African Journal of Biotechnology Vol. 10(33), pp. 6235-6240, 6 July, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2691
ISSN 1684-5315 © 2011 Academic Journals

Full Length Research Paper

Determination of mycoflora of pea (*Pisum sativum*) seeds and the effects of *Rhizobium leguminosorum* on fungal pathogens of peas

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Accepted 29 April, 2011

Mycoflora of pea seeds and the effectiveness of *Rhizobium leguminosarum* on important seed-borne pathogens were determined. Mycoflora were changed according to seed groups with or without surface sterilization. The most common isolated fungi were *Fusarium* spp., *Alternaria* spp., *Macrophomina phaseolina, Phytophthora megasperma*, *Rhizoctonia solani* and *Sclerotium rolfsii. R. leguminosarum* showed antagonistic effect on *Fusarium oxysporum* and *P. megasperma* by 66.3 and 62.1%, respectively, in dual culture test *in vitro*. *R. leguminosarum* application reduced disease severity of *P. megasperma*, *R. solani*, *F. oxysporum* and *S. rolfsii* by 52.9, 50, 67.9 and 52%, respectively. Results indicated that *R. leguminosarum* showed effects on plant pathogenic fungi and could be used as biocontrol agent.

Key words: Seed mycoflora, dual culture, *Rhizobium leguminosarum*, biocontrol pathogenic fungi, disease severity.

INTRODUCTION

Pea (*Pisum sativum*) which is the most widely grown legume in Turkey is consumed when dried, fresh or frozen. Production of peas has been restricted due to various reasons. Seed and soil-borne diseases are considered as important factors for yield loss. Nowadays, different non-chemical approaches are adopted in disease management which has benefits to plant growth by using microorganisms (Dar et al., 1997). Particularly, the establishment of beneficial bacteria on root system has long been of major interest in agricultural production. *Rhizobium* spp. are symbiotic microorganisms that form nodules fixing of free nitrogen of air in roots of legumes and improve soil fertility.

The rhizobial bacteria species including *Rhizobium leguminosarum* is able to maintain long-term viability in natural conditions and can be easily established around the plant roots (Jones et al., 2007). It has been reported that symbiotic relationship enhance plant growth as in the case of inoculation of *Rhizobium* species which affected seed germination and promoted the plants growth under controlled and field conditions (Pena-Cabriales and Alexander, 1983; Önder and Özkaynak, 1994; Chabot et al., 1996; Noel et al., 1996). Antoun et al. (1998) revealed that *Rhizobium* and *Bradyrhizobium* species had a potential of growth promoting rhizobacteria on even non-legumes, especially radishes. *Rhizobium species* are known to affect the plant growth directly by producing phytohormones like indole acetic acid (Wang et al., 1982; Wiehe et al., 1994). It was also reported that *Rhizobium* species increased tolerance to abiotic stress such as drought (Athar, 1998). In addition, different studies revealed that inoculation with *Rhizobium* species increased plant tolerance to some plant pathogens (Rabie, 1998; Sturz et al., 2000; El-Batanony et al., 2007; Huang and Erickson, 2007; Mazen et al., 2008).

In this context, it is important to control these diseases timely, especially seed-borne ones and it is necessary to develop different control strategies such as supportive and traditional methods. The aim of this study was to determine the seed-borne fungal pathogens in peas and to evaluate the effects of *R. leguminosarum* on some of these seed-borne fungal pathogens in controlled

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conditions.

MATERIALS AND METHODS

Mycoflora isolations were performed using four different groups of seeds. Peas (*P. sativum*) cv Araka was used in all experiments. *R. leguminosarum* was isolated from nodules of bean root according to Mostasso et al. (2002) for effective studies.

Mycoflora isolations and identifications in pea seeds

Potato dextrose agar (PDA) was used for fungi isolations. Four different groups of seeds were divided into two parts and thirty seeds were randomly selected in each. One group of seed was cultured on PDA directly without surface sterilization using 5 seed for one Petri dish with 6 replicates and the other groups were surface sterilized with 1% NaOCl for 5 min. Cultures were incubated for one week at 24°C. Number of fungal colonies were determined and rates of isolation were calculated for each species. Fungal colonies were identified using macroscobic and microscobic criteria (Alexopoulos, 1966; Nelson et al., 1983; Barnett and Hunter, 1998; Simmons, 2008).

