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Full Length Research Paper

Rosemary wilting disease and its management by soil solarization technique in Iran

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Wilting disease on Rosemary (Rosmarinus officinalis), an ornamental-medical plant, was studied from 2007 - 2009 in Mashhad, north-east Iran. Different Rosemary fields in this area were visited and root samples of the infected plants and soil around the roots were collected and transferred to laboratory. Samples were cultured on CMA, PDA and WA media and isolated fungi were identified. Three fungal pathogens including *Phytophthora citrophthora*, *Rhizoctonia solani* and *Fusarium oxysporum* were determined, whereas Helicotylenchus spp. was also associated. Pathogenicity tests proved that they were wilting pathogens, although *P. citrophthora* was the major pathogen in the field and glasshouses. This is the first report on Rosemary disease in the country. The disease caused losses from 30 – 60% in the fields where the suitable conditions allowed the disease to build up. Soil solarization technique was carried out to control pathogens before planting of seedlings. Application of this method reduced population density of *P. citrophthora* and *F. oxysporum* from 1300 - 1800 cfu –g/soil to 500 - 700 after 4 weeks and then 200 - 300 cfu (colony forming propagules) after 6 weeks. Solarization is a simple, economic and effective technique in managing Rosemary wilting disease before plant planting in new established orchards.

Key words: Rosemary, Phytophthora citophthora, wilting, Iran.

INTRODUCTION

Rosemary (Rosmarinus officinalis L.) as an ornamental and medicinal plant is a very significant crop all over the world (Türe et al., 2009). It can be used not only as an ornamental plant, but also as a medical or industrial plant all over the world (Singh et al., 2009; Tironi et al., 2009).

Rosemary is mostly cultivated in northeast Iran (Mashhad and Semnan provinces), but is expected to be planted in other places of the country as well. Since the plant is adapted to different climatic areas, it is cultivated in some places to make drugs (Chiej, 1988; Zargari, 1990). The drug of Rosemary can be used for controlling spasm and emphysema disease, which reduces human peace (Ghannadi et al., 1992; Hosseinzadeh et al., 2006).

Chemical products of Rosemary can be used for managing many human disorders such as headache, hair and skin problems, and cosmetic industries (Oji-Ardabily et. al., 2006). The oil of Rosemary can be distilled from the flowering tops, and the superior oil is obtained from the stem and leaves which has flavoursome cooking ingredient. However, large amounts of the commercial oil are distilled from the stem and leaves of the wild plant before they get to the flower (Hosseinzadeh et al., 2006; Tironi et al., 2009). Although, Rosemary is a native plant of the Mediterranean and Asia, it is still reasonably found in cooler areas. Also, it will stand severe frosts if conditions are not windy and wet as well (Verhoeven et al., 2008; Türe et al., 2009).

Due to considerable application of Rosemary for purposes such as ornamental, medical and cosmetic industries (Hosseinzadeh et al., 2006; Tironi et al., 2009), it is widely grown in the field and under greenhouse condition. However, this most important economic plant suffers from wilting disease by some soil born fungal pathogens. Normally, ornamental plants showed root rot,

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Abbreviations: PDA, Potato dextrose agar; **CMA,** corn meal agar; **WA,** water agar; **CLA,** carnation leaf agar.

which resulted wilting in some countries (Verhoeven et al., 2008). The main serious soil borne fungal pathogens including *Rhizoctonia solani*, *Phytophthora citrophthora* and *Phytophthora cinnamomi* were reported as root rot causal agents on ornamental trees and flowers (Atkinson, 1982; Veghe and Berre, 1982; Alvarez et al., 2007; Verhoeven et al., 2008).

It has been reported that several soil borne fungi are involved in causing root rot of trees, seedlings and flowers in Iran. Usually, soil borne pathogens including Phytophthora and Fusarium species make heavy disease for major crops and trees by affecting yield productions (Mirabolfathy and Ershad, 1993; Saremi, 2000). Generally, the Phytophthora spp. are the important plant pathogens in many trees in diverse countries (Hall, 1996). By the way, root rot and wilting of Rosemary trees causes yield losses under various conditions. The infections of Rosemary plants are facilitated by wet conditions, but the fungus can grow rapidly through the plant roots when the plants were moisture stressed. However, there is no general perfect method to be used in all instance of soil borne pathogens control. Therefore, any new method of control (even if restricted in its use) is of value, because it adds to the study's rather limited arsenal of control methods. So, soil solarization or solar heating, which is relatively a new approach, can be a good way for controlling soil borne pathogens (Harender and Sharma, 2009; Bonanomi et al., 2008; Triki et al., 2001). Anyhow, soil solarization or solar heating has been applied before as a conventional way for controlling soil borne pathogens (Tjamos et al., 2000; Annesi and Motta, 1994; Pinkas et al., 1984; Pullman et al., 1981). The aim of soil solarization is to harness solar energy to raise the temperature of moistened soil, which results in the control of soil borne pathogens (Scopa et al., 2008).

The present study has been carried out to assess the etiology of wilting disease of Rosemary, caused by fungal soil borne pathogens, in Mashhad province, north-east Iran. Accordingly, soil solarization or solar heating, as a relatively new approach, was applied for its management.

