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Studies on *Pseudomonas* and *Trichoderma*-Mediated Root Exudation Pattern in Chickpea against *Fusarium oxysporum* f. sp. *Ciceris*

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ABSTRACT

Plant growth-promoting microbes are known for protection of plants against a number of phytopathogens. In the present study, we used *Pseudomonas fluorescens* OKC and *Trichoderma asperellum* T42 singly as well as in combination in chickpea (*Cicer arietinum*) under challenge of *Fusarium oxysporum* f. sp. *ciceris* (Foc). Two cultivars of chickpea were used, viz., L550 susceptible to Foc and Avarodhi resistant to Foc. Root exudates obtained from the two cultivars of chickpea were analyzed and showed higher amount of Total Phenolic Content (TPC) accumulated in the microbe-treated plants compared to microbe un-treated control. Maximum phenolic content was observed in exudates of OKC treated plants in both cultivars. Interestingly, in OKC treated plants, TPC was higher in the exudates of the susceptible cultivar L550 compared to the resistant cultivar Avrodhi. Gallic acid content in the exudates also confirmed the pattern of TPC in the treatments. Wilting due to Foc was significantly reduced in the OKC and T42 treated plants compared to the untreated plants and thereby showed a positive correlation between TPC in exudates and reduction in Fusarium wilt. Higher TPC in the susceptible cultivar compared to the resistant cultivar clearly indicates the roles of OKC and T42 in reducing the wilt symptom whereas no wilting in the resistant cultivar may be explained due to genetic factor. High total chlorophyll content in the OKC and T42 treated plants of L550 also show the role of the microbes in maintaining a good health of the Foc challenged plants.

Keywords: *Cicer arietinum*, Chlorophyll, Plant phenolics, Wilting.

INTRODUCTION

Chickpea (*Cicer arietinum*) is a leguminous crop infected by many fungal pathogens throughout its growing stages. Among them, wilt of chickpea caused by *Fusarium oxysporum* f. sp. *ciceris* (Foc) is one of the most devastating diseases, infecting usually the roots. Successful management of *Fusarium* infection in chickpea is achieved through AM fungi (Sohrabi *et al*., 2015) and Plant Growth-Promoting Rhizobacteria (PGPR) in field (Singh *et al*., 2002). PGPR are known to induce resistance systemically in plants and check the pathogen ingress into the plants (van Peer *et al.*, 1991; Wei *et al.*, 1991). Plants root exudates affect rhizosphere microbial communities that contain beneficial, neutral and pathogenic microbes. Interactions between the different elements of these communities have been studied in

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relation to biological control of plant pathogens. One of the mechanisms of disease control is Induced Systemic Resistance (ISR). Studies on biological control of plant diseases have focused on ISR in the last decades, because ISR is effective against a wide range of pathogens and offers serious potential for practical applications in crop protection. *Pseudomonas* spp. are generally known to promote plant growth either directly by production of hormones or indirectly by production of anti-microbial compounds which act against pathogens. Recent attempts to protect plants from pathogen through PGPR have gained worldwide attention due to eco-friendly and sustainability in agriculture (Sarma *et al*., 2015). Phenolic compounds are the natural constituents in all plants investigated till now. Besides several other classes of compounds, antibiotic phenols have been implicated in plant defense mechanisms (Singh *et al*., 2002; Kuc, 1995; Nicholson and Hammerschmidt, 1992). Among them, some occur constitutively and are thought to function as inhibitors and associated with non-host resistance (Millar and Higgins, 1970; Stoessl, 1983). Others are formed in response to the ingress of pathogens, and their appearance is considered as part of an active defense response (Nicholson and Hammerschmidt, 1992; Matta *et al.*, 1988). The activation of phenol metabolism was found at a site functional for the lateral confinement of pathogens in the xylem vessels of tomato. This observation was later supported by two phenolic compounds, a triterpenoid, and elemental sulfur in cells associated with vascular pathogens in disease-resistant genotypes of *Theobroma cacao* (Cooper *et al.*, 1996). Accumulation of phenolic compounds in carnation by a *Pseudomonas* sp. and thereby decreased Fusarium wilt has been reported (van Peer *et al.*, 1991). Phenolic compounds are plant secondary metabolites, which play important roles in disease resistance and provide protection against pests and species dissemination (Servili and Montedoro, 2002). Previous studies have reported that the phenylpropanoid pathway plays a critical role during the plant defense response to *V. dahlie* (Smit and Dubery, 1997; Pomar *et al.*, 2004; Gayoso *et al.*, 2010). These compounds are of the most widely occurring groups of phytochemicals that have considerable physiological and morphological importance in plants. These compounds play an important role in growth and reproduction, along with providing protection against pathogens and predators (Bravo, 1998). Since pathogens such as Foc invade plants through roots, the aims of the present study were to analyze: (i) The status of phenolic compounds in root exudates of a susceptible and a resistant chickpea cultivars after inoculation with two rhizospheric microbial strains against Foc challenge, and (ii) The impact of the root exudate phenolics in reducing chickpea wilt due to Foc.

