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Inoculation Techniques for Assessing Pathogenicity of *Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium oxysporum* and *Fusarium solani* on Pepper Seedlings*

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Abstract: In this study, surveys were carried out during 2015 and 2016 for wilt and root rot diseases caused by *Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium oxysporum*, and *Fusarium solani* in pepper fields in Adıyaman, Diyarbakır, Mardin and Şanlıurfa provinces of Turkey. The purpose of this study was to evaluate the effects of different inoculation methods (root dip, soil infestation with wheat bran and soil infestation with rice grain) on pathogenicities of *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani* on pepper seedlings. Inoculated pepper seedlings (cv. İnan-3363) were left to grow for three months after transplanting under growth chamber conditions. Inoculation of infective rice-grain was used to test pathogenicity of all four fungi. Root dip inoculation method was used for *F. solani* and *F. oxysporum* when the soil was infested with wheat bran method for *R. solani* and *M. phaseolina* inoculation. All tested isolates resulted in the stem and root rot, leaf chlorosis and bruising. To test the pathogenicity of fungi, soil infestation with rice grain inoculation was the most suitable method. All tested fungi induced similar foliar symptoms, root rot severity and caused a similar reduction in dry root weights when rice-grain inoculum was used. With other inoculation methods, all pathogens similarly affected root rot severity. Whereas, *F. oxysporum* was the least virulent pathogen among tested fungi affecting foliar symptom severity; for fresh root and plant weights, and dry root and plant weights; *R. solani*, *M. phaseolina* and *F. solani* were similarly virulent when these parameters were used. The results of the present study may have a useful connotation to monitor pepper seedlings against these pathogens. In conclusion, we recommend rice-grain inoculation to test pathogenicities of *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani*on various pepper cultivars.

Keywords: Pathogenicity, soil-borne pathogens, pepper

1. Introduction

Turkey is the world's third largest pepper producing country after Mexico producing 2.072.132 tones (Anonymous, 2012). Soil-borne pathogens such as *Rhizoctonia solani* Kühn., *Macrophomina phaseolina* (Tassi) Goid. *Fusarium solani* (Mart.) Sacc., and *F. oxysporum* Schlecht. in association with wilt are recurring problems in the pepper production regions of Adıyaman, Diyarbakır, Mardin, and Şanlıurfa, *Capsicum annuum* L. is a commercial spice crop cultivated in Turkey. The economic significance of sweet pepper cultivation in the world could be explicated by its superior nutritional value of vitamins, antioxidants, and some other compounds. Therefore, improving the yield of this crop is one of the objectives in agriculture in many countries (Akram et al., 2013).

Pepper is exposed to many pathogenic organisms wherever it is cultivated. Many soil-borne fungal root rot and wilt pathogens such as *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani* are reported to be widespread and attack pepper roots and stem causing severe losses in seed germination, plant growth and yield.

R. solani induces disease by tainting roots and stems from which it attains nutrients. It has a broad

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host range including pepper (Velásquez et al., 2001). It survives in soil by colonizing organic material and producing sclerotia and is an aggressive pathogen to young pepper plants and a minor pathogen to older plants causing root or crown rot (Sneh, 1991; Hane et al., 2014; Wang et al., 2015).

M. phaseolina is one of the most disruptive seed and soil-borne pathogens causing diseases in a wide range of hosts (Chidambaran and Mathur, 1975; Dhingra and Sinclair, 1977; Reuveni et al., 1983). It causes charcoal rot disease in water-deficient areas of the world (Das et al., 2008). It produces black sclerotia (Wheeler, 1975) and its occurrence can be enhanced by diverse ecological and physiological factors (Papavizas, 1977; Dhingra and Sinclair, 1978). Dark lesions become visible on the epicotyls and hypocotyls pursued by death due to obstruction of xylem. In plants, the pathogen causes reddish-brown lesions on roots and stems with the growth of dark mycelia and microsclerotia. Eventually, the plant defoliates and wilts (Abawi and Pastor-Corrales, 1990).

F. solani can attack a wide variety of plants including most common vegetables such as potato, tomato, pepper, and eggplant causing crown, foot, and root rot diseases. Many physiologic races adapted to specific hosts have been reported. It causes root and/or foot rot of the host plant. Above-ground portions' symptoms can vary based on *F. solani* species complex and plant species, and the disease may be visible as stunting, wilting and lesions on the leaves and/or stem (Abdel-Monaim, 2013; Coleman, 2016).

