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Full Length Research Paper

Cultivation of Rosemary as an ornamental-medicinal plant and managing its root rot disease in northeast of Iran

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Productions of Rosemary as an ornamental-medical plant have recently developed in eastern parts of Iran. However, root rot disease have resulted serious problems such as yield loss on these plants especially in newly planted fields, where the suitable conditions allows the disease to build up. The present study has been carried out to assess the managing of root rot and wilting disease of Rosemary, caused by fungal soil-borne pathogens. For this mean, Rosemary fields in north eastern parts of Iran were visited, infected plants and soil were collected and their fungal pathogens were isolated. Three isolated fungi were identified as the main pathogen including *Phytophthora citrophthora*, *Rhizoctonia solani* and *Fusarium oxysporum*. Pathogenicity tests also proved that these isolated soil borne fungi were the casual agents of root rot disease. In addition for managing this disease, soil solarization treatments were applied to *P. citrophthora* and *F. oxysporum* for 6 weeks, in pre-planted field soils. Population density of *P. citrophthora* and *F. oxysporum* were decreased from 1800 and 1300 cfu/g to 300 and 200 cfu/g, respectively. This technique is recommended for medicine produce in managing Rosemary soil-borne disease before planting them in fields.

Key words: Rosemary, medicine, root rot, disease, managing.

INTRODUCTION

Evergreen shrubby plant, 'Rosemary (*Rosmarinus officinalis* L.)' is an ornamental-medicinal significant crop all over the world and originally native to the Mediterranean region (Evelyne et al., 2011; Tuberoso et al., 1998; Ture et al., 2009). Rosemary has a woody texture but, like other herbs, does not actually contain woody tissues. This plant is an aromatic plant that grows up to three feet tall and has green leaves and blue-violet flowers. The leaves are dark green above and paler beneath, about one inch long and very aromatic (Figure 1). Rosemary leaves are used as herbal medicine and are known to have powerful antioxidant properties which make them as medical or industrial plants all over the world (Doolaege et al., 2007; Evelyne et al., 2011; Singh et al., 2009; Tironi et al., 2009). The oil of Rosemary can be distilled from the flowering tops which can be used as a remedy for premature baldness, scurf and dandruff (Tuberoso et al., 1998). Practically, all the commercial oil is extracted from the stem and leaves of the wild plant before it is in flower (Abdel-Monaim et al., 2011; Doolaege et al., 2007; Hosseinzadeh et al., 2006; Tironi et al., 2009). Oil of Rosemary is also used as an ingredient in eau-de-cologne and there is high iron, calcium and vitamin B6 in Rosemary plant.

Rosemary compounds assist the acetylcholine breakdown, a brain chemical that allows the cells related with reasoning and memory to communicate with one another (Balch, 2002). Acetylcholine activates muscles, and is a major neurotransmitter in the nervous system. Commonly, chemical products of the Rosemary can be used for managing many human beings disorders such as headache, hair, skin and cosmetic industries (Oji-

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(a) (b)

Figure 1. (a), Rosemary plant in the field with dark green and very aromatic leaves; (b), the small, pale blue flowers inconspicuous.

Ardabily et al., 2006). Taking Rosemary bath can help chronic circulatory weakness counting varicose veins, bruises, and low blood pressure. The plant can be used as both fungicidal and diuretic which causes the “yeast” to be flush out of the body before it can cause an infection (Kennedy and Scholey, 2006; Marie-Elisabeth et al., 1996; Oji-Ardabily et al., 2006). Despite its many healthful uses, Rosemary can be quite toxic if consumed in large quantities, with coma, spasm and vomiting (Evelyne et al., 2011; Marie-Elisabeth et al., 1996).

There are several diseases that may infect Rosemary plants, some which may be fatal for these plants, and are often very difficult to get rid-off once they infect the shrub. One of the significant diseases is known as the root rot which can cause wilting in Rosemary plants and kill them in nearly all growing fields. This disease begins to decay and spread the rot to healthy roots, possibly killing the entire plant (Mirabolfathy and Ershad, 1993; Saremi, 2000). Fungal cases of root rot are caused by dormant fungus in the soil that takes hold when excess water is added to the plant.