MATERIALS AND METHODS

Pseudomonas fluorescens strain OKC, *Trichoderma asperellum* strain T42 and pathogen *Fusarium oxysporum* f.sp. *ciceris* (Foc) were obtained from the Hoffmann Laboratory, Department of Mycology and Plant Pathology, Banaras Hindu University, and used in the current study. The culture of *P. fluorescens* strain OKC was maintained on King's B agar (Protease peptone: 20 g, $K_2HPO_43H_2O$: 2 g, $MgSO_47H_2O$: 1.5 g, Glycerol: 15 mL, Bacteriological agar 2%, Distilled water: 985 mL and pH: 7.2±0.2) medium and *Trichoderma asperellum* strain T42 and pathogen Foc were maintained on potato dextrose agar (Peeled potato: 200 g, Dextrose: 20 g, Agar: 20 g, Distilled water: 1,000 mL, pH: 6.8±0.2).

Seed Priming

Seeds of chickpea cultivars "Avrodhi" and "L-550" were surface sterilized with 0.1% $HgCl_2$ for 30 seconds in a conical flask and washed three times with sterilized distilled

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water. On the other hand, King's B broth was prepared, sterilized, and inoculated with the bacterial strain OKC. The inoculated flasks were incubated in incubator shaker at 28±2°C and 100 rpm for two days. Thereafter, cells in exponential phase were centrifuged, supernatant was decanted, and the cells were collected by pouring sterile distilled water. Similarly, T42 was grown on Petri plates containing PDA (Potato Dextrose Agar) for 5-7 days at 28±2°C. Spores of T42 were harvested by pouring distilled water in the plates followed by gentle agitation with help of a L-shaped glass rod spreader. Spore suspension was then filtered through fine mesh of sterilized cotton to separate the mycelium fragments. Cell suspensions of the microbial strains were prepared and maintained at 1.6×10^8 CFU mL^{-1} and 2×10^7 spores mL^{-1} for OKC and T42, respectively. CarboxyMethyl Cellulose (CMC)@ 1% was added as sticker to the cell suspensions (Yadav *et al.*, 2013). Seeds were dipped in these suspensions for 6-8 hours and air dried for an hour at room temperature before sowing.

Growth Conditions

Treated seeds of both chickpea cultivars as described above were grown in culture tubes containing sterilized sand (with neutral pH)**.** Culture tubes were incubated in the plant growth chamber at 21±2°C with 16:8 light and dark hours.

Determination of Chlorophyll Content in Leaves

Fresh leaf samples were selected for determination of chlorophyll content in the treated plants with bio-control agents OKC and T42 individually and in combination. The samples were crushed in 80% acetone, left overnight and then filtered. The absorption of filtrates was recorded at wave lengths of 663 nm (D663) and 645 nm (D645) with the help of a spectrophotometer. The concentrations of Chlorophyll a (Chl-a), Chlorophyll b (Chl-b) and total Chlorophyll (Chl-t) were calculated using the following equations (Arnon, 1949):

Chl-a= C12.72A663–2.59A645

Chl-b= C22.9A645–4.67A663

Chl-t= 20.31A645–8.05A663

Pathogen Application and Collection of Root Exudates

Fusarium oxysporum f. sp. *ciceris* (Foc) was grown on PDA medium. The fully sporulated plates of Foc were used for the preparation of spore suspensions in distilled water and diluted to 1×10^7spores mL^{-1}. One-week-old chickpea plants grown in sand medium were selected for Foc inoculation aseptically. One mL of spore suspension was inoculated in each culture tube of both resistance and susceptible cultivars of chickpea pre-treated with the bio-control agents either individually or in combination. The spore suspension was inoculated near the collar regions and five replicates were maintained for each treatment. After a week of pathogen inoculation, root exudates were collected separately from all treatments. The plants were uprooted from the culture tubes and 100% ethyl acetate was added to the sand medium and kept for 2 hours. Afterwards, it was fractionated with ethyl acetate and collected by filtration in screw capped bottles and kept for evaporation of ethyl acetate. The residue was dissolved in one mL HPLC grade methanol and filtered through 0.45 µ filters before HPLC analysis.

