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The Effect of Temperature and Culture Media on Mycelial Growth of *Phytophthora citrophthora* Causing Gummosis, Crown and Root Rot on Lemon Seedlings

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ABSTRACT
Infected plant materials with *Phytophthora citrophthora* were collected from the outer and inner bark of lemon seedlings showing gummosis, crown and root rot in citrus orchard in Hatay province. *Phytophthora* isolates were obtained from infected five different lemon seedlings and purified on modified PARPH and PDA with different concentrations of antibiotics in vitro conditions. On the basis of colony morphology, five isolates were identified as *P. citrophthora*. The effect of culture media and temperature on mycelial growth of *P. citrophthora* were investigated. Colony patterns of *P. citrophthora* were performed on PDA, CMA, OMA, CA and HSA media and identified their microscopic structures. The radial mycelial growth of isolates was not dependent on media. In general, five different colony patterns of *P. citrophthora* were observed on the culture media; light and dense rosette pattern, slightly stellate pattern, stellate striated pattern, slightly petaloid pattern and non-pattern colony. The rate of the mycelial growth increased as temperature increased up to 25 °C and then decreased rapidly as temperature increased. Isolate Pc3 was determined the slowest while Pc1 and Pc5 were the fastest-growing isolates on agar media. The best mycelial growth of *P. citrophthora* was obtained on HAS medium while the least mycelial growth was obtained on CMA medium. Optimum temperature and the rate of mycelial growth were determined as 24.6 °C and 9.7 mm d^{-1} respectively.
Keywords: Citrus limon, Culture medium, Colony pattern, Mycelial growth, Temperature

ÖZ
Limon Fidanlarında Zamklanma, Kök ve Kök Boğazı Çürüklüğüne Neden Olan *Phytophthora citrophthora*'nın Miselyal Gelişimi Üzerine Sıcaklık ve Besi Ortamlarının Etkisi
Hatay ili narenciye bahçesinde *Phytophthora citrophthora* ile infekteli limon fidanlarının gövde ve kök boğazında zamklanma ve kök çürüklüğü belirtisi gösteren iç, dış kabuk dokuları ile kökler kesilerek alınmıştır. *P. citrophthora* izolatları, infekteli beş farklı limon fidanından elde edilmiş ve modifiye PARPH ve PDA üzerinde farklı konsantrasyonlarda saflaştırılmıştır. In vitro koşullarda koloni morfolojisine göre beş izolatın hepsi *P. citrophthora* olarak tanımlanmıştır. Kültür ortamının ve sıcaklığın *P. citrophthora*'nın misel gelişimi üzerindeki etkisi araştırılmış, PDA, CMA, OMA, CA ve HSA ortamlarında koloni şekilleri incelenmiş ve mikroskobik yapıları tanımlanmıştır. İzolatların miselyal gelişiminin besi ortamı ile ilişkili olmadığı saptanmıştır. Genel olarak, kültür ortamında *P. citrophthora*'nın beş farklı koloni modeli; hafif ve yoğun rozet deseni, hafif yıldız deseni, yıldız çizgili desen, hafif petaloid desen ve desensiz koloni gözlenmiştir. Misel büyüme hızının, sıcaklığın 25 °C'ye kadar yükseldikçe arttığı, daha sonra sıcaklık arttıkça büyümenin hızla azaldığı gözlenmiştir. İzolat Pc3 agar besiyerinde en yavaş büyüyen izolat iken Pc1 ve Pc5 en hızlı büyüyen izolat olarak belirlenmiştir. *P. citrophthora*'nın en iyi misel gelişimi HSA besi ortamında elde edilirken iken, en az misel gelişimi CMA besi ortamında sağlanmıştır. Optimum sıcaklık ve misel gelişim oranı sırasıyla 24.6 °C ve 9.7 mm/gün olarak belirlenmiştir.
Anahtar kelimeler: Citrus limon, Kültür ortamı, Koloni deseni, Miselyal gelişim, Sıcaklık