The fungal genus *Fusarium* predominantly contains soil-borne pathogens that collectively cause disease on a number of significant agricultural crops (Beckman, 1987; Farr et al., 1989; Scandiani, 2011). *F. oxysporum* is the most widespread and destructive species causing vascular wilt diseases on many plants including pepper (Mushtaq and Hashmi, 1997). This fungi infects plant from roots and grow from inside towards the cortex to stele (Beckman, 1987; Tjamos and Beckman1989; Bowers and Locke, 2000; Nandhini et al., 2012).

The purpose of the present paper was to evaluate inoculation techniques for assessing pathogenicity of representative isolates of *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani* from pepper (*C. annuum*) seedlings.

2. Materials and Methods

2.1. Sample collection

Peppers cultivated from four provinces namely Adıyaman, Diyarbakır, Mardin, and Şanlıurfa were selected for sample collection to detect wilt and root rot diseases. Individual pepper plants with infected roots were collected in the pre-flowering stage and brought to the laboratory in an ice box and stored at 4 °C.

2.2. Fungal isolates

Isolates of *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani*were obtained from Adıyaman, Diyarbakır, Mardin, and Şanlıurfa. These fungi were isolated from stem and root tissues of infected pepper plants. Sections of tissues were surface sterilized with 3% NaOCl for 5 minutes, washed three times with distilled water and placed on sterile filter paper to remove excess water. Tissue pieces were incubated on potato dextrose agar (PDA) plates at 28 °C. Single spore culture technique was followed to obtain pure cultures of *F. oxysporum* and *F. solani*. The cultures were identified at Department of Plant Pathology, Harran University.

2.3. Pathogenicity tests

Capsicum annuum cultivar Inan-3363 was used in this study. Bioassays on healthy peppers at 4-5 leaf stage were carried out with one control and six replications for each combination. Totally seven replications were used for every pathogen, each planted in 28 pots, and repeated two times. A total of 112 pots were used; 56 for infested rice inoculation and 56 for the other inoculation techniques.

2.4. Inoculation techniques

2.4.1. Root dip method for *Fusarium oxysporum* and *Fusarium solani* inoculations; preparation of conidial suspensions

Twenty-day-old seedlings were inoculated by standard root dip method (Herman and Perl-Treves, 2007; Karimi et al., 2010). The spore suspensions were prepared from 7-10 days old isolates cultured on the PDA at room temperature. The roots of seedlings were trimmed with a sterile scissor and submerged into tubes containing 30 ml of *F. oxysporum* and *F. solani* spore suspensions (1×10^6 spore ml^{-1}) for 30 mins. And the inoculated seedlings were transplanted into mini pots, 15 cm

diameter, surface sterilized with 0.1% mercuric chloride (Dubey and Singh, 2008), containing soil and sand at 1:1 ratio and incubated in a growth chamber. Inoculated and non-inoculated (control) pepper seedlings were incubated at 22 °C. The control plants were treated with 30 ml sterile distilled water.

2.4.2. Soil infestation with wheat bran for *Rhizoctonia solani* and *Macrophomina phaseolina* inoculations

Wheat bran (100 g) and distilled water (200 ml) were mixed in an Erlenmeyer flask, autoclaved at 121 °C for 40 min. Each flask inoculated with 10 discs (0.8 cm diameter), containing the pathogen, cultured for 4 days at 25 °C under dark light condition on PDA. After 30 days of incubation, infested wheat brans were applied to the soil rhizosphere around roots (Zhang et al., 2014).

2.4.3. Soil infestation with rice-grain for *Rhizoctonia solani, Macrophomina phaseolina Fusarium oxysporum* and *Fusarium solani* inoculations

Rice-grain inoculum was prepared by transferring three to four PDA medium disks of *F. solani*, *F. oxysporum*, *M. phaseolina, and R. solani* isolates to flasks containing 25 g of long-grain rice and 18 ml of deionized water (Holmes and Benson, 1994). Subsequently, the flasks autoclaved for two consecutive days prior to use. *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani* cultures were shaken daily by hand. Seven to 10 days after preparation 100% colonization of rice grains were obtained on *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani*.