MATERIALS AND METHODS

Sample collection

The study was carried out in the Rosemary orchard in Mashhad from 2007 to 2009. Root samples of the infected plants and soil around the roots were collected and transferred to laboratory. Infected root samples were cut into 0.5 - 1.0 cm pieces and then dipped in sodium hypochlorite (0.5%) for 2 min. The pieces were rinsed in sterile distilled water, dried on sterile filter paper and plated on CMA (Corn Meal Agar), PDA (potato dextrose agar) and WA (water agar) media to isolate fungal pathogens. Soil samples from root vicinity of the infected plants were also extracted and the hyphal tip and single spore techniques were employed. The currently valid manuals in each concerned subject were used for identification of the isolated fungal pathogens. (Burgess et al., 1994; Saremi, 2005).

Identification of pathogens

Phytophthora species

General characteristics morphology including shape of sporangium, sporangiophore, hyphal diameter, optimum temperature for growth rate and others were studied for identification of Phytophthora species as the main soil borne pathogens that were isolated (Mirabolfathy et al., 2001). About 5 mm from isolated fresh mycelium was cultured in Petri dish with grain hemp and sterile water for sporangium production of Phytophthora species. The cultures were incubated in a room lighted with wave lengths (white light tube, 40 w) and fluctuating temperatures regime (25°C during the day and 20°C in the night) under 12 h photoperiod. Sporangiums were produced after 3 days incubation and then, they were morphologically studied. Mycelium tip was also cultured in carrot media, agar, CMA, WA and 10 ppm cholesterol for oospore production (Ershad, 1971). Isolated Phytophthora species were anastomosis on darkness conditions and they produced sporangiums that were considered. Colony diameters of isolates were measured in 2 and 4 days growth at 5, 7, 13, 22, 25, 28, 30, 32 and 35°C temperatures.

Rhizoctonia species

For obvious identification of Rhizoctonia species isolated from infected samples and soil around the roots, the nucleus stain method was used, rather than using the other ways (Sneh et al., 1996). Two small wood pieces were placed on the filter paper in Petri dish and two lams were located on them, then a piece of mycelium was put on lams. The mycelium was grown and it reached under the lams after two days; then, lams were separated and their myceliums were pigmented with one drop of safranin (0/5%) and one drop of fresh KOH (3%). Consequently, they were visited by a light microscope. Average hyphe diameters were 50 µ for isolated Rhizoctonia species that are just close to the right-angled branch point. Width and length of 50 monoloide cells plus colony diameter at different temperatures (5, 10, 15, 28, 30, 33, 37 and 40°C) were also studied in darkness conditions (Kim et al., 1994).

Fusarium species

Fusarium pathogens isolated from infected plants were cultured in PDA as a common medium and CLA (carnation leaf agar) as a selective medium. As a matter of fact, production of macro-conidia by Fuasrium species, which is the key factor for valid identification, is favored by CLA medium (Nelson, 1983; Burgess et al., 1994). Soil dilution technique was also used to isolate inoculums from soil in the surrounding area of infected plant roots. Characteristics of cultures including colony growth rate, shape of microconidium, macroconidium, Phialide and chlamydospore were considered for identification.

Pathogenicity test

Test for Phytophthora species

Three Rosemary seedlings were collected and planted in pots with sterile soil, separately. The mycelium of pathogen was prepared on prelate and hemp juice and then, 10 ml of inoculums were poured around the crown of each seedling. By the way, 60 g of hemp grain was boiled in 1 L water, then it was filtrated and 120 cc was added to 200 g of autoclaved prelate. Finally, Phytophthora species were placed on Petri dish and kept at a dark condition for 4 weeks to allow the consummation of all hemp juice by the pathogen as reported

Figure 1. Coverage of the infested field soils by soil borne fungi with a clear plastic to trap the sun's heat and raise soil temperatures.

already (Banihashemi, 1989).

Test for Rhizoctonia species

Propagules of Rhizoctonia species were prepared by placing 10 sterile wheat grains and a piece of mycelium (4 cm2) on a flax and kept for 4 - 5 weeks at 28°C temperature to have adequate time for colonization. Seven colonized grains were put around the roots of each seedling in each pot. Totally, three Rosemary seedlings were placed in pots that contain sterile soil and were watered once a week, separately.

Test for Fusarium species

A piece of fresh Fusarium culture was transferred from PDA media to a flax with 50 ml PDB (250 g potato, 20 g dextrose and 1 L sterile water), and was then put on a shaker for 3 days. The suspension in 106 cfu (colony forming unit) density was prepared and 200 ml of that was used for inoculating Rosemary seedling. Three out of four month old seedlings were laid in the suspension (106 cfu) for 10 min before planting in pots. All three pots were kept in glasshouse with normal conditions and watered each week.

Interaction of pathogens

Interactions of these soil borne pathogens on the Rosemary wilting disease were evaluated. Three pots with sterile soil were collected and Rosemary seedlings were planted on the pots separately. The suspension of *P. citrophthora* spores (10 ml 106 cfu), which is same for *Fusarium oxysporum* were located on the seedling crowns. This method was followed by the interaction of *P. citrophthora* with *Rhizocotina solani*. *Also*, it was followed by the interaction of *F. oxysporum* with *R. solani*. Regarding the usage of *R. solani* inoculums, seven colonized wheat grains were added rather than spore suspension. Three pots were also treated by adding only pathogen to each, independently, to assess the effect of solitary pathogen and their interaction to wilting disease. All pots were kept in glasshouse in ordinary conditions and watered each week. All Rosemary plants were sampled after two months and the results were investigated.