INTRODUCTION
Phytophthora citrophthora (R.E. Sm. & E.H. Sm.) Leonian (1925) is an oomycete plant pathogen that causes of the gummosis, foot rot, fibrous root rot, brown fruit rot on citrus, and numerous economically important other crops such as strawberry, pepper, melon, squash, apricot, cherry, walnut, apple, kiwifruit and forest trees (Erwin and Ribeiro, 1996; Akıllı *et al.*, 2012). *P. citrophthora* was first isolated by Smith and Smith (1906) from rotted lemons, and also several species of *Phytophthora*, such as *P. nicotianae* (sny = *P. parasitica*), *P. palmivora* and *P. syringae* have been reported as a pathogen in citrus growing regions of the world (Erwin and Ribeiro, 1996; Erkılıç and Canıhoş, 1999). It has been known that the most common and

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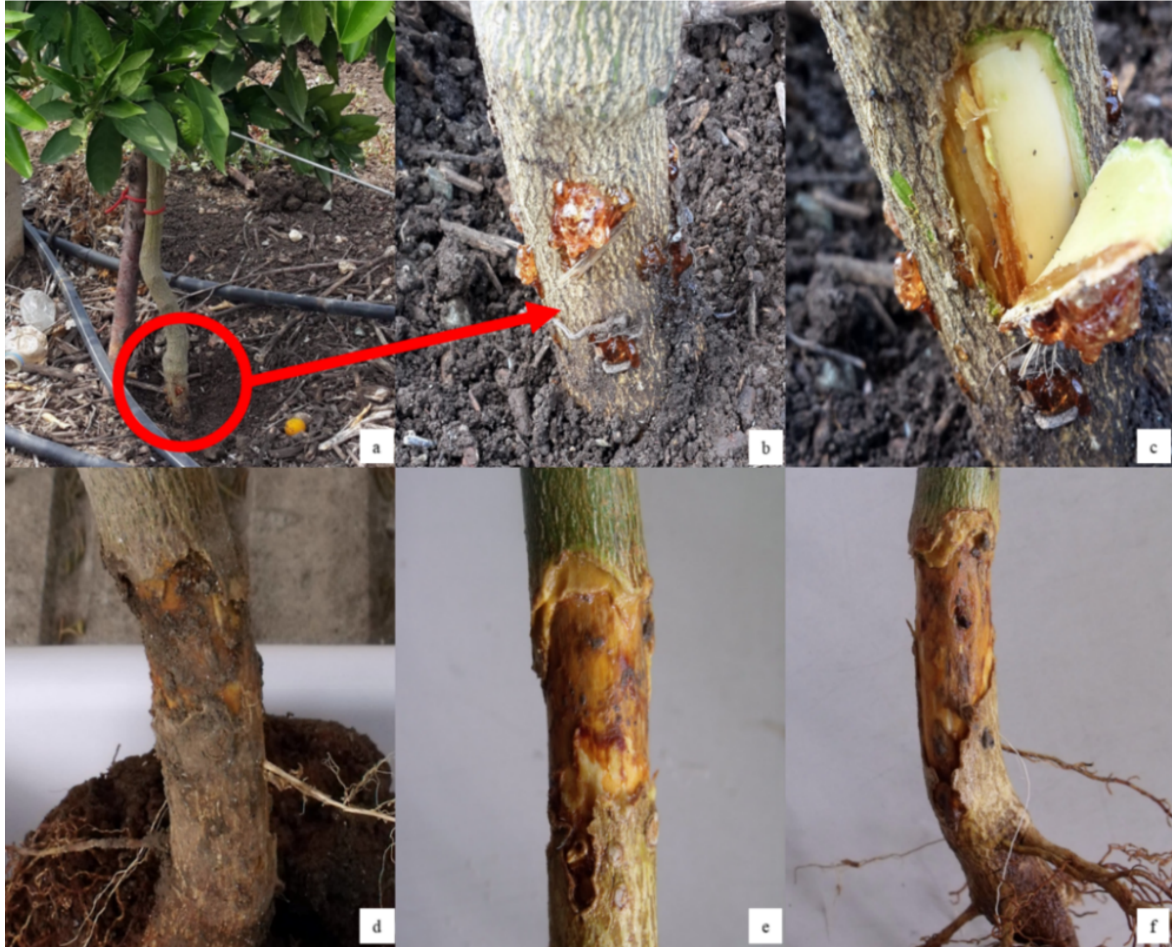