Calculation of Wilting%

Wilting% was calculated by counting the number of wilted plants and using the following formula:

Wilting%= (Total number of wilted plants×100)/Total number of plants

HPLC Analysis

High performance liquid chromatography of fractionated material was performed in a

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HPLC system equipped with two Shimadzu LC-10 ATV Preciprocating pumps, a variable UV- VIS detector, an integrator and Winchrom software for data recording and processing (Winchom, Spinco Biotech, Pvt. Ltd., Chennai, India). Running conditions included a mobile phase of Acetonitrile and water (60: 40 v/v), with 1 mL min^{-1} flow rate. The injection volume was 20 µL and detection was done at wave length 290 and 254 nm. Fractionated material (1 mg mL^{-1}) and phenolic acids dissolved in HPLC grade methanol were injected into the sample loop and the means of peak areas of individual compounds were taken for quantification. Gallic acid was used as internal and external standards. Gallic acid present in the sample was identified by comparing Retention Time (RT) of standard of Gallic acid (3.592 minutes). Amount of Gallic acid was calculated by comparing peak areas of reference compounds with fractionated in the samples run under similar conditions. Three biological replicates were taken for the collection of root exudates.

Estimation of Total Phenolic Content (TPC)

Plant leaf samples were crushed in methanol (50%) and filtered. To the filtrate, a little amount of charcoal was added for adsorption of pigments and it was again filtered. From the aliquot, 100 µL of sample was taken and 900 µl of distilled water was added. Then, 500 µL of Folin-Ciocalteu's phenol reagent (1:1; Folin-Ciocalteu's phenol reagent: Distilled water) was added followed by 1 mL of sodium carbonate (20%) and mixed thoroughly, till the color of mixture changed to blue. Ten mL of distilled water was added and the mixture was incubated for 20 minutes. After incubation, absorbance of samples was taken at 725 nm wavelength and the concentrations were determined against a standard curve prepared from Gallic acid (Zheng and Shetty, 2000).

Statistical Analysis

Experiments were performed using Completely Randomized Design (CRD). The one-way variance of analysis was performed to test the significance of the observed differences using SPSS version 16 (SPSS Inc., Chicago, IL, USA). The differences between the parameters were evaluated by means of the Duncan's test and P values$\leq$0.01 were considered as statistically significant.

RESULTS

Total Gallic Acid

HPLC analysis of root exudates of two chickpea cultivars (Avrodhi and L-550) treated with *Pseudomonas fluorescens* (OKC) and *Trichoderma asperellum* (T42) individually and in combination challenged with pathogen indicated that chickpea root exudation was rich in secondary metabolites. The number and amount of phenolic compounds slightly increased in both cultivars treated with the microbial strains either individually or in combination as compared to un-treated plants during pathogen inoculation. Gallic acid was at maximum in microbe treated plants as compared to the control and the plants under the pathogen stress. Maximum accumulation of Gallic acid was observed in OKC and T42 treated plants of the resistant cultivar as compared to the susceptible cultivar under pathogen stress. However, the combination of both strains (OKC+T42) showed maximum accumulation of Gallic acid in the susceptible cultivar as compared to the resistant cultivar under the pathogen stress condition (Figure 1).

Total Phenolic Content (TPC)

TPC in root exudates of chickpea plants varied in different treatments in both resistant (Avrodhi) and susceptible (L-550) cultivars treated with *Pseudomonas*

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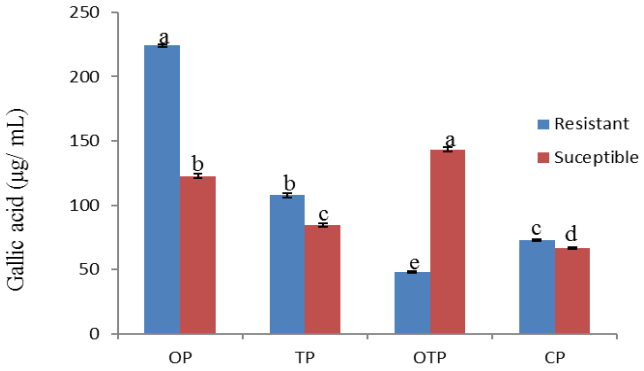

