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Preliminary investigations on suppression of ***Meloidogyne incognita*** (Kofoid & White) Chitwood (Nematoda: Heteroderidae) by antagonistic rhizobacteria*

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Summary

Fluorescent ***Pseudomonad*** strains have been obtained from diseased second juveniles from ***Meloidogyne incognita*** (Kofoid & White, 1919) Chitwood, 1949 (Nematoda: Heteroderidae) infected tomato roots. After the screening in vivo made by 5 different isolated strains, Strain Pat1 has been selected to test the antagonistic effects on the root-knot nematodes. Strain Pat1 has been identified as non plant pathogenic ***Pseudomonas fluorescens*** Migula, 1895 (Proteobacteria: Pseudomonadaceae) according to the test result. Cucumber (***Cucumis sativus***) cv **Sardes** has been used as test plant. Bacterial suspension (10^9 cfu/ml) was applied by three different ways: 1) Seed bacterization; 2) Seed bacterization plus seedling drenching; and 3) Seedling drenching. The eggs of root-knot nematodes have been obtained from diseased tomato roots by blender-sieve method for infection cucumber seedlings with root-knot nematodes. 20 000 eggs/plant have been given to cucumber seedlings during transplanting. The plants have been observed for symptom development for 10-11 weeks in climatized room under controlled conditions. Symptom development has been evaluated by using 0-4 scale for upper parts of plants, and 0-10 Zeck's root-knot scale for the roots. When seed bacterization and seed bacterization plus seedling drenching applications compared with positive control; plants infected with root-knot nematodes alone, it was determined that disease symptoms on upper parts of the plants were reduced 35 % and 21 %; gal formation on the roots were reduced 44 % and 39 %,

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respectively. The experiment has been repeated twice to observe the colonization and population dynamic of antagonistic bacteria on the roots.

Key words: Root-knot nematodes, cucumber, ***Meloidogyne*** spp., antagonistic rhizobacteria, ***Pseudomonas fluorescens***

Anahtar sözcükler: Kök-ur nematodları, hıyar, ***Meloidogyne*** spp., antagonist kök bakterileri, ***Pseudomonas fluorescens***

Introduction

Plants carry a wide range of micro organisms in their phyllosphere and rhizosphere which not only cause large variety of diseases but also control of pathogens. Nematodes have an important niche in agro-ecosystem, causing reduction in plant productivity and growth. Especially Root-knot nematodes (***Meloidogyne*** spp.) are very common and the most important nematode species of greenhouse-growing plants in Southern and Western Anatolia of Turkey (Yüksel, 1974; Elekçioğlu et al., 1994; Kaşkavalcı & Öncüer, 1999).

Indiscriminate use of chemical pesticides causes great harm to human being, animal, vegetation and to environment as a whole due to their non target effect, hazardous nature besides they are expensive. So with the increasing awareness of possible deleterious effects of the chemicals, biological controls of plants pathogen have received considerable attention (Garima et al., 2005). Biological control of soilborne plant pathogens with bacteria has been studied as an alternative or complementary approach to physical and chemical disease control measures for over 70 years (Weller, 1988).

In these bacteria, Plant growth promoting rhizobacteria (PGPR) have been identified as an important biological control agent (Johnsson et al., 1998). Fluorescent ***Pseudomonas*** spp. are among the most effective rhizosphere bacteria in reducing soil-borne diseases in disease suppressive soils, where disease incidence is low, despite the presence of pathogens and environmental conditions conducive to disease prevalence (Weller, 1988). Many species of ***Pseudomonas*** promote plant growth and reduce populations of deleterious rhizoplane fungi and bacteria when used as seed or root inoculants (Schroth & Hancock, 1981). These bacteria can antagonize soil-borne pathogens through various mechanisms (Bakker et al., 1991). For example, bacterial siderophores inhibit plant pathogens through competition for iron (De Meyer & Hofte, 1997), antibiotics suppress competing microorganisms or hydrogen cyanide (Ahl et al., 1986), and chitinases and glucanases lyse microbial cells; and these compounds have been implicated in the reduction of deleterious and pathogenic rhizosphere microorganisms, creating an environment more favorable for root growth (Leong, 1986). Recent studies have demonstrated that some rhizobacteria can also act indirectly by inducing systemic resistance in the plant towards soil-borne fungi and plant-parasitic nematodes (Wei et al., 1996; Hasky-Günther et al., 1998; Reitz et al., 2000; Siddiqui & Shaukat, 2002 a, b).

