NATURAL, ENVIRONMENTAL AND PRACTICAL BIOLOGICAL CONTROL OPTIONS FOR FUSARIUM WILT DISEASE OF CARNATION (*FUSARIUM OXYSPORUM* F. SP. *DIANTHI*)

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Abstract. Carnation (*Dianthus caryophyllus* L.) is one of the most important cut flowers in the world. Some fungi, bacteria and viruses lead to diseases that affect carnation plants, and most of these severe diseases are caused by Fungi. It is considered that *Fusarium oxysporum f. sp. dianthi (Fod)* has induced one of the most serious and detrimental diseases that impair carnation. In this study, the effect of some biological preparations [*Trichoderma harzianum Pseudomonas fluorescens; Bacillus subtilis* QST 713 (SERENADE), *Mycorrhiza* spp., *Trichoderma* spp., *Bacillus* spp. (PANORAMIX), *Lactobacillus acidophilus* + *Lactobacillus paracasei* (VITANAL), tea tree oil extract (TIMOREX GOLD), orange oil extract (PREV-AM), plant extracts of Reynoutria spp. (REGALIA) against *Fod* were investigated. Experiments were performed using a randomized plots design with five replications (five pots and one plant per pot) in a growth chamber. After 30 days, carnation plants were evaluated with a scale of 0-5 values. The effect of biological preparations was calculated based on root size, root number, plant height and number of nodes, then the acquired data were evaluated. The best results were obtained with *T. harzianum* (96%), SERENADE (96%) and PANORAMIX (88%) against *Fusarium oxysporum* f. sp. *dianthi*. All of the control plants (+) were completely dead due to the disease. In conclusion, the biological control improves the Fusarium wilt suppression capabilities of carnation.
Keywords: *Pseudomonas fluorescens, Bacillus subtilis, Trichoderma harzianum, plant extract, orange oil extract*

Introduction

Carnation (*Dianthus caryophyllus* L.) belongs to the Caryophyllaceae family and has been extremely popular amongst cut flowers since the 18th century. Carnation is one of the most demanded cut flower in the world. Netherlands is a leading country which has the biggest market share of carnations in 2015 with around 47% of all imports in the European Union followed by The United Kingdom, Spain, Poland, Bulgaria, Slovenia, Romania and the Baltic states (https://www.cbi.eu/market-information). Turkey also has a substantial place among producers of carnation plant, which is the major horticultural product of that country. Turkey has introduced a variety of popular standards and spray for carnations to marketplace for a long time. Sixty percent of the cut flower production has comprised of carnation plant in Turkey..In addition, most of the grown cut flowers are chrysanthemums, gerberas, solidange, gladiolus and freesia. The lion's share of ornamental plant production in Turkey has been churning out between the cities of Antalya, İzmir and Yalova, and Isparta (TSI, 2016).

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Some diseases and pests lead to economic damage during the process of carnation cultivation. The most important disease is Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend: Fr. f. sp. *dianthi* (Prill & Delacr.) W. C. Snyd. & H. N. Hans., which is a soil-borne fungus disease. Fusarium wilt disease is common in 79% of carnation production areas and affects 45% of total production (Anonymous, 2006). The most important phytopathological problem affecting carnation in most areas of the world is Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *dianthi*. (*Fod*). Fusarium wilt is prevalent in 79% of the national production areas of carnation and affects 45% of its total production (Anonymous, 2006). The symptom of *Fod* is characterised by wilting of shoots, discolouration of leaves, and brown streaks on vascular tissue in stem. The infected leaves turn out chlorotic and finally wilt (Sohi, 1992). *F. oxysporum* f. sp. *dianthi* has various biological races and occurs in different carnation cultivars due to their selective virulence. Race 2 of *Fod* is the most prevalent in carnation cultivars (Denmik et al., 1989; Sarrocco et al., 2007). Fusarium wilting disease is controlled by systemic fungicides. This situation has a negative impact on human health, causes pollution and toxicity and also reduces the population of beneficial microorganisms in the soil. Therefore, alternative methods must be found to reduce the use of chemical fungicides. For this reason, biological control studies are of great importance. Mahalakshmi et al. (2015) investigated the effect of different organic inputs viz., neem cake, mauha cake, coipith and vermicompost against the wilt disease in carnation. As a result, Neemcake (10%) effectively prevented the growth of Fod. Hanudin et al. (2017) reported that *Bacillus subtilis* and *Pseudomonas fluorescens* suspended in the vermicompost extract and molases on the concentration level of 0.5% were consistenly effective in suppressing Fusarium wilt on carnation. In addition, fungicides adversely affect human and environmental health. Hence, alternative control methods as biological control which are more environmentally friendly are necessary.