Pathogenicity tests

Pea seeds were sown in 15 cm diameter pots containing autoclaved growing medium with a mixture of soil, sand and pumice (1/1/1, v/v/v). Inoculation procedure were performed for *Fusarium* spp. using wheat culture of 5 g, for *Phytophthora megasperma,* zoospore suspension of 10 ml in each pot, for *Rhizoctonia solani*, sand-corn meal medium of 5 g, for *Sclerotium rolfsii* and sclerot directly (50 to 55 mg) for each pot. *Macrophomina phaseolina* was maintained on PDA for 10 days, spore suspension was prepared from formed microslerote with 10^6 microsclerotes ml^{-1} and 10 ml suspension was added to each pot. After maintaining on PDA, *Alternaria, Aspergillus* and *Penicillium* species were inoculated, preparing spore suspensions with 10^6 spores ml^{-1} and 10 ml in each pot.

In pathogenicity test, 5 pots were used for fungal agents with 3 seeds in each. Control plants with only the seed planted were included in the test and same maintenance procedures were applied. Pots were placed at 24 ± 2°C with 16 h photoperiod in a growth room. Seed germination rate, dead plants in subsequent periods, non-germinated seeds and necrosis on root and crown were recorded.

Dual culture tests

Dual culture tests consisted of two parts which are reciprocal culturing and volatile compond test. Effect of *R. leguminosarum in vitro* were performed on PDA in 9-cm Petri plates by applying a dual culture technique (Sadfi et al., 2001; Fakhrunnisa and Ghaffar, 2006). *R. leguinosarum* were streaked across the centre of the plate, with a second streak made at right angles to the first. Four discs, 5 mm in diameter cut from the edge of a 7 day-old culture of *P. megasperma*, *R. solani, Fusarium oxysporum* and *S. rolfsii* were placed at each side of the *R. leguminosarum* and 2.5 cm distance was left between the two microorganisms. Plates were incubated at 25°C for one week. Percent growth inhibition of fungal agents after 7 days was calculated by the formula (R1 - R2) / R1 x 100, where, R1 is the fungal radial growth (mm) in the direction opposite to the antagonist and R2 is the radial growth toward the antagonist (Whipps, 1987). Growth inhibition (GI) was measured on a scale of 0 to 3 (Korsten et al., 1995), where 0 = no growth inhibition, 1 = 1 to 25% growth inhibition, 2 = 26 to 50% growth inhibition and 3 = 51 to 75% growth inhibition.

To determine the effect of volatile compounds, sealed plate method was used (Fernando et al., 2005). *Rhizobium* isolate was maintained in yeast mannitol agar and incubated at 25°C for two days. *P. megasperma*, *R. solani, F. oxysporum* and *S. rolfsii* were maintained on PDA in the centre of the plate with a 6 mm disc in 9 cm diameter Petri dishes. Petri dish containing microorganisms were inverted and placed over the rhizobial culture. The two plates were sealed together with parafilm and incubated at 25°C. Petri dishes containing yeast mannitol agar without *Rhizobium* was placed over the PDA medium inoculated with the microorganisms as a control. The experiment was conducted with 5 replications. The radial growth was measured after five days.

Pot experiment

Pea seeds were placed on the two layers of blotting paper moistened with sterile distilled water in Petri dishes and it facilitated germination at 24°C. The mixture of soil : sand : pumice (1:1:1, v:v:v) used as growing medium was autoclaved at 121°C and 1 kPa twice for one hour each time. Germinated seeds were sown in 15 cm diameter pots including growing medium. Pots were placed at 24 ± 2°C with 16 h photoperiod in a growth room.

R. leguminosarum was cultured on yeast mannitol agar (YMA) at 24°C for one week (Ames-Gottfred et al., 1989). Bacterial suspension was prepared using *Rhizobium* cultures and the concentration was adjusted to 10^6 cells ml^{-1} using a spectrophotometer, and 10 ml suspensions were applied to the pots. *R. leguminosarum* was applied to the soil around the roots immediately after 10 days culture of the plant.

Inoculation procedures following fungal agents

P. megasperma was grown on PDA at 28°C for seven days and placed under fluorescent light for sporulation. Culture plates were incubated in sterile distilled water for 40 min at 4°C then for 30 min at room temperature. Zoospores were collected by filtering the suspension and zoospore concentration was adjusted to 10^6 spores ml^{-1}. A 10 ml suspension was applied to the soil around the root (Sunwoo et al., 1996). Symptoms were evaluated 10 days after inoculation, based on a 0 to 5 scale (Andres et al., 2006) where: 0 = no visible disease symptom, 1 = brownish lesion beginning to appear on stems and approximately 30% of the entire plants were diseased, 2 = 31 to 50% of the entire plants were diseased, 3 = 31 to 50% of the entire plants were diseased, 4 = 71 to 90% of the entire plants were were diseased, 5 = plant died.