Antagonistic bacteria have been repeatedly shown to be promising microorganisms for the biological control of plant parasitic nematodes. In a screening

program, 16 bacterial isolates out of 179 isolated from root and cysts caused a significant (>25 %) reduction in ***Globodera pallida*** (Stone, 1973) Behrans, 1975 (Nematoda: Heteroderidae) penetration of potato roots (Racke & Sikora, 1992). A 68% reduction of sugar beet cyst nematode root invasion was obtained by application of the rhizobacterium ***Pseudomonas fluorescens*** Migula, 1895 (Proteobacteria: Pseudomonadaceae) P523 to beet seeds (Oostendorp & Sikora, 1990). Soil application with ***Pseudomonas aeruginosa*** (Schroeter, 1872) Migula, 1900 (Proteobacteria: Pseudomonadaceae) significantly controlled root rot-root knot disease complex in tomato (Siddiqui et al., 2000; Siddiqui & Ehteshamul-Haque, 2000). Efficacy of ***P. fluorescens*** strain CHA0 against root-knot nematodes *(**Meloidogyne javanica*** (Treub, 1885) Chitwood, 1949 (Nematoda: Heteroderidae) and ***Meloidogyne incognita*** (Kofoid & White, 1919) Chitwood, 1949 (Nematoda: Heteroderidae) has been reported for certain crops including tomato (Siddiqui & Shaukat, 2002 a, b; Hamid et al., 2003).

Since soilborne root-infecting fungi and plant-parasitic nematodes are the common inhabitants of almost all the agricultural fields causing severe losses to crops, agents having the capabilities of controlling these diverse groups of pathogens could be of practical significance. Present study was carried out to assess the biocontrol potential of ***P. fluorescens*** against cotton root-knot nematode (***M. incognita***).

Material and Methods

Pot culture experiments on the management of cotton root-knot nematode (***M. incognita***) on cucumber (***Cucumis sativus***) cv **Sardes** through biocontrol agents ***P. fluorescens*** were conducted during November 2005-July 2006 in climatized room under controlled conditions (25±5°C, 55 % humidity, 16:8 h day light) at Plant Protection Department, Agricultural Faculty, Ege University.

Fluorescent ***Pseudomonad*** strains have been obtained from diseased ***M. incognita***'s second juveniles in the root-knot nematode infected tomato roots. After the screening in vivo made by 5 different isolated strains, Strain Pat1 has been selected to test the antagonistic effects on the root-knot nematodes. Strain Pat1 has been identified as non plant pathogenic ***P. fluorescens*** according to the LOPAT test results (Fahy & Persley, 1983; Lelliot & Stead, 1987; Klement et al., 1990).

Cucumber (***Cucumis sativus***) cv Sardes known as susceptible to root-knot nematodes has been used as test plant and the seedlings of them were planted in 2 kg capacity pots filled with sterilized sandy loam soils.

The eggs of ***M. incognita*** have been obtained from infested tomato roots by blender-sieve method for infection cucumber seedlings with root-knot nematodes. Cucumber seedlings were inoculated with 20 000 eggs of ***M. incognita*** per plant during transplanting.