The aim of this study was to evaluate the effectiveness of some biological methods against Fusarium carnation wilt disease using a randomized plots design with five replications over a 30 day period, in order to find the best biological practice to combat the disease.

Material and methods

Fusarium oxysporum f. sp. *dianthi* (*Fod*) was used as a pathogen. *Fod* was isolated from Isparta carnation greenhouses in Turkey and tested for pathogenicity). Some biological organisms and plant extracts were utilised as a biological control. Microorganisms and plant extracts used in the experiment are given in *Table 1*. All treatments were applied on the carnation cuttings by root-dipping.

Isolation and identification of Fusarium oxysporum f. sp. dianthi

Samples exhibiting wilt disease of carnation were collected from the 5 different greenhouses of Isparta, Turkey during 2017-2018 and brought to the laboratory. From these samples five isolates of *Fod* were isolated and identified (Nelson et al., 1983; Burgess et al., 1994). Pathogenicity of isolates was assessed. As a result of the pathogenicity test, ISP-3 isolate, which caused the most diseases in commercial carnation cultivar was used in the experiments.

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Table 1. *The biological microorganisms and plant extracts used in the experiment*

Treatments	Number of plant
Trichoderma harzianum	5
Pseudomonas fluorescens	5
Bacillus subtilis QST 713 (SERENADE)	5
Mycorrhiza spp., *Trichoderma* spp., *Bacillus* spp (PANORAMIX)	5
Lactobacillus acidophilus + *Lactobacillus paracasei* (VITANAL)	5
Tea tree oil extract (TIMOREX GOLD)	5
Orange oil extract (PREV-AM)	5
Plant extracts of *Reynoutria* spp. (REGALIA)	5

Isolation of Trichoderma harzianum, Pseudomonas fluorescens

Trichoderma harzianum and *Pseudomonas fluorescens* were isolated by dilution plate technique from the soil samples collected from Isparta and Aydın, in Turkey (Johanson, 1957). The fungal isolate was grown on PDA plates, the bacteria isolate was grown on Nutrient Agar (HIMEDIA, 13 g/L) media at 25 °C. The isolated species were identified by Rhodes (1959), Whipps (2001) and Chin et al. (2003). The isolates of *T. harzianum* and *Pseudomonas fluorescens* were kept on the PDA/NA medium in the Biotechnology and Plant Pathology Research Laboratory, Isparta University of Applied Sciences, Isparta.

Plant material

The commercial carnation cultivar of Picasso was used for its potential antagonistic capacity to Fusarium carnation wilt disease reaction in a growth chamber condition.

Treatment and assessment

Pseudomonas fluorescens were grown in Nutrient Agar (HIMEDIA, 13 g/L) medium and placed at 90 rpm on a rotary shaker for 48 h at room temperature. The cells were harvested by centrifugation for 15 min at 5000 rpm and the pellet was suspended in distilled water. Suspension of bacteria was adjusted to 1×10^7 cfu/ml. The isolates of *T. harzianum* and *F. oxysporum* f. sp. *dianthii* were cultured in PDA plates and incubated at 25 °C for 10 days. Sterile distilled water was poured on the mycelium of fungi and mycelia were collected with a scalpel and the liquid was taken out. Suspensions were then filtered through sterilized cotton filters to obtain pure conidial suspensions and the spores were counted by using a hemocytometer. The spore suspensions of both fungi were adjusted to 1×10^6 conida/mL concentration with sterile distilled water. When plants had 6-8 true-leaf stages, all treatments (REGULAR-200 ml/100 L, TIMOREX-200 ml/100 L, PREW-AM 200 ml/100 L, VITANOL-300 ml/100 L, PANORAMIX-600 ml/100 L, SERENADE-1000 ml/100 L, *T. harzianum*-1×10^6 conida/mL), *P. fluorescens* -1×10^7 cfu/ml.) were applied on the carnation cuttings by root-dipping for 30 min using 20 mL per plant. Treated plants were separately planted in pots with 2 torf:1 perlit: substrate and the remainder suspensions were added to the pots. After 48 h, inoculum suspension of *F. oxysporum* f. sp. *dianthii* were added using 20 ml per plant. The plants were transferred to growth chambers at 24 °C and 12-h day/12-h night for 30 days (*Fig. 1*). Experiments were performed using a randomized plots design with five