Soil solarization technique

Natural infected soils were irrigated deeply after plowing process for increasing transmission of heat through the soil. The moistened soil was covered with transparent polyethylene sheet to raise soil temperatures high enough in limiting the pathogen activities (Figure 1). The plastic edges were buried in the trench to ensure that the plastic is held in place to stable the heat. The process facilitated the rise of solar energy and increases the temperature of moistened soil, which is necessary for controlling the soil borne pathogens in soils (Figure 1). The effectiveness of soil solarization on propagule reduction of soil borne pathogens in the field (Figure 1) was assessed for 6 weeks as a common moment in time, recommended by researchers (Nafees et al., 2007; Bonanomi et al., 2008; Harender and Sharma, 2009).

Evaluation of propagules on solarized soil

Population densities of the pathogens in solarized soils were evaluated by soil samples four times. The first sample was collected at the start of soil solarization application and the other three samples were removed during 2, 4 and 6 weeks of soil solarization process. Three soil samples were taken from 5 - 10 cm depth of each treated plot by means of a soil auger, and were then mixed together and transferred to the laboratory via paper bags. Soils were air dried and sieved truly to remove stones and large particles and mixed thoroughly for analysis. Quantitative estimation of the pathogen in the soil was made with a dilution–suspension technique and expressed in propagules of the tested soil using soil dilution technique (Saremi et al., 1999).

RESULTS

Wilting disease symptoms

Different soil borne fungal species produced various wilting symptoms on the infected Rosemary plants. The main symptoms on infected plants in the studied areas were yellowing, root rot, crown rot and stem canker on

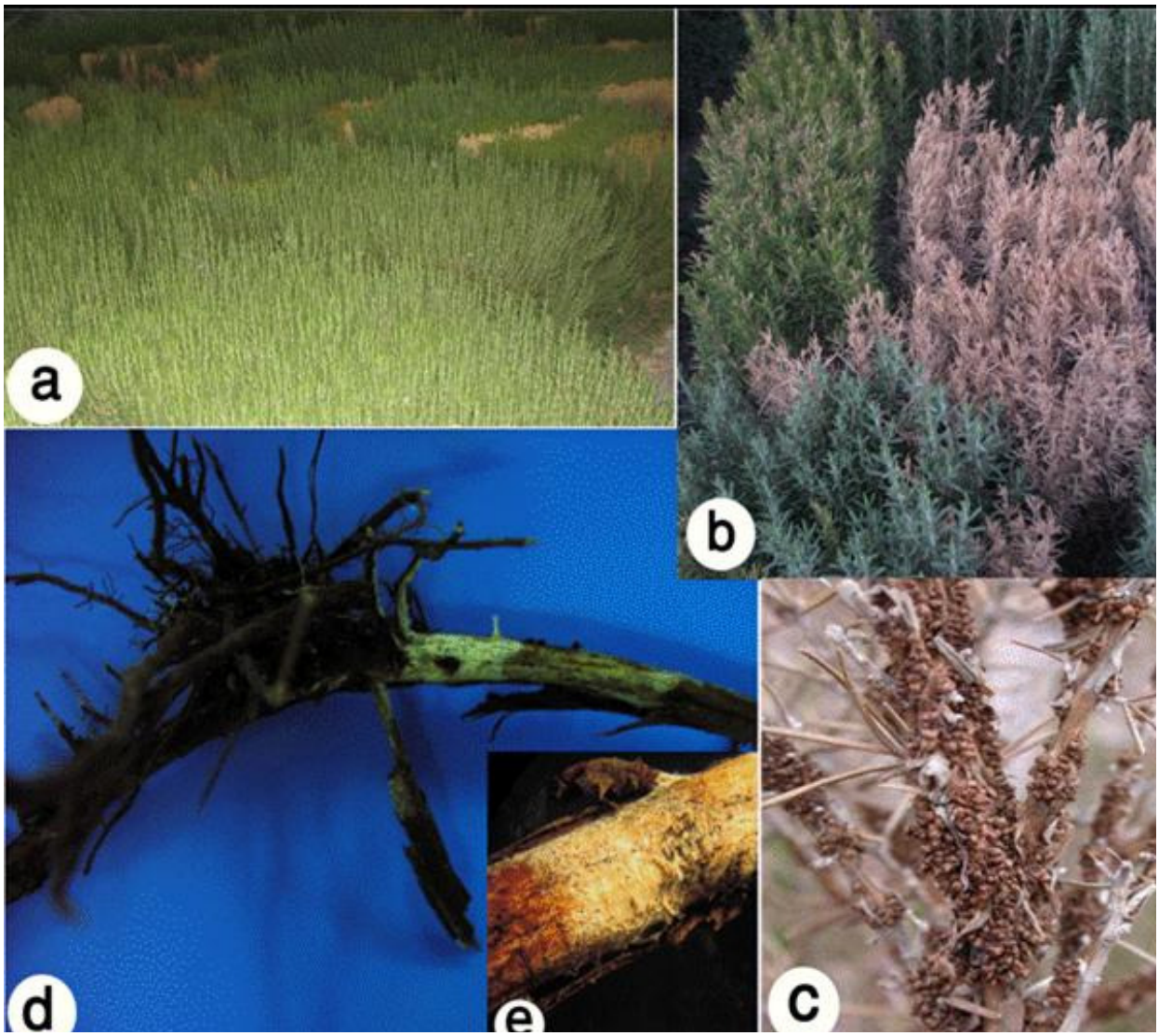

Figure 2. Symptoms on Rosemary disease showing wilting (a), whole death (b), root rot (d), canker (c) and rhizomorphe (e) in landscape at the campus of Mashhad University, Iran.