Figure 1. Disease simptoms of *Phytophthora citrophthora* on lower stem bases of lemon seedlings. Gummosis (a–b); Sharply discoloration between diseased and healthy wood (c); Reddish brown necrotic lesions as a ring appearances of inner bark (d–f).

significant *Phytophthora* species mentioned above in citrus orchards are the *P. citrophthora* and *P. nicotianae* (Erwin and Ribeiro, 1996). From these species affecting citrus trees, *P. citrophthora* can be predominant species in the Mediterranean climate (Alvarez *et al*., 2008; Yaseen *et al*., 2010).

Identification of *P. citrophthora* was based primarily on morphological features of sporangia, antheridia, oogonia, oospore and chlamydospores along with other criteria like cardinal growth temperature, colony morphology in culture medium (Waterhouse 1963; Newhook *et al*., 1978; Erwin and Ribeiro, 1996). However, this pathogen seems to have a poor development on culture medium and this contributes to the failure in identification, isolation and growth of *P. citrophthora*. In addition, it is necessary to culture the fungus in artificial medium and produce inoculum in the form of sporangium suspension. It has known that sporangia are used as inoculum in pathogenicity tests by researchers. In spite of identification of *the P. citrophthora*, researchers have very little information associated with its biology, including colony patterns, daily mycelial growth and cardinal temperature. Better management strategies can be developed to control *P. citrophthora* in orchards if its biological characters must have been well identified.

The objective of this research is to study the effects of culture media and incubation temperature on mycelial growth of five local isolates of *P. citrophthora*.

MATERIALS and METHODS

Isolation and identification of *Phytophthora citrophthora*

The root and crown tissue pieces were taken from 5 diseased lemon seedlings showing gummosis symptoms with wet, dark discolorations on the stem bases in Hatay province of the Southern Turkey in March 2020 (Figure 1). Isolates of *Phytophthora* were obtained applying the direct infected tissue isolation technique and using the modified PARPH medium according to Alvarez *et al*. (2008). Modification in the medium was addition of 100 ml/L hymexazol, 100 mg/L benomyl and 10 mg/L rifampicin to prevent development of *Fusarium* and *Pythium* species. For

isolation of the pathogen, lesions on outer and inner bark tissues were surface-sterilized by immersing in 70% ethanol for 10 to 20 s and then washed three times by autoclaved distilled water. The samples were dried on filter papers by putting in sterile bench. Then, four and five small pieces (0.5–10 mm^2) were placed onto modified PARPH medium. Plates were incubated at 25 °C in 24 h darkness for 3–4 days. Pure cultures of *Phytophthora* sp. were obtained by hyphal tip isolation method. Twenty-five isolates of *Phytophthora* sp. were obtained from infected lemon tissues. Five isolates were selected and used in all experiments.

Phytophthora isolates were identified based on standard morphology methods: types of colony morphology and pattern on culture medium, type and size of sporangia, cardinal growth temperature (Erwin and Ribeiro, 1996; Das *et al*., 2016). Firstly, the isolates of *Phytophthora* were divided by colony morphology secondly, five isolates were examined for sporangial morphology. Sporangial morphology [(lengths (l), breadth (b), length/breadth ratio (l/b), papillate or semi-papillate sporangium of each isolate] was determined from 50 conidia with a compound microscope camera (Leica, DMA 750).