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replications (five pots and one plant per pot) in a growth chamber. The experiment was repeated twice. Diseases severity was evaluated 30 days after inoculation with a 0-5 scale values as follows: 0 = no symptoms (0% disease); 1 = weakly affected plant (5%); 2 = local base-stem symptoms (20%); 3 = one-sided and well-developed symptoms (50%); 4 = severe disease symptoms throughout the plant (80%); 5 = dead plant (100%) (Baayen and van der Plas, 1992).

Figure 1. *Different treatment against Fod on carnation cv Picasso in a growth chamber*

The disease severity was monitored every week throughout experimental procedure. The disease severity was evaluated using Townsend-Heuberger's formula (Towsend and Heuberger, 1943). The percentage effect of the applications was calculated using the Abbott formula (Abbott, 1925). The effect of biological preparations (%) was calculated, root size (cm), root number, plant height (cm), number of nodes were measured. Control plant groups were used in the experiment, and control positive plants groups were applied only to Fod and control negative plants only water was applied.

All data were analyzed by analysis of variance (ANOVA) to detect differences between treatments. Mean comparisons were made by using Duncan's tests; all statistical tests were conducted at a probability level of $P \leq 0.05$. All analyses were performed using the SPSS 21 software.

Results

Trichoderma harzianum, *P. fluorescens*, *B. subtilis* QST 713 (SERENADE), *Bacillus* spp + *Trichoderma* spp. + *Endomycorrhiza* (PANORAMIX), *L. acidophilus* + *L. paracasei* (VITANAL), and plant extracts of Tea tree oil extract (TIMOREX GOLD), Orange oil extract (PREV-AM), *Reynoutria* spp. (REGALIA) were applied to carnation wilt disease caused by *F. oxysporum* f. sp. *dianthi*. All applications reduce disease symptoms significantly (*Fig. 2*).

The lowest disease severity (%) was obtained from *P. fluorescens* (4%), SERENADE (6%), and *T. harzianum* (10%) to Fod. Disease severity (%) was determined in the application of PANORAMIX (12%). The percentage of disease severity in control plants was observed as 96% (*Fig. 3*). In the experiment, it was determined that the highest percentage of efficacy values of the biological preparations were for *P. fluorescens* (96%), SERENADE (94%), and *T. harzianum* (90%) to Fod.

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Control plant groups (+) were completely dead due to the disease. The lowest percentage of efficacy values of the biological preparations were determined with the application of TIMOREX (76%), VİTANAL and REGALIA (72%). In conclusion, the biological control method improves the suppressive capacity against Fusarium wilt disease in carnation (*Table 2*).

Figure 2. *Effects of different treatments on Fod incidence on pot assay in a growth chamber*

Figure 3. *Effects of SERENADE, TIMOREX GOLD and T. harzianum on plant growth*

The effect of different treatments on plant parameters of carnation was presented in *Table 3.* Statistical analysis of figure showed significant differences in treatments at $P \leq 0.05$ levels. Application of different treatments have improved carnation growth statistically. Plant height was showed significant variation with TIMOREX GOLD application. The longest plant height was found in TIMOREX GOLD application (31.60 cm) followed by PANORAMIX (26.46 cm), REGALIA (26.44 cm) and SERENADE (25.98 cm)) applications. The shortest plant height were in *P. Fluorescens*