the health and wilted plant. Symptoms showed wilting on some parts of the plants or death of the entire infected plants (Figure 2). Commonly, the disease caused a remarkable yield reduction which resulted to an economical problem for producers.

Isolation and identification of causal agents

Phytophthora citrophthora

According to the obvious characteristics, *P. citrophthora* was the most identified isolate collected from CMA and WA media. This species showed almost a fast growing rate and irregular shape (Figure 3a). Its hyphe diameter was 4.8 µm (Figure 3b) and was found without any chlamydospre. However, sporangiophore was narrower than hyphe; whereas, sporangium demonstrated mostly apical connection (Figure 3c). Sporangium was pear, oval or circular shaped and some times they showed irregularity and germination of another sporangium (Figure 3f and g). Dimension of sporangium was 24 - 44 µm in length, 25 - 39 µm in width and optimal temperature 25 - 29°C for growth of all isolates. Microscopic figures have been taken with suitable resolution in a clear image that was actually identified by the width and height of the image (Figure 3).

Rhizoctonia solani

Colony pigmentation of this pathogen was white and flat (Figure 4a) and each cell had more than two nuclei (Figure 4b and c). The hyphe was 4 - 7 µm in diameter

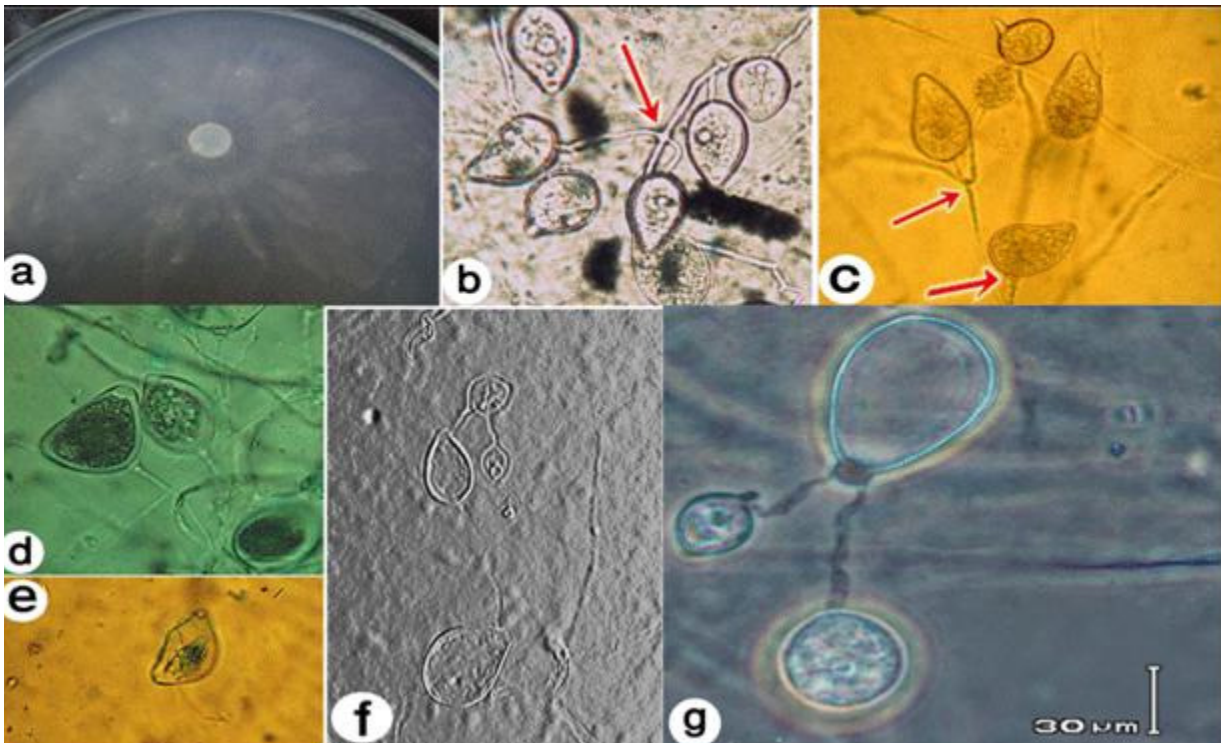

Figure 3. Characteristics of *P. Citrophthora* isolated from infected Rosemary colony (a), hyphe and sporangium morphology (b - g) in Mashhad, Iran.

