6 CFU/ml), *T. harzianum* TH12 (1.5 × 10^7 CFU/ml) and *T. viride* TH10 (1 × 10^7 CFU/ml), exhibited higher antagonistic potential as these isolates reduced the growth of pathogen *R. solani* with the inhibition zone reaching to 100%, 94.20%, 100% and 84.44% respectively. Their culture filtrates (CF) also were tested for growth inhibition of the pathogen, which reached 66.07%, 55. 72%, 63.90% and 54.00% respectively (Table 1).

Our results are compatible with a previous study which reported the *Pseudomonas* and *Bacillus* showed antagonism against different soil-borne pathogens, the colony diameter of *R. solani* reached 68mm and 69 mm, respectively and for *T. harzianum* parasitized the pathogen and produced coiling around their mycelia was reached 51mm[29]. Suppression of the growth of various pathogens by bio-elicitors and their cell-free during the present studies supports the findings showed ability TH12, TV10 and their cell-free to inhibition *Sclerotinia sclerotium* growth[3] and ability cell-free of three strains of *P. fluorescens* to reduce the germination capacity of the *R. solani* sclerotia[25].

Table 1: *In vitro* screening of bio-elicitors and their culture filtrates against *R. solani*

	Inhibition ability test	
Bio-elicitors	**Mean radial pathogen growth (mm)**	**% Inhibition (seventh day of inoculation)**
PF83	0.00	100
BS87	5.22	94.20
TH12	0.00	100
TV10	14.00	84.44
LSD	**6.19**	**3.94**
Culture Filtrates (CF)		
PF83	30.53	66.07
BS87	39.85	55. 72
TH12	32.49	63.90
TV10	41.40	54.00
LSD	**4.82**	**5.36**

Culture filtrate of *B. subtilis* MA-2 and *P. fluorescens* MA-4 inhibited the growth of phtytopathogens *Alternaria alternata, Curvularia andropogonis, Fusarium moniliformae* and *Colletotrichum acutatum* at 10 % concentration[24]. No previous reports on antagonistic activity of culture filters (CF) of these bio-elicitors are available against *R. solani* on oilseed rape, but in our studies, it successfully inhibited the growth of *R. solani*.

The ability of bio-elicitor strains and to reduce and inhibition growth of pathogen due to produce volatile and non-volatile antibiotic and produce low molecular weight diffusible compounds or antibiotics[9], and produce antifungal substances such as secondary metabolites, antibiotics iturin A, surfactin, 2,4-diacetylphloroglucinol, pyrrolnitrin [3-chloro-4-(2′- nitro-3′-chlorophenyl)-pyrrole and phenazine-1-carboxylic acid and produce lytic enzymes including chitinase, pectinase, glucanase, HCN, iron (Fe)-chelating siderophores, salicylic acid and indole-3-acetic acid (IAA)[4,8,10,22,27,28].

ISR of biotic elicitors to *R. solani* in oilseed rape

R. solani is the main pathogen of oilseed rap and other crucifers, the results showed the degree of infection and disease severity in plants infected by *R. solani* reached 82.39 %

and 74.33 % (disease score 3) respectively after 15 days of inoculation, while the non-treated plants reached 0.00 and 0.00% respectively (Fig 1). The symptom development on oilseed suggests that pathogen is able to cause significant symptoms on *B. napus* confirmed by others in their investigations of pathogenicity of *R. solani* to oilseed, the degree of infection and disease severity on oilseed rape crop is in agreement with previous reports that isolates belonging to this pathogen are highly pathogenic to Brassica species[19].

In this experiment, we studied the development of rot roots disease symptoms and correlated such symptoms with effect the bio-elicitors and their culture filtrates (CF), the results showed ability *P. fluorescen* PF 83, *B. subtilis* BS 87, *T. harzianum* TH12 and *T. viride* TH10 to induced resistance in oilseed plants infected where the degree of infection reached 5.67%, 10.33%, 7.00% and 14.67%, respectively, while for disease severity, with numbers reached 4.33%, 7.00%, 6.67% and 10.33%, respectively (Fig 1).