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(21.25 cm) application. Significant variation was observed in the case of the number of roots with different treatments. The maximum root number was in TIMOREX GOLD application with 23.20, the minimum number of root was determined in PREV-AM with 11.60. The root length was showed significant variation with different treatments. The maximum root length was determined on REGALIA application (4.28 cm). Significant variation was determined in the case of the number of nodes. It was indicated that the maximum number of node (9.00) was found VITANAL and REGALIA, the minimum number of node (5.60) was in *P. fluorescens*. Results showed that TIMOREX GOLD, REGALIA and SERENADE in generally were found effective to enhance the plant growth percentage compared to control.

Table 2. *Effectiveness of different treatments against F. oxysporum f. sp. dianthi on carnation*

Treatments	DS*	E*
Orange oil extract (PREV-AM)	20.0	80.0 ab
T. harzianum	10.0	**90.0 a**
B. subtilis QST 713 (SERENADE)	6.0	**94.0 a**
L. acidophilus + *L. paracasei* (VITANAL)	28.0	72.0 bc
P. fluorescens	4.0	**96.0 a**
Mycorrhiza spp., *Trichoderma* spp., *Bacillus* spp (PANORAMIX)	12.0	88.0 ab
Tea tree oil extract (TIMOREX GOLD)	24.0	76.0 b
Plant extracts of *Reynoutria* spp. (REGALIA)	28.0	72.0 bc
Control (+)	96.0	

*Mean values with the same letter within a column are not significantly different at the $P \leq 0.05$ probability level by Duncan, DS: Disease severity (%), E: Effect (%)

Table 3. *Effectiveness of different treatments on root length (cm), number of root, plant height (cm) and node number on carnation*

Treatments	RL*	NR*	PH*	NN*
Orange oil extract (PREV-AM)	2.40 bc	11.60 e	22.30 b	7.20 ab
T. harzianum	2.40 bc	17.20 bcd	22.56 b	8.40 a
B. subtilis QST 713 (SERENADE)	**2.82 b**	14.60 bcd	**25.38 ab**	8.20 ab
L. acidophilus + *L. paracasei* (VITANAL)	2.14 bc	18.00 abc	21.30 b	9.00 a
P. fluorescens	1.66 c	14.00 bcd	21.25 b	5.60 b
Mycorrhiza spp., *Trichoderma* spp., *Bacillus* spp (PANORAMIX)	2.54 bc	14.20 bcd	24.46 ab	8.20 ab
Tea tree oil extract (TIMOREX GOLD)	**2.82 b**	**23.20 a**	**31.60 a**	8.20 ab
Plant extracts of *Reynoutria* spp. (REGALIA)	**4.28 a**	15.66 bcd	**26.44 ab**	9.00 a
Control (-)	1.59 c	19.60 ab	22.00 b	8.00 ab

**Mean values with the same letter within a column are not significantly different at the $P \leq 0.05$ probability level by Duncan, RL: Root length (cm), NR: Number of root, PH: Plant height (cm), NN: Node number

Discussion

In this study, *F. oxysporum* f. sp. *dianthi* was significantly reduced by several microorganisms and plant extracts. In the experiment, it was determined that the highest percentage of efficacy values of the biological preparations were for *P. fluorescens*, SERENADE and *T. harzianum* to Fod. In addition, the application of SERENADE, TIMOREX GOLD and REGALIA increased plant height. SERENADE, *T. harzianum* and *P. fluorescens* are very common biocontrol agents against the pathogen. Biocontrol

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bacteria and fungi produce multiple antibiotics that eliminate plant pathogen bacteria and fungi (Strange, 2007; Wang et al., 2016). *Trichoderma* spp. produce various antibiotics, such as viridin, gliotoxin, polyketides and pyrones, against fungal phytopathogens (Howell, 2003; Harman et al., 2004; Naher et al., 2014). *Bacillus* spp. include bacitracin, mycosubtilin polymyxin, bacillomycins and gramicidin and they inhibit fungal germination, suppress of some plant pathogen. One of the most important antibiotics compound produced by *P. fluorescens* is pyrollnitrin, which inhibited growth of some plant fungal pathogens (Karimi et al., 2012; Santoya et al., 2012; Chapelle et al., 2016).