Effect of culture medium on mycelial growth of *P. citrophthora*

Growth characters of *P. citrophthora* were presented on the five different media (Table 1). Potato Dextrose Agar (PDA, Merck) and Cornmeal Agar (CMA, Sigma) were prepared according to label directions. Carrot Agar (CA): Peeled and sliced carrots (200 g) were washed with running tap water. Eight hundred milliliter de-ionized water was added in the sliced carrots and boiled for 20 minutes. Then carrots were crushed and blended mixture was passed through four layer of cheesecloth. After that the filtrate was brought to 1 L with de-ionized water and 15 g of agar (Merck) was added on to filtrate and autoclaved at 121 °C at 15 psi pressure for 20 minutes. Hemp Seed Agar (HSA): Hemp seeds (50 g L^{-1}) were autoclaved as described above in 1 L of de-ionized water. The hemp seeds were removed by straining the liquid through four layer of cheesecloth, and 15 g of agar (Merck) was added to the filtrate. Total volume was adjusted to 1L before re-autoclaving. Oatmeal Agar (OMA): Single-grain oatmeal (60 g L^{-1}) autoclaved as described above. in 1 L of de-ionized water. The single-grain oats were removed by straining through four layer of cheesecloth, and 15 g of agar (Merck) was added to the filtrate. Total volume was adjusted to 1 L before re autoclaving.

To carry out the experiment, a 5-mm-diameter fungal plug from a week old colony was placed in the center of plates containing 20 ml of medium. Isolates were incubated on all of media at 25 °C in darkness for 5 days. Colony diameter of all isolates in each plate was measured along two axes perpendicular (crossing) at 5 d after inoculation, and the two measurements were averaged. The complete randomized design with four replications was used for this trail and the colony morphology also was noted.

Effect of temperature on mycelial growth of *P. citrophthora*

In order to determine the effect of different temperatures (5 °C intervals from 5 to 35 °C) on radial mycelial growth, a 5-mm-diameter fungal plug from a week old colony was placed in the center of plates containing 20 ml of PDA. The colony diameter in each plate was measured as described above. The complete randomized design with four replications was used for this trail. Regression curves were obtained according to Vial *et al*. (2006). Two parameters were calculated in the fitted equation for each isolate: optimum temperature for radial growth and maximum daily radial growth (millimeters per day).

Statistical analysis

The data were analysed using ANOVA (SPSS v.20) to determine the significance of differences between treatments and means were compared using Tukey's HSD multiple comparison test (P = 0.05).

RESULTS and DISCUSSION

Isolation and identification of *P. citrophthora*

Gummosis, crown and root rot diseases in citrus was determined in the past in Turkey (Karel, 1958) and it was observed on lemon, mandarin and orange trees (Erwin and Riberio, 1996). Symptoms of *Phytophthora* root and crown rot disease first appear in early spring. Diagnostic symptoms were found also bellow the grafting zone as large dark spots clearly defined from healthy tissues (Figure 1). Reddish brown lesions with wet and gummosis appearance were found on crowns and roots of the infected lemon trees or seedlings (Figure 1a–b). There was a discoloration distinct margin between diseased and healthy wood (Figure 1c) and infected lower stem becomes dark brown and lesions could spread as rings and also upwards on the trunk of the seedlings (Figure 1d–f). Observed symptoms in this study were exactly identical to those described earlier by Alverez *et al*. (2011) and Zouaoui *et al*. (2016). Moreover, disease symptoms, Brentu and Vicent (2015) reported that gummosis, crown and root rot disease on citrus trees infecting by *P. citrophthora* in Ghana.

The isolates obtained in our laboratory on modified PARPH medium were belonged mainly to the genus *Phytophthora*. Identification of the first five isolates obtained from 4–5 years old lemons with symptoms of collar rot. On the basis of colony morphology, five sterile isolates were identified as *P. citrophthora* (Newhook *et al*., 1978; Erwin and Ribeiro, 1996).