This medicinal plant is mainly cultivated in northeast of Iran and it is expected to be planted in other locations of the country, since it is adapted to different climatic areas (Chiej, 1988; Kennedy and Scholey, 2006; Türe et al., 2009; Verhoeven et al., 2008). However, as previously mentioned, this economic plant suffers from wilting disease by soil-borne fungal pathogens. The main serious soil-borne fungal pathogens which causes root rot in Rosemary are reported to be *Rhizoctonia solani, Phytophthora citrophthora* and *Phytophthora cinnamomi* (Alvarez et al., 2007; Atkinson, 1982; Veghe and Berre, 1982; Verhoeven et al., 2008).

Generally, the *Phytophthora* spp. are indicated as the main plant pathogens, in many trees in various countries (Hall, 1996). Many techniques have been used to manage soil-borne pathogen diseases, among all soil solarization or solar heating has been received much attention in recent years (Bonanomi et al., 2008; Harender and Sharma, 2009; Triki et al., 2001). The aim of soil solarization is to harness solar energy and raise the temperature of moistened soil which results in reduction of soil-borne pathogens (Klein et al., 2011; Scopa et al., 2008).

The main purpose of this study was to identify the significant pathogens that cause root rot in Rosemary plants; in addition for the pathogens control management, soil solarization treatments were applied in pre-planted fields in northeast of Iran.

MATERIALS AND METHODS

Sample collection

Root samples of the towel infected Rosemary plants and soil around the roots were collected from four locations in Mashhad, northeast Iran. Three (3) samples from each infected tree and thoroughly 36 samples were collected then transferred to plant disease laboratory. Samples were cut into around 1.0 cm pieces, and then dipped in sodium hypochlorite for disinfection and sterilization. The pieces were rinsed in sterile distilled water, dried on sterile filter paper and plated on common media such as corn meal agar (CMA), potato dextrose agar (PDA), and water agar (WA) 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 used. The identification of the isolated fungal pathogens was based on valid diagnosis manuals for each pathogen (Alvarez et al., 2007; Burgess et al., 1994; Saremi, 2005).

Identification of fungal pathogens

General morphology characteristics including shape of sporangium, sporangiophore, hyphal diameter, optimum temperature for growth rate and others were studied for identification of *Phytophthora* species as the soil-borne pathogens 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 wavelengths (white light tube, 40 w) and fluctuating temperatures regime, 25°C during day and 20°C in night under 12 h photoperiod. Sporangiums were produced after 3 days incubation, then 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 produced sporangium’s 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 (Alvarez et al., 2007; Mirabolfathy et al., 2001).

Nucleus stain method was also carried out for evident identification of *Rhizoctonia* species isolated from infected samples and soil around the roots (Sneh et al., 1996). Two small wood pieces were placed on the filter paper on Petri dish and two lams were located on them, then a piece of mycelium was put on lams. The mycelium was grown and reached under the lams after 2 days, then lams were separated and their mycelium were pigmented with one drop of safranin 0/5% and one drop of fresh KOH 3% and visited by light microscope. Average hyphe diameters were 50 µ for isolated *Rhizoctonia* species just close to 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) also were studied in darkness conditions (Kim et al., 1994).

Fusarium pathogens isolated from infected plants were cultured in PDA as common medium and carnation leaf agar (CLA) as

selective medium. In point of fact, production of macro-conidia by *Fuasrium* species, which is the key factor for valid identification, is favored by CLA medium (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.

Soil-borne fungal pathogenicity test

Phytophthora species

Three Rosemary seedlings were planted in separate pots with sterile soil. The mycelium of pathogen was prepared on Prelate and hemp juice and 10 ml of inoculums were poured around the crown of each seedling. Before, 60 g of hemp grain was boiled in 1 L of water, then filtrated and 120 cc was added to 200 g of autoclaved prelate. Finally *Phytophthora* species were placed on Petri dish and kept at dark condition for 4 weeks to allow pathogen consumes all hemp juice as reported already (Banihashemi, 1989).