Competition for nutrients and plant surface is another mechanism for plant pathogens. Competitive exception of pathogens as the result of fast colonization of the rhizosphere or plant surface by biological fungus and bacteria may also be an important factor in the control of plant pathogens. Biocontrol species have a higher affinity for nutrients especially iron, phosphorus, nitrogen and can stimulate plant growth directly by or indirectly. They can suppress a broad spectrum of bacterial, fungal and nematode diseases (Jan et al., 2011).

Many biocontrol bacteria and fungi induced systemic resistance which is characterised by a broad spectrum of resistance against pathogens (Conrath et al., 2006; Pieterse et al., 2014; O'Brien, 2017). Beneficial microbes elicite the signalling pathways, and stimulate the host's immune system. Biocontrol bacteria and fungus induced systemic resistance (ISR), may also contribute to disease suppressiveness (Beredsen et al., 2018).

Plant extracts and essential oils appeared to be efficient to control *F. oxysporum* f. sp. *dianthi*. Some research studies reported the efficacy of REGALIA in controlling bacterial spot of tomatoes and peppers (*Botrytis* spp. of grapes and strawberries, powdery mildew of cucurbits, downy mildew of lettuce (*Bremia lactucae*), Cercospora on soybeans (*Cercospora kikuchii*), *Cercospora zeae-maydis; Podospheria leucotricha*, *Venturia inaequalis,* bacterial canker on citrus (*Xanthomonas axonopodis* pv. *citri*), *Xanthomonas campestris* pv. *vesicatoria* and *Xanthomonas euvesicatoria* (Su, 2012; Worthington et al., 2012; Harbou and Jaksen Ziems, 2015; Delong et al., 2018). Many beneficial bacteria and fungi have a general plant growth promoting effect, and produce analogues of plant growth regulatory hormones, volatile compounds to stimulate plant growth. (Harman et al., 2004; Taghavi et al., 2009; Hermosa et al., 2012; Rashid et al., 2012; Truyens et al., 2014). Some beneficial microorganism is able to establish a symbiotic relationship with plants and they increase the availability of these nutrients to plants (Gutiérrez-Luna et al., 2010; Saharan and Nehra, 2011). Its beneficial effects of *Trichoderma* sp. on beside abiotic stress have been well documented (Donoso et al., 2008; Mastouri et al., 2010, Roatti et al., 2013).

In our experiment, the application of TIMOREX GOLD significantly reduced the Fod. TIMOREX GOLD components include cineole cymene, linalool and terpinen-4-ol (Carson et al., 2006; Goni et al., 2014; Wei et al., 2018). The plant oil and plant extracts include secondary chemical compounds such as terpenes, alcohols, aldehydes and phenols, and these materials exhibit fungicidal potential (Zanellato et al., 2009). Essential oil from tea tree oil extract has shown promising results in reducing disease occurrence and severity induced by *Cercospore beticola* in sugar beets, and *Alternari solani* in potato (Caolotanski et al., 2002). It was also reported that tea tree oil was effective against Fusarium head blight in wheat, barley and oats, barley leaf stripe and powdery mildew Terzi et al., 2007). The application of tea tree oil at 2.0% inhibited the

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mycelium growth of *Botrytis oryzae, Alternaria brassicicola, Fusarium moniliforme, Aspergillus flavus, Fusarium proliferatum* (Thobunluepop et al., 2009).

Conclusion

It has been observed that the treatments had a positive effect against the *F. oxysporum* f. sp. *dianthi* and the plant growth of carnation. Biological control method seems to be safe and environmentally friendly and an alternative method with respect to fungicides. Utilization of beneficial microorganisms could be a common agricultural practice in the near future. In addition, biological control methods might be combined with other control methods, but additional research is needed to develop methods of incorporation of biological organisms into other control strategies for the carnation wilt disease management. It is helpful to conduct an extended study that identifies the microorganisms and plant extracts used in the experiment under greenhouse conditions against Fod and carnation plant growth.

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