The seedlings were inoculated by making two holes (2 cm deep and 1 cm from opposite sides of the seedling stem) with a wooden rod and placing a single colonized rice-grain into each hole (Benson et al., 1997). The medium was pushed back to cover the inoculum. Inoculated plants were placed in a growth chamber under a 10 h dark/14 h light photoperiod and 25-30 °C temperature with 80-85% RH. For each isolate, 6 replications of each cultivar were inoculated in a completely randomized design and the experiment was repeated twice. Cultures were incubated for 4 days at 25 ± 1 °C, and placed on a laboratory bench under 12 h light and 12 h dark. At the end of the experiment (90 days), all surviving seedlings were evluated for above ground disease symptoms. Each seedling was rated based on the severity of disease symptoms using the following numeral system. Disease severity was rated using a numerical scale of the 0-4 index defined by Aoyagi et al. (1998), where 0: healthy, 1: 1-25%, 2: 26-50%, 3: 51-75%, and 4: 76-100% diseased plant. Liu et al. (1995), identified a disease severity index (DSI) for each replicate similar to the one used here. It was estimated along these lines DSI = Σs/(s max* n)*100; where s is the possible disease score, s max is the maximum disease score and n is the total number of plants examined in each replicate. Seedling roots were washed, surface sterilized, and analyzed for colonization by inoculated isolates as described above. Therefore, foliar symptom indices and root-rot symptom indices were measured. Additionally, fresh plant weight (g), fresh root weight (g), dry plant weight (g) and dry root weight (g) parameters were also recorded for each plant.

2.5. Statistical analysis

Since the distribution of all data was normal, they all analyzed by SPSS program (SPSS, Inc., Chicago IL). Effect of treatments on *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani* were analyzed for each treatment using one-way ANOVA followed by the Duncan Multiple Range Test ($p<0.05$).

3. Results and Discussion

Fungal isolates of *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani* were obtained from stem and root tissues of infected pepper plants (Figure 1). Pathogenicity of *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani* isolates were evaluated on pepper under growth chamber conditions (Table 1). Inoculated plants were left to grow for three months after transplanting (Diyarbakır Plant Protection Research Institute). The plants were examined monthly for the severity of infections caused by the fungi based on disease assessment and the averages were recorded. Pepper seedling mortality due to Fusarium inoculation was low However, significant differences ($p<0.05$) were found among different isolates for the other measured parameters, including seedling foliar symptom, root rot symptom, fresh plant weight, fresh root weight, dry plant weight and dry root weight (Table 1). When all parameters were collected, isolates were ranked as to their relative virulence on inoculated seedlings (Table 1). All pathogen-infected pepper plants grew less vigorously compared to control (Figure 2).

Four different soil-borne fungi isolated from roots of pepper plants, collected from different fields in Adıyaman, Diyarbakır, Mardin and Şanlıurfa provinces, showed root rot and wilt symptoms. These isolates were identified as *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani*. Pathogenicity test of the obtained isolates revealed that all tested isolates could infect the roots

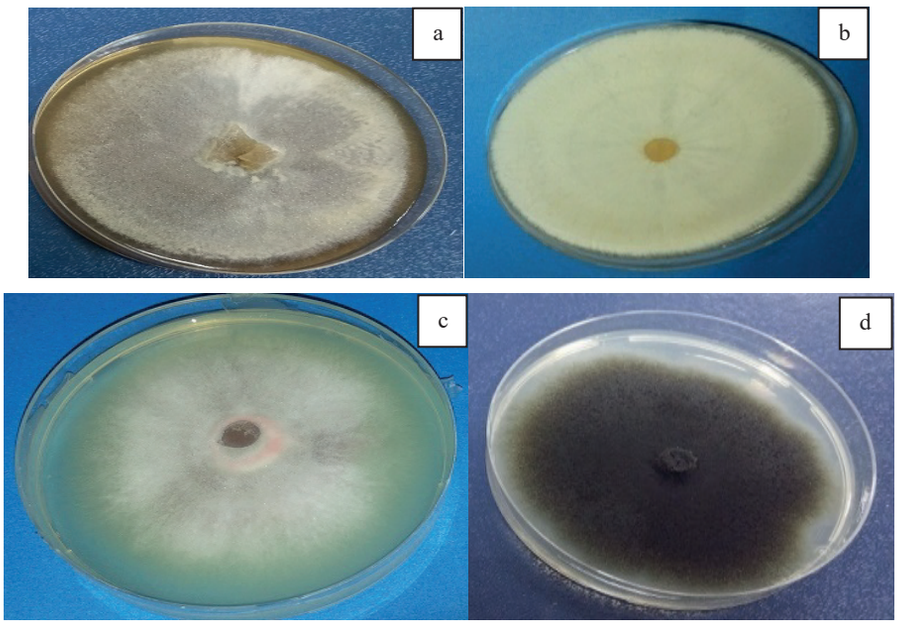

Figure 1. Pathogenic isolates of *F. solani* (a), *R. solani* (b), *F. oxysporum* (c), *M. phaseolina* (d)