Figure 1. Gallic acid content in different root exudates treatments (µg mL^{-1}) by HPLC analysis. O= *Pseudomonas fluorescens* (OKC); T= *Trichoderma asperellum* (T42); P= Pathogen (*F. oxysporum* f.sp. *ciceris*), C= Control. Different letters a,b indicate data significantly different from the other treatments ($P \leq 0.05$; Duncan's multiple rangetest).

fluorescens (OKC) and *Trichoderma asperellum* (T42) individually and in combination and challenged with the pathogen. TPC was at maximum in the microbe treated plants as compared to challenged control. Maximum phenolic content was observed in OKC treated plants in both cultivars. However, TPC in the combined treatment was higher in the susceptible cultivar compared to the resistant cultivar. In contrast, phenolic content was more in T42 treated plants in the resistant cultivar compared to the susceptible cultivar (Table 1).

Wilting and Chlorophyll Content in Plants

Wilting in chickpea plants and chlorophyll content also varied in different treatments under the pathogen stress. Highest wilted plants and least chlorophyll content were recorded in plants only inoculated with the pathogen and, in contrast, maximum chlorophyll content and least wilted plants were recorded in plants treated with OKC and under pathogen stress. In OKC treated plants, chlorophyll b and total chlorophyll were found maximum compared to the other treatments. Plants treated with the dual microbial consortium also showed very high chlorophyll content and low wilted plants. In contrast, among the various microbial treatments, T42 treated plants showed maximum wilted plants and least chlorophyll content. However, wilted plants in T42 were significantly low and chlorophyll content was significantly high in comparison to the microbe non-treated

Table 1. Total phenol content in chickpea root exudates treated with *Pseudomonas fluorescens* and *Trichoderma asperellum* under the biotic stress of *F. oxysporum* f.sp. *ciceris*.

Treatment [a]	Total phenolic content (mM Gallic acid equivalent per gram fresh weight)	
	Resistant variety (Avrodhi)	Susceptible variety (L-550)
OP	1.316 ± 0.006^{a}	2.055 ± 0.021^{a}
TP	0.974 ± 0.026^{b}	0.739 ± 0.049^{c}
OTP	0.615 ± 0.011^{c}	1.269 ± 0.006^{b}
CP	0.495 ± 0.008^{d}	0.333 ± 0.007^{d}

[a] O= *Pseudomonas fluorescens*(OKC); T= *Trichoderma asperellum*(T42); P= Pathogen (*F. oxysporum* f.sp. *ciceris*), C= Control. Different superscript letters a, b indicate data significantly different from the other treatments ($P \leq 0.05$; Duncan's multiple range test).

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pathogen challenged plants(Table 2, Figure 2).

DISCUSSION

In the rhizosphere, plant roots secrete compounds which interact with microorganisms to gain functional advantage at different developmental stages of plants (Smit and Dubery, 1997). It was previously reported that when plants are challenged by any harmful or beneficial microbes, protein secretion pattern by the plant root is altered at various developmental stages. Plants use this as a strategy for defense responses against harmful microorganisms encountered. The metabolic profiling of root exudates of *Arabidopsis* showed presence of various metabolites such as sugars, amino acids, fatty acids as well as various classes of proteins (Wei *et al.*, 1991; De-la-pena *et al.*, 2010). The root exudates affected modulation in expression of some bacterial genes that influenced the microbe and plant interactions (Mark *et al.*, 2005). Roots release exudates according to the genotype of a plant species in the rhizosphere and the exudation profile is modified during stresses (Selvakumar *et al.*, 2012; Zamioudis and Pieterse, 2012). The exudation under such circumstances contains more antimicrobial, phytotoxic, nematicidal and insecticidal compounds which are useful in defending the plants against biotic and abiotic stresses (Bais *et al.*, 2006). Various microbes utilize the carbon compounds that exude out from plant roots differently and, thus, have a great influence on the composition and quantity of soil microbes (Bais *et al.*, 2004). It was

Table 2. Total Chlorophyll content in chickpea cultivar (L-550) treated with *Pseudomonas fluorescens* and *Trichoderma asperellum* under the stress of *F. oxysporum* f. sp. *ciceris.*

Treatment [a]	Chlorophyll content (µg mL^{-1})		
	Chl-a	Chl-b	Chl-t
OP	17.06 ±0.072^{c}	39.23±0.250^{a}	53.36±0.120^{a}
TP	22.49±0.171^{a}	22.91±0.578^{c}	44.46±0.427^{c}
OTP	21.62±0.130^{b}	29.45±0.175^{b}	51.05±0.505^{b}
CP	12.06±0.699^{d}	12.29±0.165^{d}	12.52±0.160^{d}

[a] O= *Pseudomonas fluorescens*(OKC); T= *Trichoderma asperellum*(T42); P= Pathogen (*F. oxysporum* f.sp. *ciceris*), C= Control. Different superscript letters a, b indicate data significantly different from the other treatments ($P \leq 0.05$; Duncan's multiple range test).