Bacteria were routinely cultivated in nutrient broth glycerole agar (NGA). Bacteria were grown in 250 ml erlenmeyer flasks containing 100 ml King's B liquid

medium (KBLM; King et al., 1954) at 24^0C for 24 hours with shaking (140 rpm). The bacterial culture was centrifuged at 2800 x g for 20 min, the supernatant discarded and the pellet resuspended in $MgSO_4$ (0.1 M) (Mercado-Blanco et al., 2004).

Bacterial suspension (10^9cfu/ml) was applied by three different ways:

1) Seed bacterization (5 g seed/5 ml suspension);
2) Seed bacterization plus seedling drenching;
3) Seedling drenching.

Control negative (without root-knot nematodes and rhizobacteria) and control positive (only infested with root-knot nematodes) characters were also placed in the experiment. The experiment has been repeated two times: First trial was done the date between 18.11.2005-02.02.2006 and second trial was done the dates between 16.05.2006-14.07.2006. Eight replications were maintained for each treatment and the pots were arranged in a randomized complete block design. The plants had been observed for symptom development for 10-11 weeks in climatized room under controlled conditions. Symptom development has been evaluated by using 0-4 scale for upper parts of plants, and Zeck's (1971), 0-10 gall mass index for the roots. Wilt development on each plant was rated using the following scale (0-4) (Table 1):

Table 1. Wilt symptoms scale for evaluating the affect of ***Meloidogyne incognita*** (Kofoid & White) Chitwood on the cucumber plants

Wilt symptoms scale	Symptoms
0	Healthy
1	¼ of plants were wilted and withered
2	½ of plants were wilted and withered
3	¾ of plants were wilted and withered
4	Completely breakdown, dried and dead plants

The disease index was calculated from the disease rating by the formula:

$$Disease\ \ index = \frac{\sum (Rating\ number \times Number\ of\ \ plants\ in\ the\ rating)}{Total\ number\ of\ \ plants \times The\ highest\ rating} \times 100$$

Root colonization assay

An experiment was conducted to determine the ability of Str-mutant of ***P. fluorescens*** strain Pat1 to colonize cucumber roots. Bacterial suspension (10^9cfu/ml) was applied by seed bacterization (5g seed/5 ml suspension) and seed bacterization plus seedling drenching as mentioned above. There were five replicated plants for bacterial treatment in a randomized complete block design. Plants were incubated under climatized conditions 25±5°C for 60 days. To determine colonization of root tissue by bacteria, plants were uprooted from pots

and the root systems were washed under running tap water, dried with sterile filter paper and cut into 1 cm-long pieces with 20 days intervals. For each treatment, samples of 0.5g of root pieces were ground in 49.5 ml 1M $MgSO_4.7\ H_2O$. Serial dilutions were plated onto modified KB amended with 100μg Str ml^{-1} and incubated 25°C for 48 h. Then, bacterial colonies were counted and bacterial populations were expressed as colony-forming units (cfu) g^{-1} of fresh root tissue (Stockwell et al.,1998)

Statistical analyses

The data were analyzed by ANOVA using SPSS version 12 statistical software (SPSS Inc. Chicago, Illinois). Before the analyses were carried out, data on percentage of wilt severity were transformed using the arcsin transformations (arcsin of the square root). Also, the data of gall mass index were transformed by using log10 (X+1) transformation prior to statistical analyses. Differences between treatments were determined by Duncan's Multiple Range Test (DMRT) at 5% significance level.

Results and Discussion

The wilt incidence was recorded at 60 d after transplanting. The results are given on Table 2. In the first pot trial, seed-bacterization by strain Pat 1 of ***P. fluorescens*** significantly reduced the percentage of wilt severity caused by ***M. incognita*** of cucumber by 37,49 %, compared to the Control Positive treatment that was treated with root-knot nematodes alone, in 60 d after transplanting of seedlings (Table 2). Seed bacterization + seedling drenching of biocontrol agent resulted in lower suppression of ***M. incognita*** (29,16 %) (Table 2). In the second pot trial, the efficacy of both treatments on wilt incidence of cucumber plants were found similar and to be slightly less effective than results obtained in the first trial (Table 2).