Rhizoctonia species

Propagules of *Rhizoctonia* species were prepared by placing 10 sterile wheat grains and a piece of mycelium (4 cm^2) on a flax and kept for 4 to 5 weeks at 28°C temperature to have adequate time for colonization. Seven colonized grains were put around roots of each seedling in each pot. Totally, three Rosemary seedlings were placed in pots containing sterile soil and watered once a week separately.

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) then put on a shaker for 3 days. The suspension in 10^6 colony forming unit (cfu) density was prepared and 200 ml of that was used for inoculating Rosemary seedling. Three of 4 months old seedlings were laid in the suspension (10^6 cfu) for 10 min before planting in pots. All three pots were kept in glasshouse with normal conditions and watered each week.

Pathogens interaction

The general interactions of three soil-borne pathogens on the Rosemary wilting disease were also investigated. Rosemary seedlings were planted on twelve pots with sterile soil separately. The suspension of *P. citrophthora* spores (10 ml 10^6cfu) as same *Fusarium oxysporum* were located on three seedling crowns. This method was followed for interaction of *P. citrophthora* with *R. solani*, as well as for interaction of *F. oxysporum* with *R. solani*. Regarding to using *R. solani* inoculums, seven colonized wheat grains were added rather than spore suspension. Three pots also were treated by adding only each pathogen 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 2 months and the results were investigated.

Soil solarization process

Soil solarization was carried out in four plots in infested soils close to Rosemary planting area in Mashhad in northeast Iran. 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. The plastic edges were buried in the trench to ensure that the plastic is held in place to stable the heat. The process was facilitated to raise solar energy and increase the temperature of moistened soil which is necessary for controlling the soil borne pathogens in soils. The effectiveness of soil solarization on propagule reduction of soil-borne pathogens in the field was assessed for 6 weeks as a common moment in time recommended by several researchers (Bonanomi et al., 2008; Harender and Sharma, 2009; Nafees et al., 2007).

Solarized soil assessment

Samples were collected at the start of soil solarization application and during 2, 4 and 6 weeks soil solarization process. Inoculums' densities of the two *F. oxysporum* and *P. citrophthora* pathogens were evaluated by tree soil samples throughout each collected sample. Solarized soil and unsolarized soil were collected each during four times in a randomized block design to assess propagule fluctuation of pathogens. The soil samples were taken from the 5 to 10 cm depth of each treated plot by means of a soil auger, then mixed together and transfer to 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., 2011). A similar bed with no plastic sheet was also served as unsolarized soil to compare effect of soil solarization on propagules reduction.

RESULTS

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

Pathogens isolation and identification

P. citrophthora

Obvious characteristics showed that *P. citrophthora* was the most identified isolate collected from CMA and WA media. This species showed nearly fast growing rate, and irregular shape hyphe diameter was 4.8 µm without any chlamydospre. However, sporangiophore was narrower than hyphe and sporangium demonstrated mostly apical connection (Figure 3). Sporangium was pear, oval or circular shaped and sometimes they showed irregular

(a) (b)

Figure 2. Wilting symptoms in rosemary (a) and whole death of some plants (b) in one site studied in Mashhad, northeast, Iran.

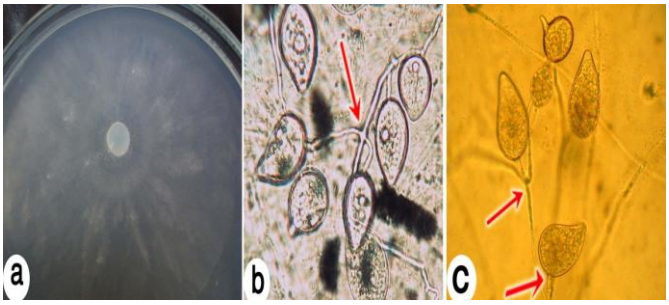

Figure 3. Morphology of P. citrophthora including (a), colony; (b), hyphe; (c), sporangium isolated from infected Rosemary, in Mashhad.