F. oxysporum was grown at 25°C for 7 days on PDA and for inoculation, wheat culture was prepared. Grains were boiled to soften, and then cooled and 200 g amounts were placed in Erlen mayer and sealed with cotton and aluminium foil. Grains were autoclaved for 30 min. After cooling, the 1 cm diameter 5 mycelial discs of *F. oxysporum* were placed on medium and incubated at 25°C for 15 days. Application of *Fusarium* wheat culture provided enough mycelial development using 5 g in each pot. *F. oxysporum* wilt symptoms was evaluated using 0 to 4 scale according to Kraft and Boge (2001), where: 0 = no disease symptoms, 1 = epinasty, shedding of primer leaves, 2 = wilting at second and third leave stage, 3 = wilting of entire plant, 4 = dead plant.

R. solani was maintained on PDA at 25°C for 7 days. For inoculation, sand-corn meal medium was used (Porter and Merriman, 1983). Sand-corn meal medium included 237 g of sand, 13 g of corn meal and 50 ml of distilled water in 500 ml Erlenmayer flasks and it was autoclaved. After cooling, five mycelial discs of *R. solani* were placed in the medium and incubated for 3 weeks at

Table 1. Fungal flora of pea seeds without surface sterilization and rate of isolations (%).

Isolated fungi	Isolation rate (%)			
	Group 1	Group 2	Group 3	Group 4
Fusarium spp.	45	30	40	20
Penicillium spp.	10	12	9	19
Aspergillus spp.	14	21	16	16
Rhizopus spp.	1	3	1	2
Alternaria spp.	15	20	18	18
Yeasts colonies	7	9	6	11
Bacteria colonies	8	5	10	14

25°C. Five grams of the medium was applied around roots for each pot. For evaluation of disease symptoms, 0 to 5 scale was used (Mc Coy and Kraft, 1984), where: 0 = no visible symptoms, 1 = <25% disease of entire plant, 2 = 25% diseased plant, 3 = 25 to 50% diseased plant, 4 = 50 to 75% diseased plant, 5 = >75% diseased plant.

S. rolfsii was maintained on PDA which facilitated the formation of sclerot. After 3 weeks, selected sclerots were added to soil (25 to 30) (approximately 50 to 55 mg). For evaluation of disease symptoms. A 0 to 5 scale was used (Lotunde-Dada 1993), where: 0 = no visible symptom, 1 = a small number of leaf wilt symptoms in plants, 2 = slight infection, mycelial mass only on the surface of the soil, 3 = moderate infection, wilting and blight, mycelial mass around stem, 4 = severe infection, advanced wilt, sclerot forming around crown, 5 = dead plant.

The following treatments with four replications and five plant in each treatment were used according to completely randomized block design: *R. leguminosarum* (Rl), *F. oxysporum* (Fo), *P. megasperma* (Pm), *R. solani* (Rs), *S. rolfsii* (Sr), *F. oxysporum* + *R. leguminosarum* (Fo + Rl), *P. megasperma* + *R. leguminosarum* (Pm + Rl), *R. solani* + *R. leguminosarum* (Rs + Rl), *S. rolfsii* + *R. leguminosarum* (Sr + Rl), and control (C).

Diseases index was calculated using scale values. The data were subjected to analysis of variance (F-test). Means were compared using Fisher's LSD test at P = 0.05 (Gomez and Gomez, 1983).

RESULTS

Fungal flora of seeds

Isolations were performed to determine the origin of the fungal flora in pea seeds with or without surface sterilization. Fungal profile and ratios were changed in seeds with or without surface sterilization. The most commonly isolated fungi were *Fusarium* species from seeds placed on PDA directly without surface sterilization for all groups with high ratio (Table 1), followed by *Alternaria* species, the other isolated fungal species belonged to the genus *Penicillium, Aspergillus* and *Rhizopus*. Apart from these fungal species, yeast and bacteria colonies were counted and determined for these seeds on the surface microflora.

Different fungal species were obtained from seeds with surface sterilization (Table 2), however, genus *Fusarium* was the most isolated. The other species isolated include *Phytophthora* sp., *S. rolfsii, R. Solani* and *Macrophomina* sp.