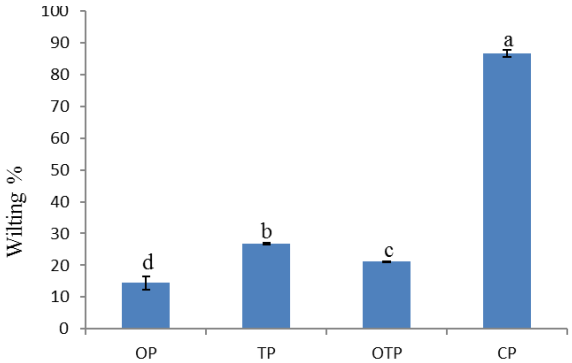

Figure 2. Wilting percent in different treatments after pathogen inoculation. O= *Pseudomonas fluorescens* (OKC); T= *Trichoderma asperellum* (T42); P= Pathogen (*F. oxysporum* f.sp. *ciceris*), C= Control. Different letters a, b indicate data significantly different from the other treatments ($P \leq 0.05$; Duncan's multiple range test).

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observed that plant root secreted ions, free oxygen, enzymes, mucilage and carbon containing primary and secondary metabolites have significant impact on the rhizosphere (Bertin *et al.*, 2003). Similarly, plant phenolics are natural products synthesized by activation of the phenylpropanoid pathway that share a major role among the families of various biomolecules responsible for imparting PGPR mediated ISR response (Singh *et al.*, 2002). A number of phenolic acids are also known to be antifungal (Bertin *et al.*, 2003) and accumulation of these compounds is positively correlated with reduction in plant mortality (Singh *et al.*, 2013). TPC in chickpea plants was estimated in all the treatments in the present study and maximum accumulation of TPC was observed in plants treated with *Pseudomonas* (OKC) individually as compared to the combination of OKC+T42, T42 treated plants, and the control ones during the pathogen stress. Moreover, maximum TPC was estimated in the susceptible cultivar treated with OKC as compared to the resistant cultivar and accumulation of TPC was low in T42 treated plants as compared to OKC ones. Earlier reports also suggest that rhizobacteria mediates accumulation of TPC in plants (Singh *et al.*, 2002). Phenolic compounds in chickpea seedlings also accumulated in higher amounts when seeds were bacterized with an endophytic strain *Bacillus megaterium* (Rangeshwaran *et al.*, 2008). The antimicrobial property of phenols and their rapid esterification into the plant cell wall is correlated with increase in plant resistance to cell wall degrading enzymes of pathogens (Nicholson and Hammerschmidt, 1992). Plants also need phenolic compounds for pigmentation, growth, reproduction, and resistance to pathogens (Lattanzio *et al.*, 2006). In the present study, higher phenolic content in the root exudation from the plants under pathogen stress was considered as chickpea plants influenced by the rhizosphere microbes OKC and T42. Accumulation of defense related compounds in PGPR treated plants during pathogen stress was also reported earlier by Wei *et al.* (1996). However, in the present study, higher accumulation of phenolics in combined application of OKC and T42 in the susceptible cultivar indicates that the microbes had a significant role in triggering defense responses in the susceptible cultivar which is otherwise susceptible to wilting. Gallic acid is a naturally occurring phenolic compound which is notable for its antioxidant and antimicrobial activity (Stoessl, 1983). The number and amount of phenolic acids slightly increased in both tested cultivars during individual treatment and co-inoculation of OKC and T42 as compared to the untreated plants after pathogen inoculation. Maximum accumulation of Gallic acid was observed in OKC and T42 treated plants of resistant cultivar as compared to susceptible cultivar under pathogen stress. Several authors previously reported that the rich amount of secondary metabolites in the host inhibits the growth and development of fungal pathogens (Zamioudis *et al.*, 2012). According to some earlier reports, plants treated with non-pathogenic microbes increased the chlorophyll content in leaves, the size of new leaves, and the number of new branches, and advanced the timing of bud break, delayed the senescence of infected tissues, and increased the nutrient uptake (Taylor *et al.*, 2004). In the present study also, less wilting of plants and high chlorophyll content were observed in plants treated with OKC and T42 individually and in combination as compared to the untreated control plants. In OKC treated plants, chlorophyll a and total chlorophyll were found maximum compared to other treatments. Similarly, T42 treatment significantly reduced the wilting of plants challenged with Foc and plants exhibited higher chlorophyll content compared to the challenged and untreated control. A study also reported the role of *Bacillus amyloliquefaciens* strainNJN-6 as important PGPR, having a role in production of important secondary metabolites, which help