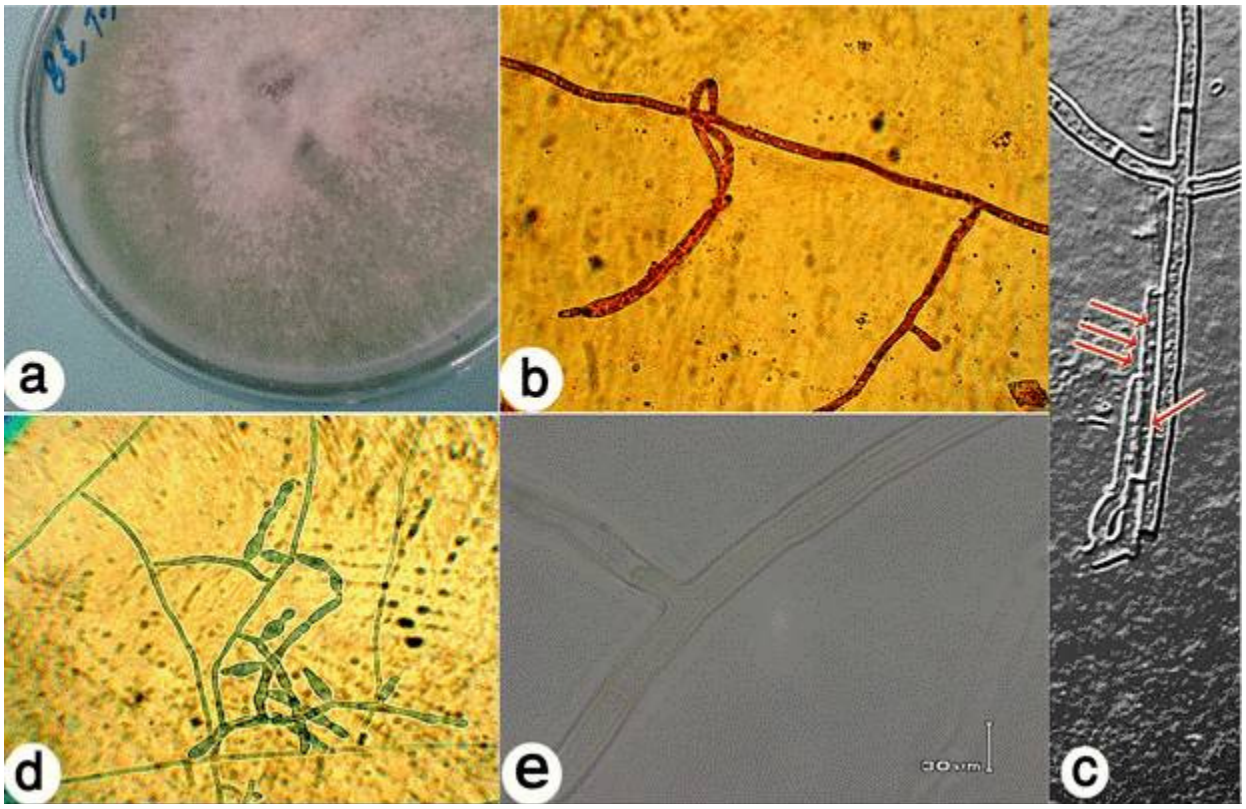

Figure 4. Characteristics of *R. solani* isolated from infected Rosemary as well as colony (a), bi nucleus cell (b and c) and right-angle branched (e and d).