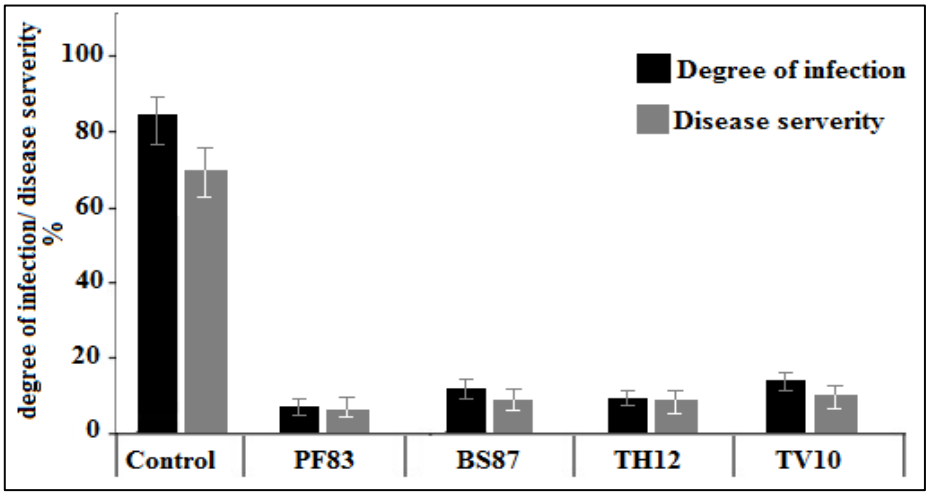

Fig. 1: Effects *R. solani* and Bio-elicitors on oilseed rape

Culture filtrates (CF) of bio-eliciors PF83, BS87, TH12 and TV10 tested to protects plant against pathogen, the degree of infection reached 15.00%, 25.33%, 19.67% and 33.00% respectively, while for disease severity, reached 12.33%, 20.00%, 17.67% and 28.33% respectively (Fig 2).

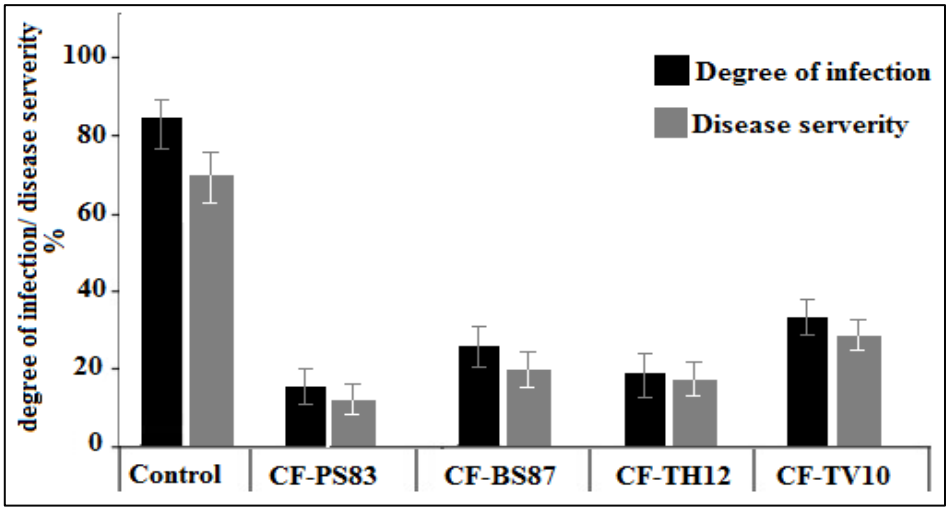

Fig. 2: Effects Culture filtrates (CF) of Bio-elicitors on oilseed rape

These results agree with the previous studies about ability of the application of bio-elicitors to control diseases, *P. fluorescens*, *B. subtilis* and *Trichoderma* sp important of microorganisms that play a major role in the plant induced systemic resistance[3,12,14,21]. Further evidence that some strains of *Pseudomonas sp.* and *Bacillus sp*. can elicit systemic protection in oilseed rape against foliar and soil-borne pathogens ex: *Sclerotinia sclerotiorum*, *Alternaria brassica*, *Leptospheria maculans*, *Botrytis cinerea* and *Verticillium longisprum*[3,11,13]. *T. hazanim* TH12 and *T. viride* TV10 were tested on *B. napus* to *S. sclerotiorum*, the degree of infection and disease index reached 7.22% and 6.67 % respectively in seedlings treated with TH12 and reached 17.78% and 11.67% respectively when treated with TV10, while reached 81.40% and 83.50% respectively in plants non-treated (control)[3].

Application of cultures filtrate of bio-elicitors of *P. fluorescens* strains and *B. subtilis* to ISR in plants were provided by many studies against phytopathogens ex: *P. putida* BTP1 to pathogen *Botrytis cinerea* in bean[26], culture filtrate of *Bacillus* sp. BS107 as an ISR determinant in tabacco against *Pectobacterium carotovorum* subsp. *carotovotrum* SCC1[32], the untreated culture filtrates (CF) and culture filtrates treated with heat (CFH) of TH12 and TV10 can stimulate resistance against phytopathogen *S. sclerotiorum* in *B. napus* AACC and *Raphanus oleracea* RRCC[3].