Table 2. The efficiency of treatments of ***Pseudomonas fluorescens*** Migula Pat1 on wilt development of cucumber plants infected by ***Meloidogyne incognita*** (Kofoid & White) Chitwood

Treatments	1st Trial			2nd Trial		
	Wilt Incidence (%)		Efficiency (%)**	Wilt Incidence (%)		Efficiency (%)
Seed bacterization	46,88	A*	37,49	31,25	AB	23,06
Seed bacterization + seedling drenching	53,13	A	29,16	31,25	AB	23,06
Seedling drenching	65,63	A	12,49	-		-
Control Positive	75,00	A	-	40,63	B	-
Control Negative	40,63	A	45,83	9,38	A	76,91

* Means followed by the same letter are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

** Percentage of reduction in wilt severity compared to the root-knot nematode alone.

The efficacy of treatments of ***P. fluorescens*** strain Pat1 on gall development of cucumber plants infected ***M. incognita*** is shown on Table 3. When seed bacterization and seed bacterization plus seedling drenching applications compared with positive control; gal formations on the roots were reduced 43,46 % and 38,45 %, respectively.

Population dynamics of *P. fluorescens* **strain Pat 1 on cucumber roots**

The experiment has been repeated twice to observe the colonization and population dynamic of antagonistic bacteria on the roots. At the first sampling time, Pat 1 $^{strR+}$ was recovered at mean detectable population sizes of 10^5 CFU per gram fresh root tissue. The mean detectable population size on the roots applied seed bacterization varied from $2x10^5$ to $9x10^5$ CFU per g root within 20 days (Fig.1). The population dynamics of Pat 1 $^{strR+}$ on cucumber roots which were treated by seed bacterization + seedling drenching varied from $6x10^5$ to $5x10^6$ CFU per g root within 20 days and were found more promising than only seed bacterization treatment (Fig.1). The mean population size of Pat 1 $^{strR+}$ on cucumber roots for two different treatments increased 10-fold within 30 days after transplanting. ***P. fluorescens*** strain Pat 1 $^{strR+}$ was effectively colonized on the cucumber roots for the both treatments.

Table 3. The efficiency of treatments of ***Pseudomonas fluorescens*** Migula Pat1 on gall development of cucumber plants infected by ***Meloidogyne incognita*** (Kofoid & White) Chitwood

Treatments	1st Repetition			2nd Repetition		
	Average Gal Index		Efficiency (%)**	Average Gal Index		Efficiency (%)*
Seed bacterization	4,75	B*	43,46	1,87	B	68,20
Seed bacterization + seedling drenching	5,17	B	38,45	2,36	B	62,48
Seedling drenching	6,83	C	18,69	-		-
Control Positive	8,40	C		6,29	C	-
Control Negative	0,00	A		0,00	A	-

*Means followed by the same letter are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

**Percentage of reduction in wilt severity compared to the root-knot nematode alone

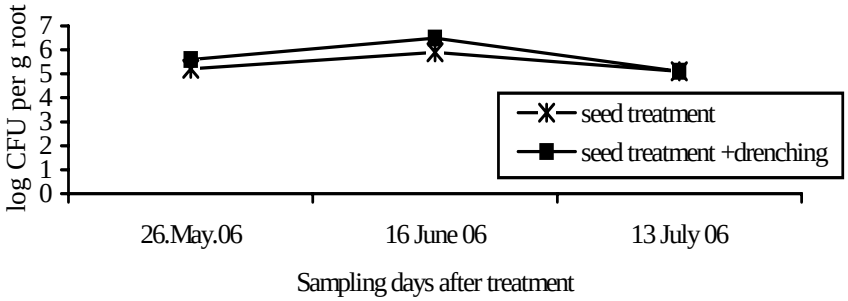

Figure 1. Mean population sizes of ***Pseudomonas fluorescens*** Migula Pat1 $^{strR+}$ on cucumber roots.