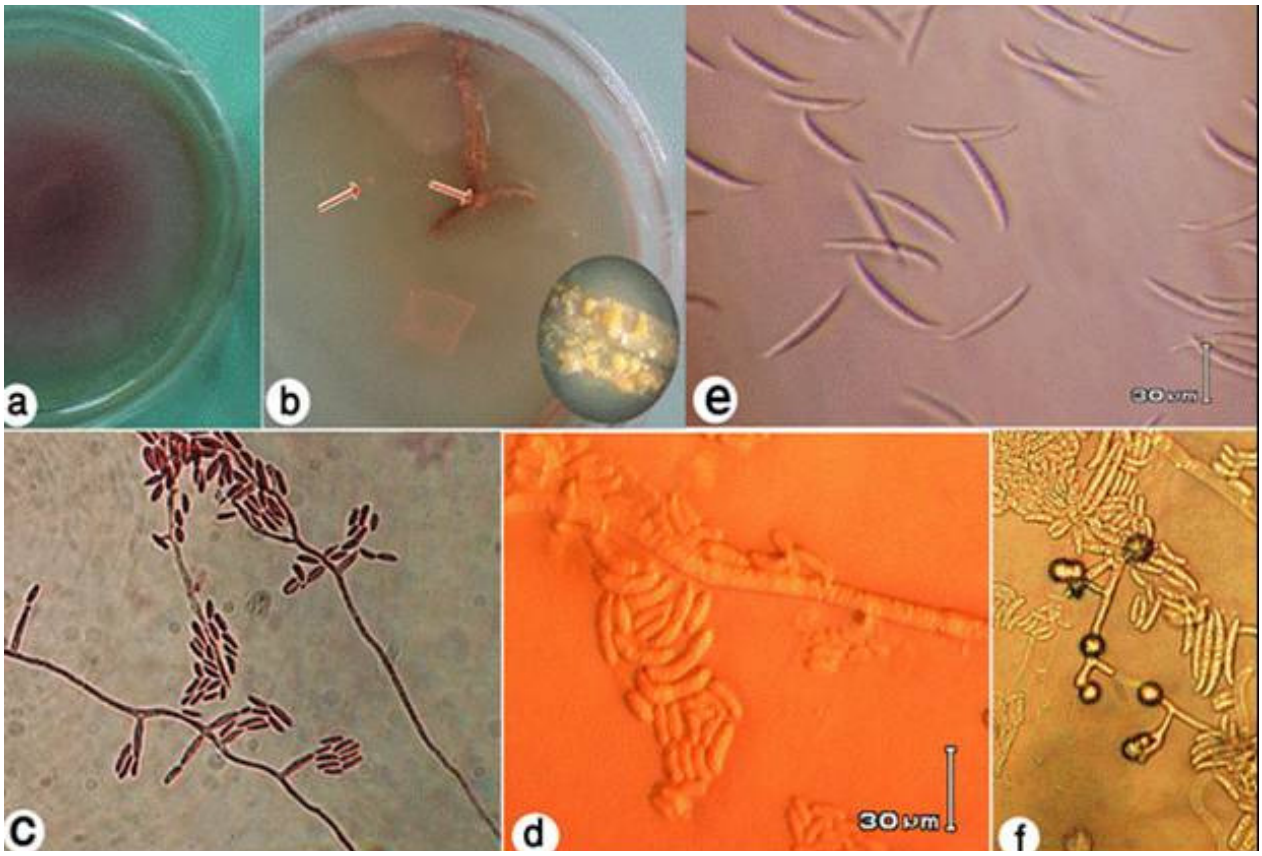

Figure 5. Characteristics of *F. oxysporum* isolated from infected Rosemary together with colony (a), spordocium (b), macroconidia and false head phialide (c and d), macroconidia (e) and clamydospore (f).

and right-angle branched, and an appropriate growth on 25 to 37°C temperature. Some other soil borne fungal species were also recovered rarely in areas studied.

Fusarium oxysporum

Commonly, *F. oxysporum* on PDA media showed colony and intense mycelium among white to purple pigmentation (Figure 5a). This species produced much light orange spordochium (Figure 5b) and mono cellular miccroconidia with single false-head phialide (Figures 5c and d). Macroconidia was sickle up long with 3-4 cells and produced by small single conidiophores (Figure 4e). This species showed the production lots of clamydospores, which would be the capability of long survival in field soil (Figure 5f). This cosmopolitan species was isolated repeatedly from collected samples.

Effect of pathogens interaction

The results of pathogens interaction during two months in glasshouse showed that the pathogens created more wilting disease than when they were in solitary. For example, *F. oxysporum* and *R. solani* showed just one complete wilting (Figures 6c and d), while they produced three complete wilting after interacting together (Table 1). Although, *P. citrophthora* caused two complete wilting as solitary pathogen (Figure 6b), they caused three complete wilting when they acted together with *F. solani*. The results also confirmed that *P. citrophthora* caused severe damage than other soil borne fungal pathogens (Table 1).

The collected eighteen Rosemary seedlings after four months old showed the various effects of the considered pathogens on Rosemary wilting disease in glasshouse. There were various influences of treated plants on pots, while the pathogens interaction showed most involvement on Rosemary wilting occurrence (Table 1).

Role of soil solarization on pathogen inoculums

Population density of *F. oxysporum* and *P. citrophthora* were decreased quickly after two weeks soil solarization application in pre planted field (Figure 7). Indigenous population density were 1300 (cfu g-1/soil) for *P. citrophthora* and 1800 cfu g-1/soil for *F. oxysporum* in the natural field at the start of soil solarization. The mean of propagule density for *F. oxysporum* was clearly reduced