Table 1. Disease indices of pepper plants inoculated with investigated isolates of *Rhizoctonia solani*, *Fusarium oxysporum*, *F. solani* and *M. phaseolina*

Tested fungi	Foliar symptom indices	Root-rot symptom indices	Fresh plant weight (g)	Fresh root weight (g)	Dry plant weight (g)	Dry root weight (g)
Soil infestation with rice grain						
Rhizoctonia solani	2.00 a	2.50 a	12.57 a	1.64 a	1.43 a	0.26 a
Fusarium oxysporum	1.83 a	2.17 a	16.99 c	2.38 b	1.78 c	0.31 a
Fusarium solani	2.17 a	2.17 a	16.09 bc	1.85 ab	1.72 bc	0.30 a
Macrophomina phaseolina	2.00 a	2.33 a	13.48 ab	1.84 ab	1.49 ab	0.27 a
Control	0.00 b	0.00 b	18.80 c	3.52 c	2.21 d	0.52 b
Root dip method inoculation[1] / Soil infestation with wheat bran[2]						
Rhizoctonia solani[2]	2.67 ab	2.67 a	11.41 a	1.77 a	1.31 a	0.25 a
Fusarium oxysporum[1]	2.50 b	2.17 a	15.17 b	2.82 b	1.74 b	0.39 b
Fusarium solani[1]	3.00 a	2.50 a	12.05 a	1.96 a	1.42 a	0.29 a
Macrophomina phaseolina[2]	2.83 ab	2.50 a	11.43 a	1.88 a	1.45 a	0.28 a
Control	0.00 c	0.00 c	18.80 c	3.52 c	2.21 c	0.52 c

Within the columns, the values following a common letter do not differ significantly from Duncan's Multiple Range Test ($p<0.05$)

of pepper (cv. Inan-3363) inducing typical symptoms of foliar and root rot and reducing fresh shoot and root weight in growth chamber conditions, using the root dip and soil infestation inoculation with rice-grain and wheat bran methods. Roots of the infected plants developed less compared to control plants (Figure 3). When rice-grain inoculum was used, all pathogens caused similar disease foliar and root rot severity. However, when fresh plant weight was used as a disease parameter *R. solani* was more effective in reducing fresh plant weight than other pathogens. Dry plant weight reduced more by *R. solani* and *M. phaseolina* than other pathogens. Finally, all pathogens affected dry root weight similarly to control (Table 1). With other inoculation methods, root dip or wheat bran inoculations, *R. solani*, *M. phaseolina* and *F. solani* similarly affected foliar symptom severity, fresh plant and root weights and dry plant and root weights. While *F. oxysporum* was the least virulent pathogen, all pathogens similarly affected root rot severity (Table 1).

Our results found that *F. solani* were more aggressive for inducing foliar symptoms than *F. oxysporum* using the root dip method, but all isolates appeared to be similarly aggressive for inducing both foliar root rot symptoms when inoculated using the soil infestation with the rice-grain method. In relation to the infection process of *F. oxysporum* and *F. solani* and the expression of