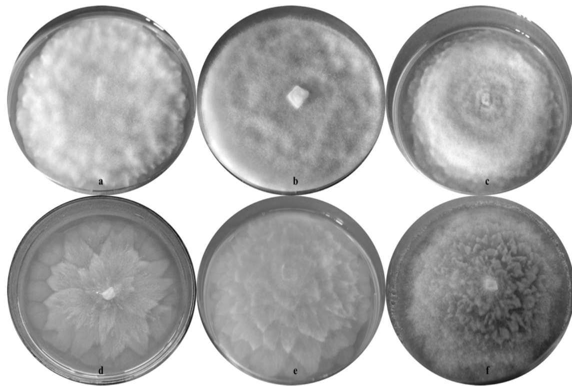

Figure 2. Colony morphology of *P. citrophthora* isolate Pc1 on agar media after 14 day of incubation. Dense cottony mycelium without pattern on PDA (a); cottony mycelium without pattern on PDA (b); fluffy cottony mycelium with slightly stellate pattern on OMA (c); light rosette pattern on CA (d); dense rosette pattern on CMA (e); stellate striated pattern on HSA (f).

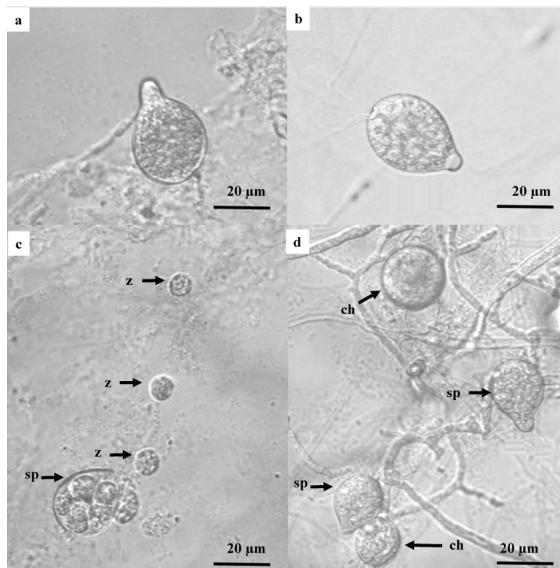

Figure 3. Sporangial characteristic of *P. citrophthora* isolate Pc1. Papillate (a), semi-papillate sporangium (b), zoospore releasing from sporangium (c = z: zoospore; sp: sporangium), chlamydospore (d = ch: chlamydospore; sp: sporangium). Bar = 20 µm.

These isolates were showed uniform, stellate and rose petal-like patterns and slightly white cottony colonies on culture media (Figure 2). Hyphae were 5 to 8 µm. Sporangium shapes were ranged from ovoid to lemoniform (Figure 3), but one papillated (Figure 3a) and semi-papillated (Figure 3b). Sporangia were of about 43.5 ± 9.5 × 30.7 ± 7.9 (L average ± SD × B average ± SD) µm and length / breath ration of sporangia was 1.4 ± 0.2 µm. Results of this study were in agreement with the report of Mounde *et al.* (2012) and intercalary or terminal chlamydospores and mycelial swellings were produced on culture medium (Figure 3d). As in earliar study sexual mating of *P. citrophthora* was rare (Erwin and Riberio, 1996). In addition, Cohen *et al.* (2003) reported that about 70% of the isolates of *P. citrophthora* formed a large group of predominantly sterile isolates, and also Alvarez *et al.* (2011) reported that only one of 134 *Phytophthora* isolates produced oospores. However, this study determined that all isolates of *P. citrophthora* were sterile because they did not produce oospores. Our study supported the results of researches by Vial *et al.* (2006) and by Mounde *et al.* (2012).