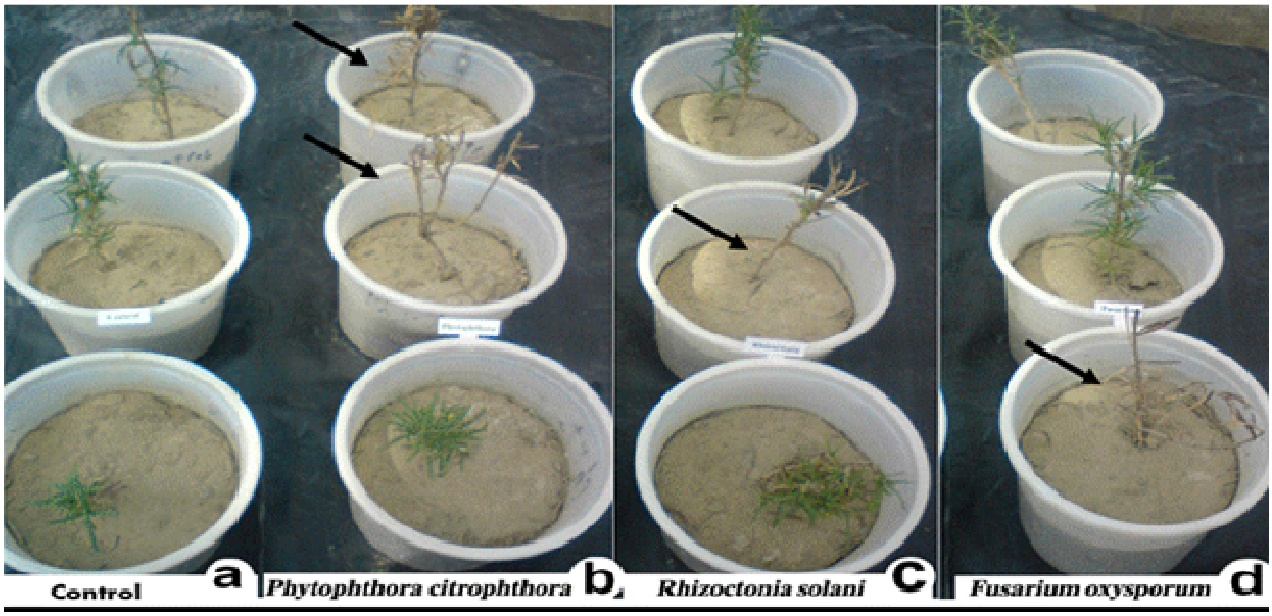

Figure 6. Wilting of Rosemary on infested pots with isolated pathogens in glasshouse, *P. citrophthora* (b) *R. solani* (c) *F. oxysporum* (d) Mashhad, Iran.

Table 1. Effect of isolated pathogens on Rosemary plants at solitary and interacted pathogens at glasshouse in Mashhad, Iran.

Pathogens	Rosemary wilting number	Rosemary colorless
P. citrophthra	2	0
R. solani	1	1
F. oxysporum	1	1
P. citrophthora + R. solani	3	0
P. citrophthora + F. oxysporum	3	0
R. solani + F. oxysporum	3	0

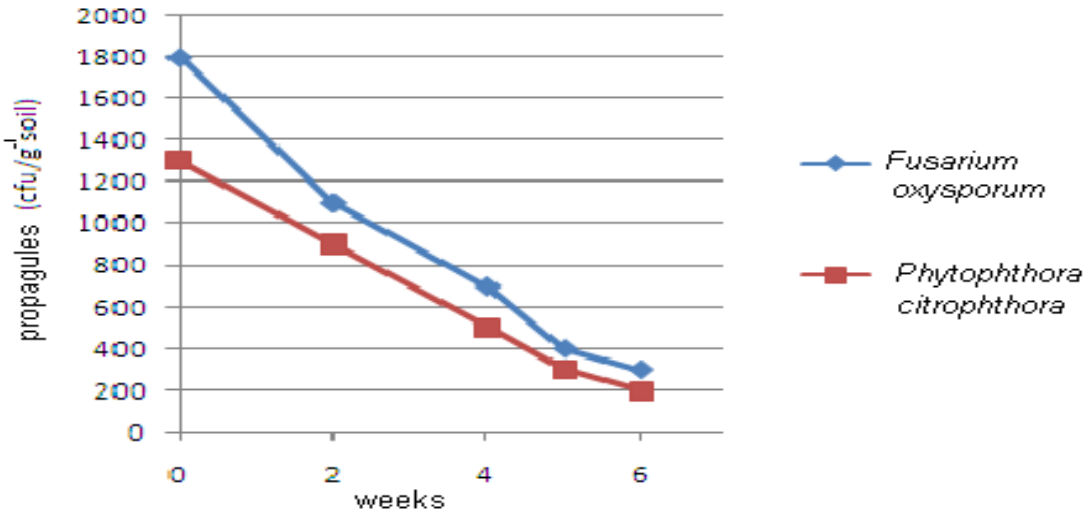

Figure 7. Propagule reduction of *P. citrophthora* and *F. oxysporum* throughout the 6 weeks soil solarization process in the fields.

Table 2. Reduction propagules of *F. oxysporum* and *P. citrophthora* in non planted solarized soils.

Date (week) after soil solarization	Inocolum of *F. oxysporum* (cfu/g^{-1} soil)	Inocolum of *P. citrophthora* (cfu g^{-1} /soil)
0	1800	1300
2	1100	900
4	700	500
5	400	300
6	300	200

up to 1100 cfu g-1/soil after 2 weeks and was then reduced to 700 cfu g-1/soils after 4 weeks. The population of the pathogen was also reduced to 400 and 300 cfu g-1/soil after 5 and 6 weeks solarization (Table 2). The average inoculums density of *P. citrophthora* was also reduced to 900, 500, 300 and 200 cfu g-1/soil during 2, 4, 5 and 6 weeks solarization application in field soil, respectively (Table 2). As a matter of fact, the solarization practice heated the covered soil through the repeated daily cycle; hence, temperature of solarized soil was higher than non treated soils.

DISCUSSION

Commonly, soil borne fungal pathogens caused wilting diseases on various plants in Iran for a long time past (Saremi, 2000; Saberi et al., 2004). Therefore, Phytophthora species, Fusarium species and Rhizoctonia species cause diseases in a variety of plants including Rosemary as an economic tree. Recently, these soil borne pathogens have been severely increased due to development of Rosemary plantation in the country. These soil borne pathogens caused root rot and wilting disease that resulted yield losses on infected plants all over the world (Singleton et al., 1992; Yangui et al., 2008; Deeksha et al., 2009). The findings of the study confirmed that these major soil borne pathogens, *P. citrophthora*, *F. oxysporum* and *R. solani*, were the main casual agents of Rosemary wilting disease of collected samples in Mashed region, Iran.