Many different bio-elicitors can mediate plant protection. Most important are *Pseudomonas*, *Bacillus* and *Trichoderma* strains are through acting as bio-control agents and can induce systemic resistance ISR and systemic acquired resistance SAR by involves salicylic acid (SA), jasmonic acid (JA) and ethylene (Et) signaling within the host and these hormones induce the host plant's defense responses against different of plant pathogens[15]. PGPR and PGPF Bio-elicitors can induce ISR and SAR in different ways too, some depending on pathogenesis-related proteins (PR) proteins and some on only JA/Et[2]. This induced plant protection is associated with an increase of defence related marker genes in either SA, JA and Et dependent genes after bio-elicitors or their CF treatment, oilseed rape infected by *S. sclerotiorum* and treated with TH12 and CF suspension showed a greater effect, wherein the *AOC3*, *PDF 1.2* and *ERF2* (genes with related JA/ETH) expression levels were up-regulated in plants treated with TH12, and the *PR-1*, *TGA5* and *TGA6* (genes with related SA) expression levels were up-regulated in genotypes treated with CF[2].

Effect bio-elicitors on oilseed rape growth under greenhouse conditions

The results showed that the pathogenic *R. solani* effect is significant in oilseed rape (*B. napus*) plants compared to non-infected plants (water treatment) (Table 2). The growth of oilseed rape as root fresh and dry weight, shoot fresh and dry weight and height were determined and reached 3.624 g, 0.069 g, 7.382 g, 0.894 g, and 21.67 cm in plant healthy non-infected (water treatment), while reached 1.513 g, 0.018 g, 3.965 g, 0.193g and 15.37 cm in infected plant (pathogen treatment) (Table 2).

Amendment with bio-elicitors increased the fresh and dry weights of root and shoot and height of oilseed rape plants and differed significantly compared to plants infected by the pathogen (pathogen treatment) (Table 2). The PF83 treatment ranked as most effective on root fresh and dry weight, shoot fresh and dry weight and height of oilseed rape plants with 3.413 g, 0.057 g, 6.827 g, 0.776 g and 20.94 cm respectively (Table 2). This was followed by TH12, which reached 3.150 g, 0.045 g, 5.618 g, 0.747 g and 20.33 cm, respectively. For culture filter (CF) The CF of PF83 provided the height protection against the disease (2.911 g, 0.044 g, 5.341 0.715 g,19.28 cm, respectively), this was followed by CF of TH12, which reached 2.753 g, 0.039 g, 5.260 g, 0.635 g and 19.42 cm, respectively (Table 2).

Table 2: Effect of bio-elicitors and their CF on growth of oilseed rape (*B. napus*) infected by *R. solani* in greenhouse conditions

Treatments	Root weight (g)		Shoot weight (g)		Shoot lengt (cm)
	Fresh	Dry	Fresh	Dry	
Water	3.624	0.069	7.382	0.894	21.67
pathogen	1.513	0.018	3.965	0.193	15.37
PF83	3.413	0.057	6.827	0.776	20.94
BS87	2.178	0.038	5.356	0.690	19.56
TH12	3.150	0.045	5.618	0.747	20.33
TV10	2.173	0.029	5.134	0.712	19.33
PF83- * CF	2.911	0.044	5.341	0.715	19.28
BS87-CF	2.025	0.031	5.127	0.580	18.61
TH12-CF	2.753	0.039	5.260	0.635	19.42
TV10-CF	1.972	0.021	4.237	0.562	17.93
L.S.D	**0.512**	**0.104**	**0.419**	**0.113**	**1.38**

Tables with the some treatments are not significantly different (P = 0.05).

***CF= culture filter of resistant biotic elicitor. LSD = least significant difference.**

PGPR and PGPF and their CF which are environment-friendly approach, and are directly or indirectly involved in promoting plant growth and development via production and secretion of various regulatory chemicals in the vicinity of rhizosphere, direct mechanisms of plant growth promotion as Nitrogen fixation, Phosphate solubilization, Siderophore production, Phytohormone production and 1-Aminocyclopropane-1-carboxylate (ACC) deaminase[1]. The effects of plants inoculation with PGPR and PGPF are the enhancement in rhizobial nodulation, the plant root elongation, promotion of shoot and root growth and N, P and K uptake[15].

Trichoderma spp. can produce organic acids, such as gluconic and fumaric acids that permit the solubilisation of phosphates and decrease soil pH, micronutrients, and mineral cations, such as Mg, Mn, and Fe which are useful for plant metabolism[7,17]. Culture filtrates of TH12 and TV10 were tested to *S. sclerotiorum* in oilseed rape were tested to stimulate plant growth and led to increased shoot and root system of plants treated compared to plants infected, molecular characterizations of the mechanisms of the diseases control effects of culture filtrate of bio-elicitors proved that multiple signaling pathways are involved in ISR by culture filtrate and are mainly mediated by SA/JA-ET signals[2].

CONCLUSION

The application of bio-elicitors and their culture filtrates of bio-elicitors to stimulate resistance and increase plant growth indicates that it contains activated substances and can be used in disease control and protect the plants from different pathogens.

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