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in inhibiting several soil-borne pathogens, plants growth promotion, and suppression of banana wilt (Yuan *et al.*, 2013). Plant protection from pathogen through enhancement of plant vigor was reported by Nicholson and Hammerschmidt, (1992). The priming of seeds with PGPR increase the germination rate, root growth, yield, leaf area, chlorophyll content, magnesium, nitrogen and protein content, hydraulic activity, tolerance to drought and salt stress, shoot and root weights, and delayed leaf senescence (Yadav *et al.*, 2013).

CONCLUSIONS

There is enhancement in the plant development after bio-priming of seeds with beneficial rhizospheric microbes. Bio-agents have specific roles to play in plant protection by stimulating production of secondary metabolites and these metabolites especially phenolics were secreted in the form of exudates during root infection by pathogens like *Fusarium*. Looking into the results from the present study and earlier reports, it can be concluded that seed bio-priming with bio-agents such as *Trichoderma* and *Pseudomonas* have beneficial roles in enhancing plant secondary metabolites such as phenolics and some of which are also secreted through root exudation. Such mechanisms help plants to resist infection by pathogens such as *Fusarium*. However, more studies are needed, particularly at molecular level, to understand the mechanisms at a greater depth.

Abbreviations

C= Control, Foc= *Fusarium oxysporum*,O= *Pseudomonas fluorescens* (OKC), P= Pathogen (*F. oxysporum* f.sp. *ciceris*), RT: Retention Time, T= *Trichoderma asperellum* (T42), TPC= Total Phenolic Content.

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بررسی الگوی تراوشات ریشه به میانجی سودوموناس و تریکودرما بر علیه *Fusarium oxysporum f.* sp. Ciceris در نخود

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چکیده

میکروب های افزایش دهنده رشد گیاه به عنوان حافظان گیاه در برابر تعدادی از پاتوژن های گیاهی شناخته شده اند. در این پژوهش، از دو میکرب *Pseudomonas fluorescens* OKC و *Trichoderma asperellum* T42 به طور مجزا و نیز در ترکیب با هم در برابر آلودگی به *Fusarium oxysporum* f. sp. *ciceris* (Foc) در نخود (*Cicer arietinum*) استفاده شد. دو کولتیوار نخود به نام های L550(حساس به Foc) و Avarodhi(مقاوم به Foc) مطالعه شدند. تراوشات ریشه این دو کولتیوار مورد تجزیه قرار داده شد . بر اساس نتایج تجزیه، مقدار کل مواد فنلی (TPC) انباشته شده در گیاهان تیمار شده با میکرب ها بیشتر از نخود های تیمار نشده بود. مقدار بیشینه مواد فنلی در هر دو کولتیوار در تراوشات بوته های تیمار شده با OKC مشاهده شد. جالب بود که در بوته های تیمار شده با OKC ، مقدار TPC در تراوشات ریشه کولتیوار حساس به Foc (کولتیوار L550) در مقایسه با کولتیوار مقاوم (Avarodhi) بیشتر بود. محتوای گالیک اسید هم در تراوشات ریشه از الگوی TPC در تیمارها تبعیت میکرد. در بوته های تیمار شده با OKC و T42 در مقایسه با بوته های تیمار نشده، پژمردگی ناشی از Foc به طور معناداری کم شده بود که این امر همبستگی مثبتی بین TPC موجود در تراوشات ریشه با کاهش پژمردگی فوزاریمی نشان می داد. بالاتر بودن میزان TPC در کولتیوار حساس در مقایسه با کولتیوار مقاوم، به وضوح به نقش OKC و T42 در کاهش علایم پژمردگی اشاره دارد در حالیکه عدم وجود علایم پژمردگی در کولتیوار مقاوم را می توان به عوامل ژنتیکی نسبت داد. بالا بودن میزان کلروفیل کل در بوته های کولتیوار L550 تیمار شده با OKC و T42 نیز نقش میکروب ها را در حفظ سلامت بوته های آلوده به Foc نشان می دهد.

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