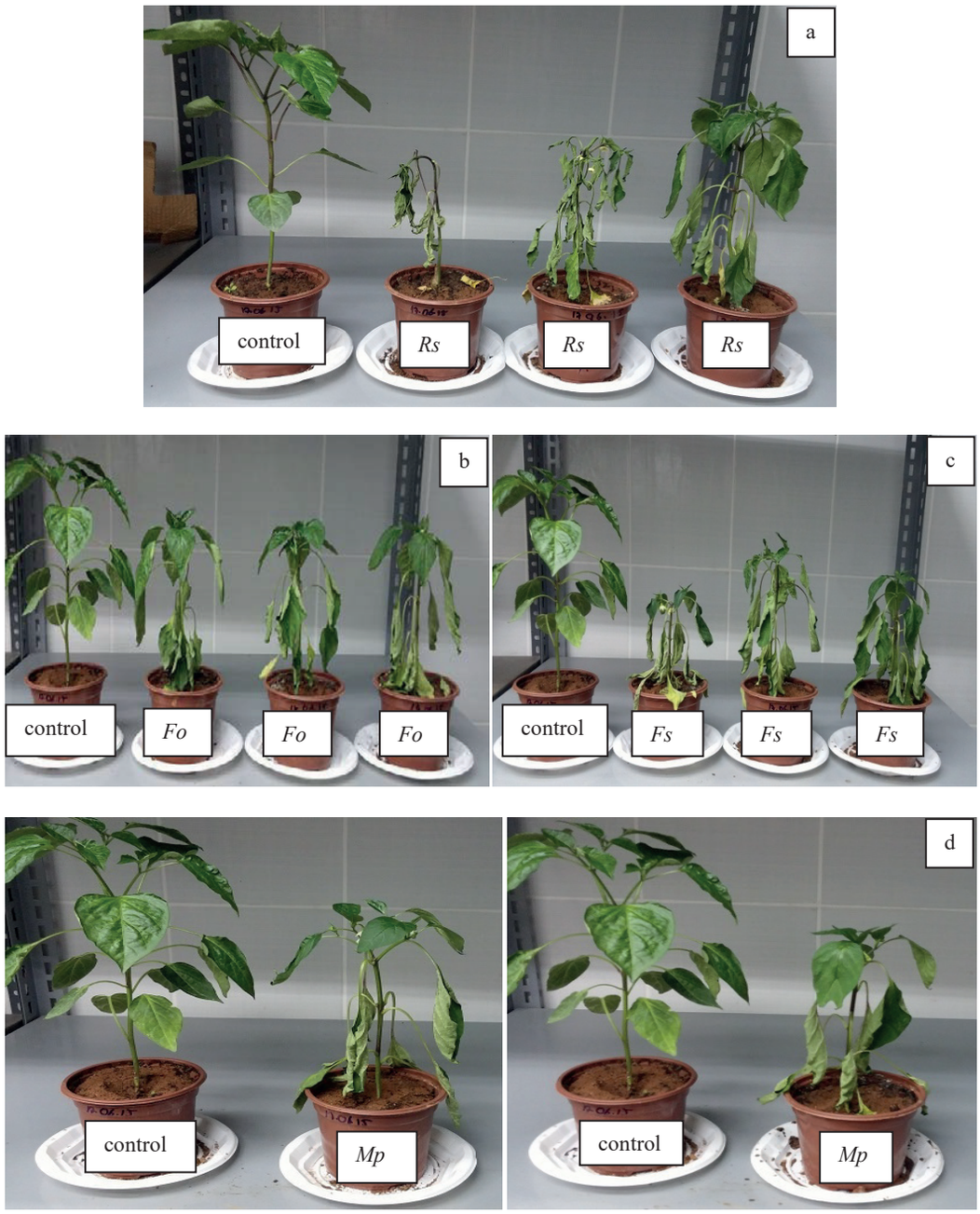

Figure 2. *Rhizoctonia solani* (Rs), *Fusarium oxysporum* (Fo), *Fusarium solani* (Fs) and *Macrophomina phaseolina* (Mp) pathogenicities by the use of soil infestation method with rice-grain inoculum on peppers under a growth chamber conditions. a) Symptoms caused by *R. solani*, b) Symptoms caused by *F. oxysporum*, c) Symptoms caused by *F. solani*, d) Symptoms caused by *M. phaseolina*

foliar symptoms, there is effective and ineffective zones of infection in the roots. Although the reasons for these individual differences are unknown, one reason might be the difference between infection process by *F. oxysporum* and *F. solani* on pepper roots (Romberg and Davis, 2007). Using different inoculation methods might also result in different concentrations of conidia. Disease ratings made by determining fresh plant and root weight have been suggested as a useful tool for comparing aggressiveness of many pathogen isolates. In this current study, there was a reduction in fresh plant and root weight when both inoculations were employed.