Fusarium species were identified as *F. Oxysporum* and *F. solani.* Chromist *Phytophthora* sp obtained from two groups of seeds were identified as *P. megasperma*. Three *Aspergillus* spp. were obtained and determined as *Aspergillus niger, Aspergillus flavus* and *Aspergillus ochraceus.* All examined *Alternaria* colonies isolated from the all groups of seeds were identified as *Alternaria alternata*.

Pathogenicity tests

Pathogenicity tests were conducted using pea seeds and evaluating seed germination and symptoms in plant after germination, and results are summarized in Table 3. Ratio of germinated seeds and infected plants were recorded. *F. oxysporum* and *F. solani* influenced the germination of seeds by 46.6 and 33.3% in plants and caused infection in plants by 53.3 and 50%, respectively.

R. solani and *S. rolfsii* also reduced the rate of seed germination by 66.6% and germinated seeds also had infection depending on the disease development. *Alternaria* species were isolated from all seed samples and reduced the seed germination, and caused the disease by 26.6 and 20%, respectively.

Aspergillus spp. and *Penicillium* spp showed signs of infection in pea plants, but a negative impact on seed germination was not observed in these plants. Seeds germination rate was only 20% for both fungal species.

In vitro studies

R. leguminosarum tested were the most effective in dual culture against *F. oxysporum* and *P. megasperma* with 66.3 and 62.1% growth inhibition belonging to scale 3, respectively. The effectiveness was found to be lower than *R. solani* and *S. rolfsii* belonging to scale 2 and 1, respectively (Table 4). In volatile compound test, *R.*

Table 2. Fungal flora of pea seeds with surface sterilization and rate of isolations (%).

Isolated fungi	Isolation rate (%)			
	Group 1	Group 2	Group 3	Group 4
Fusarium spp.	65	67	60	45
Phytophthora sp	7	-	5	-
Sclerotium rolfsii	5	2	-	-
Rhizoctonia solani	5	7	10	8
Alternaria spp.	5	5	8	3
Macrophomina phaseolina	-	5	-	7
Aspergillus spp.	3	6	5	10
Others	10	8	12	27

Table 3. Summary of fungal inoculation test.

Inoculated fungi	Seed germination rate (%)*	Infected plant (%)	Symptom
Fusarium oxysporum	46.6	53.3	Dead plants
F. solani	33.3	50	Yellowing and wilting
Phytophthora megasperma	33.3	66.6	Yellowing and wilting
Rhizoctonia solani	66.6	33.3	Dead plants
Sclerotium rolfsii	66.6	33.3	Dead plants
Machrophomina phaseolina	33.3	26.6	Necrosis on root and stem
Alternaria alternata	26.6	20	
Aspergillus spp.	20	-	-
Penicillium spp.	20	-	-
Control (-)	10	-	-

*The seed germination rate were evaluated using 15 seeds and ratio of infected plants were determined after germination of seeds.

Table 4. Antagonistic effect of *R. leguminosarum* on fungal pathogens in dual culture.

Fungi	Growth inhibion (%)	GI value	Effect of volatile compound (%)
Pm	62.1	3	44.4
Rs	57.4	2	40.9
Fo	66.3	3	63.2
Sr	42.5	1	43.75

Pm, *P. Megasperma*; Rs, *R. Solani*; Fo, *F. Oxysporum*; Sr, *S. Rolfsii*.

leguminosarum showed maximum effect on *F. oxysporum* by 63.2%. The effects on the other microorganisms changed between 40.9 and 44.4%.

Determination of the effects of *R. leguminosarum* on fungal pathogen under pot conditions

The effects of *R. leguminosarum* on fungal pathogens are summarized in Table 5. The disease severity of *F. oxsporum* and *P. megasperma* inoculated plants were 70 and 68%, respectively. For Rhizobium treated plants, the disease severity was lower with 32 and 22.5%, respectively. The disease severity of *R. solani* and *S. rolfsii* were 60 and 50%, respectively, while in *Rhizobium* treated plants, the disease severity decreased to 30 and 24%.

DISCUSSION

Fusarium species were found to be importantly obtained from seeds cultured on PDA directly and with surface sterilisation in isolations. Saprophyte flora were also obtained from the surface of seeds. More pathogenic fungi were isolated from the seeds with surface sterilization and they include, *R. solani, S. rolfsii* and a chromist *P. megasperma* in addition to *Fusarium* species. In pathogenicity test, *A. alternata*, *Aspergillus* spp. and *Penicillium* spp. were non-pathogenic, whereas *F. oxysporum* and *M. phaseolina* were pathogenic. *R. solani, P. megasperma* and *S. rolfsii* showed typical

Table 5. The effects of *R. leguminosarum* on diseases in peas.