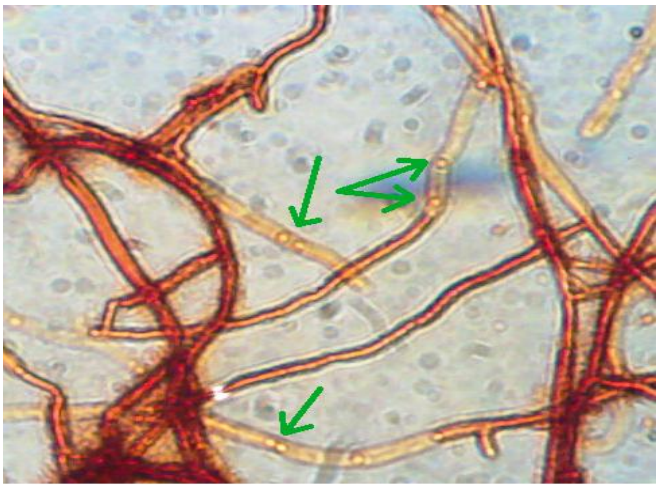

Figure 4. R. solani isolated from infected Rosemary showing binucleus cell and right-angle branched hyphe Mashhad, Iran.

and germinating another sporangium (Figure 3b and c). Dimension of sporangium was 24 to 44 µm length, 25 to 39 µm width and optimal temperature 25 to 29°C for growth of all isolates. Microscopic figures have been

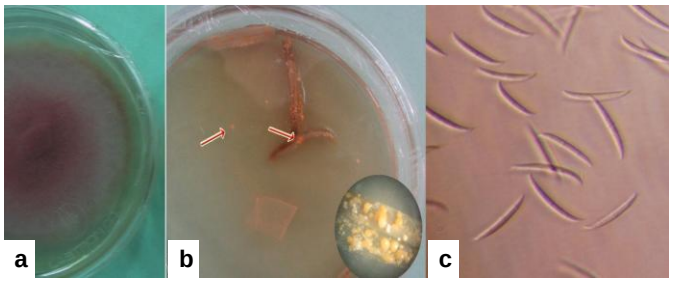

Figure 5. Morphology of *F. oxysporum* counting colony (a), spordochium and microconidi (b), macroconidia (c) isolated from infected Rosemary from Mashhad.

taken with suitable resolution in clear image, where they were identified by the width and height of the image (Figure 3).

R. solani

The most popular pathogen *R. solani* showed white and flat colony pigmentation with more than two nucleuses in each cell (Figure 4). The hyphe showed 4 to 7 µm in diameter and right-angle branched as well as appropriate growth on 25 to 37°C temperature. Some other soil-borne fungal species were also recovered in areas studied.

F. oxysporum

F. oxysporum on PDA media showed cottony and intense mycelium among white to purple pigmentation. This species produced much light orange spordochium and mono cellular microconidia with single false-head phialide (Figure 5a and 5b). Macroconidia was sickle up to long, with 3 to 4 cells and produced by small single conidiophores (Figure 5c). *F. oxysporum* showed some morphological criteria, including the shape of macroconidia, the structure of microconidiophore and the formation of chlamydospores (Figure 5). Microconidia were formed abundantly in false heads on short monophialides (Figure 6). This species showed the production lots of clamydospores which would be the capability of long survival in field soil. This cosmopolitan species was isolated repeatedly from collected samples recovered from areas studied.

Pathogens interaction

Pathogens interaction during 2 months in glasshouse created more wilting disease than when they were solitary. Namely, *F. oxysporum* and *R. solani* showed just one complete wilting (Figure 7b and 7d), while they produced three complete wilting after interacting together (Table 1). Although *P. citrophthora* caused two complete

Figure 6. Microconidia in false heads on short monophialides on hyphae of *F. oxysporum* from area studied.