Although, these three pathogens caused wilting on Rosemary plant, the study's survey in both field and glasshouse showed that *P. citrophthora* caused more damage than other pathogens. Normally, *P. citrophthora* causes root rot disease on many plant all over the world (Mirabolfathy et al., 2001, Alvarez et al., 2007). However, this is the first time that this species is reported as a causal agent on Rosemary wilting disease in Iran. The study's pathogenicity test in glasshouse proved susceptibility of the Rosemary shrub to this pathogen. On the other hand, yield production of Rosemary as an ornamental-medical plant is significant for growers. Therefore, the pathogen which causes lot of economic problem in the country should be managed in a simple and economic way.

Unfortunately, Rosemary plants suffer from wilting disease which resulted yield reduction and economic problem recently. Although three mentioned fungal pathogens were identified as major agents of wilting on Rosemary, other soil borne pathogens such as bacteria and nematode species can also be associated in the wilting disease (Modupe et al., 2007). Experimental test and field survey showed that Helicotylenchus spp as a main nematode assist wilting disease on plants in some locations (Barbercheck and Broembsen, 1986; Nico et al., 2003; Nafees et al., 2007). This study showed that the most frequent soil borne pathogen caused by the Rosemary wilt was *P. citrophthora*. Actually, this species has been reported as a causal agent of root rot and wilting disease of many other ornamental shrub, flowers and trees (Mirabolfathy and Ershad, 1993; Alvarez et al., 2007; Pettitt et al., 2008). Management of root rot and wilting disease in Rosemary is significant in the most mounting areas. However, chemical control and biological control process were not effective in managing soil borne pathogens in the fields (Mojibur et al., 2006; Modupe et al., 2007; Kotan et al., 2009). A favorite way should be introduced for managing this problem and promoting growers in Rosemary cultivation. Nevertheless, the favorite way would be soil solarization method, in particular for newly established orchard. The idea of control soil borne pathogens by soil solarization was carried out on plant trees in several countries (Nico et al., 2003; Saremi et al., 2007; Bonanomi et al., 2008; Scopa et al., 2008). The managing Verticillium wilt by soil solarization in the newly established olive orchards has been tried in southern Spain and other countries (Rodríguez et al., 2004). The success of this method is based on the fact that plant pathogens and casual agents on Rosemary wilting, are unable to survive for long periods at high temperature.

Generally, plants growing in solarized soil suffered less from wilt disease than plants in untreated soil. On the other hand, the technique is simple and economic without any negative side on natural environment. Populations of *F. oxysporum* were greatly reduced in a soil artificially infested with the highly pathogenic following solarization. Application of solarization for 8 weeks completely controlled wilt disease in plants and gave a yield production almost more than the plants in untreated soil (Tjamos et al., 2000; Saremi et. al., 2008). Normally, plant performance

was significantly better in solarized soil than in uninfected control soil, so the beneficial effects of this treatment would be suggested to wilt control (Gonzalez-Torres et al., 1993; Saremi et al., 2007). It has been reported that even after 40 days of solarization; the solarized plots contain significantly less number of fungi and nematodes as compared to the non solarized plots, which confirmed the durability of this process (Nico et al., 2003; Nafees et al., 2007).

Solarization causes physical, chemical and biological changes in the soil by raising soil temperatures from 7 - 15°C above the temperatures of untreated soil (Stapleton and DeVay, 1984; Chen and Katan, 1980). It has been reported that with solarization during July and August, the temperature achieved at levels between 5 and 20 cm below the soil surface were 45 - 55 and 39 - 45°C, respectively, in California (Katan, 1981). In Florida, the temperatures at depths of 5, 15 and 25 cm were recorded as 49.5, 46.0 and 41.5°C, respectively, in solarized soil (Chellemi et al., 1994). Soil solarization is an environmentally friendly soilborne pathogen control method utilized in agriculture, while the use of plastic films for soil solarization provides for an increased level of agricultural productivity and a reduction in the use of chemicals. Actually, using soil solarization for three weeks was previously shown to effectively control soil borne pathogens and sufficient to eliminate 91% of the P. cinnamomi population, while achieving total elimination after six weeks (Pinkas et al., 1984; Nafees et al., 2007; Harender and Sharma, 2009).

According to the authors' studies, the use of soil solarization would be suggested for the newly established orchard before planting Rosemary. Naturally, plant seedlings are more susceptible to soil borne pathogens than the growth plants. Consequently, we should try to protect them from early infection. The study's experiences showed that solarized soil with no high pathogen propagules were suitable condition for seedling growth, after which, plants would have a nice natural competition. However, pre planting application encourages agricultural growers to extend mostly this ornamental and economic plant and establish almost new healthy orchards.

Predominantly, the study on etiology of Rosemary wilting disease assisted in introducing a way for managing the limitation of Rosemary production in the case of an economical point of view. Any knowledge on Rosemary wilting and reduction in its damage would improve the Rosemary planting regarding to ornamental-medical productions. This project was the first experimental work on wilting disease as the main problem of Rosemary plant in Iran. The authors would also try to search out any other ways including biocontrol, nano technology and resistant variety for managing the wilting disease. Recognition of the casual agent of Rosemary wilting guides the study to work on the control management of disease and gives confidence to farmers for developing Rosemary plantations in different locations.

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