Treatment	Disease index	Disease severity (%)	Effect (%)*
Pm	3.4	68	-
Pm + Rl	1.6	32	52.9b
Rs	3.0	60	-
Rs + Rl	1.5	30	50b
Fo	2.8	70	-
Fo + Rl	0.9	22.5	67.9a
Sr	2.5	50	-
Sr + Rl	1.2	24	52b

Pm, *P. Megasperma*; Rs, *R. Solani;* Fo, *F. Oxysporum*; Sr, *S. Rolfsii*.
*Means within column followed by different letters are significantly different (P = 0.05) according to Fisher's LSD test.

symptoms on plants. Esen and Erkiliç (2004) determined the fungal flora of first and second crops of soybean, showing that *Fusarium verticilloides* (*Fusarium moniliforme*), *Fusarium avenaceum, A. alternata*, *A. niger, A. flavus, A. ochraceus* and *Penicillium* spp. were obtained by isolation from harvested seeds. According to pathogenicity tests, *Aspergillus* spp. and *Penicillium* spp were not pathogenic, however, seed germinated and plant growth was adversely effected by fungus. Results indicated that *Fusarium* spp. and *M. phaseolina* were the potential pathogens in Çukurova soybean growing areas (Esen and Erkilic, 2004).

In this study, *R. leguminosarum* was the most effective on *F. oxysporum* and *P. Megasperma*, reducing the mycelial growth *in vitro*. Similarly, volatile compounds of *R. leguminosarum* had the same effect on both pathogens. Volatile compound had certain effect against *S. rolfsii* in contrast to lower effect of dual culture. Arfaoui et al. (2006) revealed that some isolates of *Rhizobium* were more effective against *F. oxysporum* f.sp *ciceris* of chickpea with 60% growth inhibition *in vitro*. In another study, *Rhizobium* spp. reduced the mycelial growth of *F. oxysporum* f.sp *phaseoli in vitro* (Buonasisi et al., 1986). *Rhizobium meliloti* inhibited growth of *M. phaseolina, R. solani* and *F. Solani*, while *Bradyrhizobium japonicum* inhibited *M. phaseolina* and *R. Solani*, producing zones of inhibition *in vitro* (Ehteshamul-Haque and Ghaffar, 2008). *Rhizobium* isolates were effective not only for soil borne pathogen but also, the leaf disease as indicated *in vitro* antibiosis assays that showed that *Rhizobium* strains from chickpea nodules are effective via antagonism against blight disease of chickpea caused by *Ascocyhta rabiei* (Küçük and Kivanç, 2008).

In this study, *Rhizobium* application reduced the disease severity of all tested fungi under pot conditions. These results are similar to those obtained by Buonassisi et al. (1986) which indicated that *Rhizobium* spp. reduced the disease severity of *F. oxysporum* f.sp *phaseoli* in bean. *Rhizobium* can be effective when it is not alone and also the increasing effect was obtained when dual inoculation was done with mcorrhizal fungi and other beneficial microorganisms (Rabie, 1998). Another study was conducted under field conditions, and *R. meliloti, R. leguminosarum* and *B. japonicum* were used either as seed dressing or as soil drench to reduce infection of *M. phaseolina, R. solani* and *Fusarium* spp., in legumes such as soybean, mungbean and non-legumes such as sunflower and okra (Ehteshamul-Haque and Ghaffar, 2008). In addition, Sheikh et al. (2006) reported that *Bacillus thuringiensis* and *R. meliloti* with and without available nursery fertilizers were used to study their effect on the growth and suppression of soil borne root infecting fungi which include *M. phaseolina*, *R. solani* and *Fusarium* spp., on mungbean and okra plants, and applications gave a significant decrease in infection by root infecting fungi by providing better plant growth. In another study, *R. meliloti* and *R. leguminosarum* had complete control against *Fusarium* wilt in bushbean (Khalequzzaman and Hossain, 2007).

Consequently, *Fusarium* species were isolated predominantly and may also be important in field conditions with *P. megasperma*, *R. solani* and *S. rolfsii* from pea seeds. Existing *R. leguminosarum* isolate was significantly effective against pathogens *in vitro* and in pot conditions.

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