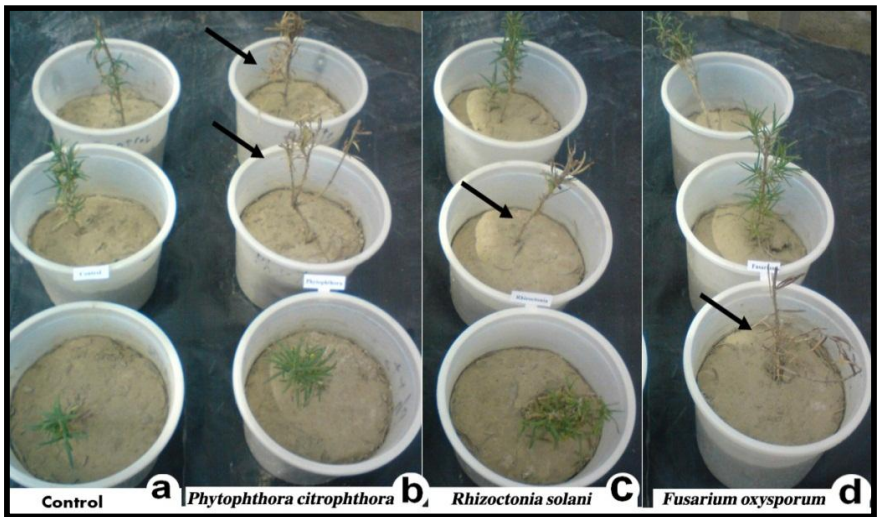

Figure 7. Wilting of Rosemary seedling plants on infected pots by isolated pathogens, (a), Control plants; (b), *P. citrophthora*; (c), *R. solani*; (d) *F. oxysporum* in glasshouse in Mashhad.

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

Pathogen	Rosemary wilting number	Rosemary colorless
P. citrophthora	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

* Treatments were replicate in a randomized block design.

wilting as solitary pathogen, but they caused three complete wilting when it act together with *F. solani*. The results also confirmed that *P. citrophthora* caused severe damage than other soil-borne fungal pathogens (Table 1).

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

Weeks after of soil solarization	*F. oxysporum*		*P. citrophthora*	
	Non-solarized (cfu/g)	Solarized (cfu/g)	Non-solarized (cfu/g)	Solarized (cfu/g)
0	1800	1800	1300	1300
2	1700	1100	1300	900
4	1800	700	1200	500
5	1800	400	1300	300
6	1700	300	1200	200

*Treatments were replicate in a randomized block design.

The 4 months old collected Rosemary seedlings showed the various effects of considered pathogens on Rosemary wilting disease in glasshouse. There were various influences on treated plants on pots, the pathogens interaction showed most involve on Rosemary wilting occurrence (Table 1).

Inoculum of pathogens in solarized soils

Propagules of *F. oxysporum* and *P. citrophthora* were decreased quickly after 2 weeks soil solarization application in pre-planted field. Indigenous population density were 1300 (cfu/g) for *P. citrophthora* and 1800 cfu/g for *F. oxysporum* in the natural field at the start of soil solarization. The mean of propagule density for *F. oxysporum* was clearly reduced up to 1100 cfu/g after 2 weeks, and then reduced to 700 cfu/g after 4 weeks. The population of the pathogen was also reduced to 400 and 300 cfu/g 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 during 2, 4, 5 and 6 weeks of solarization application in field soil (Table 2). In point 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

The positive functions of Rosemary plant have led producers to develop its plantations in many parts of the world. Component of Rosemary oil are favourite roles consequently researchers attempt to get their metabolites all over the world (Doolaege et al., 2007; Tironi et al., 2009; Abdel-Monaim et al., 2011). However, several diseases can be serious problems for Rosemary plants and are often very difficult to manage. One of these diseases which Rosemary is dealing with is the wilting disease, causing high amount of yield reduction in fields. Generally, soil-borne fungal pathogens have been reported to cause wilting diseases on various vegetations in Iran from long time ago (Saberi-Riseh et al., 2004).