Effect of the culture media on colony morphology

The mycelial growth ratio and colony pattern of *P. citrophthora* isolates were significantly ($P < 0.05$) affected by content of culture media (Table 1). Generally, HSA and OMA were most favorable for fast radial growth of mycelium of all isolates tested. At 25 °C, colonies on these two media reached the edge of the plates after 5 days of inoculation. Radial mycelial growth on HAS and OMA were initially poor with scanty and sparse mycelium. Isolates of *P. citrophthora* formed fluffy cottony mycelium with slightly stellate pattern an OMA (Figure 2) and stellate striated pattern on HAS after 14 d of inoculation. On PDA, all of isolates formed mostly cottony mycelium with slightly petaloid pattern. On CA and CMA the isolates of *Phytophthora* formed a generally submerged white or cream colored rosette colony patterns. Consequently, there was a significantly difference among isolates ($F_{(4, 75; 0.05)} = 4.5$, $P < 0.05$) and media ($F_{(4, 75; 0.05)} = 944.1$, $P < 0.05$) on radial mycelial growth. However, the interaction ($P > 0.05$) between media and isolates was not founded (Table 1). In other words, the radial mycelial growth of *P. citrophthora* isolates were not depend on content of the media. In general Pc1 and Pc5 was the fastest growing isolate and HSA medium was determined as the medium that provided the fasted growth of the isolates (Table 1).

These results agreed with those reported by Alvarez *et al.* (2011) demonstrated that colony patterns of *P. citrophthora* isolates were ranged from chrysanthemum (stellate), rosette and without patterns (uniform) on different culture media. Besides, It was determined in previous studies that all isolates of *P. citrophthora* formed stellate, petalloid (Ann *et al.*, 2010) and stellate, rosette, slightly cottony and non-patterned colonies (Mounde *et al.*, 2012) on PDA medium. On the other hand, it has been reported in recent studies (Vial *et al.*, 2006; Das *et al.*, 2016) as well as previous studies (Waterhouse *et al.*, 1983) that *P. citrophthora* isolates form the same colony patterns on CMA and V8. When the results obtained from this study were

Table 1. Influence of culture media on daily radial growth of *P. citrophthora* isolates at 25 °C in darkness for 5 days

Media[a]	Isolates					Main Effect Media	Colony Morfology[b]
	Pc1	Pc2	Pc3	Pc4	Pc5		
PDA	44.6[c]	43.3	45.1	41.6	46.4	44.1 c	Cottony, no pattern
CMA	42.8	42.0	40.9	41.3	42.8	42.0 d	Dense rosette pattern
OMA	54.5	53.3	52.4	54.1	55.0	53.9 b	Cottony, Slightly stellate pattern
HSA	62.6	62.5	61.1	63.4	64.1	62.7 a	Stellate striated pattern
CA	35.4	34.8	34.6	34.6	34.8	31.8 e	Light rosette parrern
Main Effect Isolates	48.8 ab	47.2 b	46.8 b	47.0 b	48.6 a		

[a] PDA = Potato dextrose agar; CMA = Cornmeal agar; OMA = Oatmeal agar; HSA = Hemp seed agar; CA = Carrot agar
[b] Colony patterns were formed for 14 days after inoculation.
[c] Values are the means of repetitions (four replicate plates of each medium for each isolate). Values within a row and column with the same letter are not significantly different according to Tukey's HSD multiple comparison test (P = 0.05).

compared with the literatures, it was determined that *P. citrophthora* isolates formed the same colony patterns on different culture media. Especially, considering that *P. parasitica*, *P. nicotianae* and *P. syringae* form similar colony patterns, it can be said that colony patterns are characteristic at genus level, but not at species level (Mounde *et al*., 2012; Prasad *et al*., 2017).

Effect of temperature on radial mycelial growth

The radial mycelial growth of five *P. citrophthora* isolates followed similar trends in response to changes at different temperatures (Figure 4 and 5). *P. citrophthora* isolates grew at 5 and 30 °C, but exhibited no growth at 35 °C on PDA for 5 days (Figure 4). The rate of mycelial growth increased as temperature increased up to 25 °C and then decreased rapidly as temperature increased. There was a significant interaction ($F_{(24,\ 105;\ 0.05)}$ = 10.4; P < 0.0001) between incubation temperature and isolate. Namely, the mycelial growth rates of *P. citrophthora* isolates were incubation temperature dependent. Generally, all temperatures, Pc3 was the fastest growing isolate and Pc5 was the slowest growing isolate on PDA (Figure 4). Optimum temperature and the rate of mycelial growth was determined as 24.6 °C and 9.7 mm d^{-1} respectively.