Most of the studies used sorghum grain inoculums for testing pathogenicity of *R. solani* and *M. phaseolina* (Aly et al., 2007; Mikhail et al., 2009; Mahmoud and Abo-Elyousr, 2014; Prasad et. al., 2014). To test the pathogenicity of

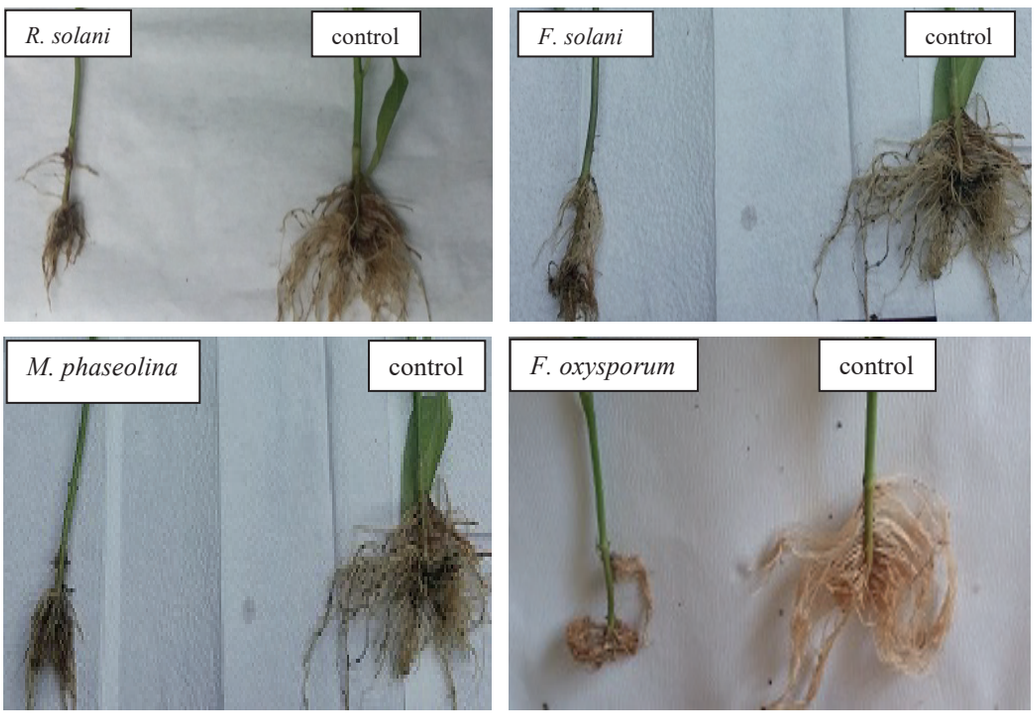

Figure 3. Root rot of infected pepper seedlings with pathogens

F. oxysporum, root dip (Meinhardt et al., 2002; Eken and Demirci, 2003; Amatulli et. al., 2010; Chehri et. al., 2011; Nirmaladevi et. al., 2012; Abada and Ahmed, 2014) and soil infestation with wheat bran (Shi et al., 2000; Zhang et al., 2014) methods were generally preferred.

Rice-grain inoculum had an advantage for testing all soil-borne pathogens as well as simultaneous pathogenicity evaluation of these fungi. Since rice-grain inoculum was routinely used for *Phytophthora* inoculations (Holmes and Benson, 1994), this growth medium was applied for the first time for testing pathogenicity of *R. solani*, *M. phaseolina*, *F. oxysporum,* and *F. solani* taking advantage of previous studies of soil-borne pathogen Phytophthora inoculations (Holmes and Benson, 1994). Rice-grain cultures yielded excellent opportunity to test pathogenicity of all these fungi. We conclude that rice-grain cultures are sensitive and specific for the inoculation of pepper root tissues and testing pathogenicity and virulence of *R. solani*, *M. phaseolina*, *F. oxysporum* and *F. solani* on pepper. Until now, only Amusa et al. (2007) used rice-grain methods to test pathogenicity of *M. phaseolina* on cowpea plants. However, as far as we know, there is no report to test the pathogenicity of these fungi on Solanaceous crops. The main advantages of this method reside in its sensitivity and selectivity, which enabled the simultaneous identification of these fungi.

4. Conclusions

The results of this study can provide practical contributions to monitoring pepper seedlings against these pathogens. As a result, we recommend rice grain inoculation to evaluate the pathogenic effects of *R. solani*, *M. phaseolina*, *F. oxysporum*, and *F. solani* on various pepper cultivars in Turkey.

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