Three main soil-borne fungal pathogens, *Phytophthora*, *Fusarium* and *Rhizoctonia* species have been reported to cause wilting 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 have caused root rot and wilting disease that resulted yield losses on infected plants all over the world (Deeksha et al., 2009; Singleton et al., 1992; Yangui et al., 2008). Our findings 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 the study areas.

This is the first time that *P. citrophthora* is reported as causal agent on Rosemary wilting disease in Iran and apparently this pathogen caused more damage than the other pathogens. Several studies reported *P. citrophthora* as the main cause of root rot disease on many other ornamental shrub, flowers and trees plants, all over the world (Alvarez et al., 2007; Mirabolfathy et al., 2001; Mirabolfathy and Ershad, 1993; Pettitt, et al., 2008). Our pathogenicity test in glasshouse proved susceptibility of the Rosemary shrub to this pathogen. Although three mentioned fungal pathogens were identified as major agents of wilting on the Rosemary, other soil-borne pathogens such as bacteria and nematode species can also associate in the wilting disease (Modupe et al., 2007). Experimental test and field survey showed *Helicotylenchus* spp. as a main nematode which assists wilting disease on plants in some locations (Barbercheck and Broembsen 1986; Nafees et al., 2007; Nico et al., 2003).

As previously mentioned, 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 by a simple and economic approach. Numerous chemical control and biological control process were reported as non-effective in managing soil-borne pathogens in the fields (Modupe et al., 2007; Mojibur et al., 2006). Nevertheless, soil solarization method in particular for new established orchard has showed reasonable results in many studies (Barbercheck and Broembsen, 1986; Bonanomi et al.,

2008; Klein et al., 2011; Nico et al., 2003; Scopa et al., 2008; Saremi et al., 2007). The managing *Verticillium* wilt by soil solarization in new established olive orchards has been tried in south Spain and other countries (Rodríguez et al., 2003). The success of this method is based on the fact that plant pathogens, casual agents on Rosemary wilting, are unable to survive for long periods at high temperature.

Using soil solarization for 3 weeks have shown to control soil-borne pathogens in several studies, for example eliminating 91% of the *P. cinnamomi* population after 3 weeks of application and a total elimination of the pathogen after 6 weeks of application (Harender and Sharma, 2009; Nafees et al., 2007; Pinkas et al., 1984). In this study, similar trend were seen for the *F. oxysporum* and *P. citrophthora,* where significant reduction were seen after 6 weeks of solarization. Soil solarization increases the soil temperature. Using solarized soil showed a symptom reduction of wilting in newly established pistachio and olive orchards in the treated trees compared with the untreated trees on other project (Saremi et al., 2010). Frequently, by raising soil temperatures, solarization causes physical, chemical, and biological changes in the soil (Chen and Katan, 1980; Stapleton and DeVay, 1984).

We suggest the use of soil solarization for new established orchard before planting the Rosemary plants. Naturally, plant seedlings are more susceptible to soil-borne pathogens than the growing plants, consequently we should try to protect them from early infection. Our experiences showed that solarized soils with no high pathogen propagules were suitable for seedling growth and expect to have natural competition. Pre-planting application encourage agricultural growers to extend this most ornamental and economic plant and establish new nearly healthy orchards.

The study on etiology of Rosemary wilting disease was an assistance of introducing a way for managing the limitation of Rosemary production. Any knowledge on Rosemary wilting and reducing its damage would improve Rosemary ornamental-medical productions. This project was the first experimental work, on wilting disease as main problem of Rosemary plant in Iran. Our further approach is to search and explore other ways including biocontrol, nano technology and resistant variety for managing the wilting disease. Isolation and identification of causal agent of Rosemary wilting disease direct us to manage the disease and give confidence to farmers for developing Rosemary plantations in different locations.

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