The results of the present study agree with the earlier findings by several investigators in crops such as tomato (Jothi & Sivakumar, 2003; Verma, 2005), beet seeds (Oostendorp & Sikora, 1990), certain crops including tomato (Hamid et al., 2003; Siddiqui & Shaukat, 2002 a, b).

Such enhanced nematode suppression and resultant improvement in plant growth in cucumber due to soil application of ***P. fluorescens*** may be due to the enhanced root colonizing ability of ***P. fluorescens***. Supporting this view, Shanthi & Sivakumar (1995) had related the nematode suppressing ability of Pseudomonads bacterial strains to their rest colonizing ability. Also similarly, Ramakrishnan et al. (1998) had viewed that the nematode suppressing ability of ***P. fluorescens*** strains related mainly to their root colonizing ability. Also, our results on population dynamics of ***P. fluorescens*** strain Pat1 confirmed the mentioned findings.

The possible reason of this result could be the rapid multiplication and colony formation of ***P. fluorescens*** in soil as it is most conducive substrate with adequate organic matter for their development. Further soil application could help for easy colonization in the root system.

Because the rhizosphere provides the front line of defense for roots against attack by pathogens, microorganisms that can grow in the rhizosphere are ideal for to use as biocontrol agents. These rhizosphere microorganisms encounter the pathogens during primary infection and also during second spread on the roots.

Özet

Meloidogyne incognita **(Kofoid&White) Chitwood (Nematoda: Heteroderidae)'nın antagonist kök bakterileri ile mücadelesine yönelik ön çalışmalar**

Floresan ***Pseudomonad*** ırkları nematod ile bulaşık domates köklerinde bulunan hastalıklı ***Meloidogyne incognita*** (Kofoid & White, 1919) Chitwood, 1949 (Nematoda: Heteroderidae) 2. dönem larvalarından elde edilmiştir. In vivo'da 5 farklı ırk izole edilmiş, Kök-ur nematodlarına antagonist etkilerini test etmek amacıyla bu ırklar içinden patojen olmadığı tespit edilen ***Pseudomonas fluorescens*** Migula, 1895 (Proteobacteria: Pseudomonadaceae) Strain Pat1 seçilmiştir. Denemelerde hıyar (***Cucumis sativus***) cv **Sardes** test bitkisi olarak kullanılmıştır. Bakteri süspansiyonu (10^9 cfu/ml) üç farklı şekilde uygulanmıştır: 1) Tohum bakterizasyon; 2) Tohum bakterizasyon ve fide içirme ve 3) Fide içirme. Hıyar bitkilerinin fidelerini kök-ur nematodları ile bulaştırmak için, kök-ur nematodlarının yumurtaları bulaşık domates bitkilerinin köklerinden blender-elek yöntemiyle elde edilmiştir. Şaşırtma esnasında hıyar fideleri 20 000 yumurta/bitki ile bulaştırılmıştır. Bitkiler, kontrollü koşullara sahip olan klimatize edilmiş odalarda belirti gelişimi için 10–11 hafta gözlenmiştir. Belirti gelişimi, bitkilerin toprak üstü kısımları için 0–4 skalası, kökleri içinde 0–10 Zeck Kök-ur skalası kullanılarak değerlendirilmiştir. Tohum kaplama ile tohum kaplama ve fide daldırma, sadece kök-ur nematodları ile bulaşık bitkilerin bulunduğu pozitif kontrol ile karşılaştırıldığında, bitkilerin toprak üstü kısımlarındaki hastalık gelişiminin sırasıyla % 35 ve % 21, köklerdeki ur oluşumunun % 44 ve % 39 oranlarında düştüğü belirlenmiştir. Deneme, köklerdeki antagonist bakterilerin kolonizasyonu ve populasyon dinamiğini gözlemek amacıyla iki kez yinelenmiştir.

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