Moreover, small differences in colony morphology of all isolates were observed at 10, 15, 20 and 30 °C on PDA. The isolates formed a mostly submerged and cream colored light and dense rosette colony patterns at 10 and 15 °C. Conversely, the they formed fluffy cottony mycelium with slightly stellate colony pattern, stellate striated colony pattern and cottony mycelium with slightly petalloid colony pattern at 20 and 30 °C. Overall, five different colony patterns of *P. citrophthora* were observed on PDA. Similar to this study, Alvarez *et al*. (2011), demostrated that the isolates of *P. citrophthora* grew at 5 and 32 °C, but exhibited no mycelial growth at 35 °C on PDA medium. As well as this study, Vial *et al*. (2006) reported that the optimum growth temperature of *P. citrophthora* isolates causing gummosis, crown and root rot disease on citrus was

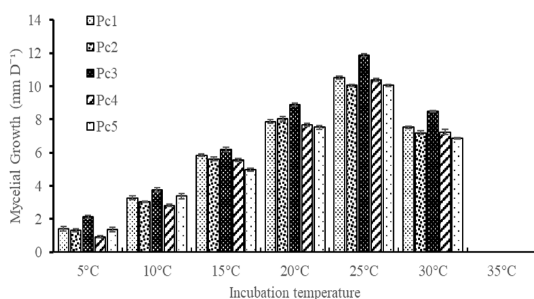

Figure 4. Effect of temperature on the daily radial mycelial growth rate of five isolates of *P. citrophthora* on PDA medium for five days. Bar = standard error of mean.

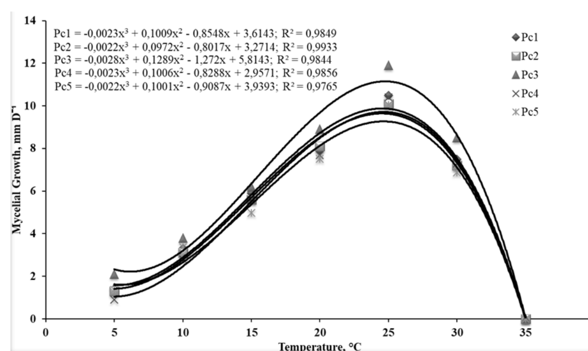

Figure 5. Mycelial growth temperature curves obtained with isolates of *P. citrophthora* obtained from lemon seedlings.

between 25 and 30 °C, and they showed no growth at 35 °C. The temperature requirements all of five isolates of *P. citrophthora* (range 5–30 °C; optimum 24.6 °C) as defined in this study agreed with earlier study in Taiwan where *P. citrophthora* was identified as a pathogen on fruit rot of strawberry by Kao and Leu (1979). In addition, Alvarez *et al*. (2011) reported that the optimum growth temperature of *P. citrophthora* isolates was variable, ranging from 22.9 to 28.9 °C.

The results of this study indicated that the mycelial growth of *P. citrophthora* isolates were not depend on content of media, but incubation temperature dependent. Isolates of *P. citrophthora* formed submerged and cream colored colonies at low temperatures (10 and 15 °C), but at high temperatures (30 °C) dense and light cottony mycelium with slightly stellate, rosette, petalloid or without patterns were observed. Totally, five different colony patterns of *P. citrophthora* were observed on culture media. On the other hand, there was a significant interaction between temperature and isolates on mycelial growth. The Pc3 from *P. citrophthora* isolates was the fastest growing isolate and Pc5 was the slowest growing isolate on PDA. Results are also useful for epidemiology and pathology *of P. citrophthora* under natural conditions. Such studies may improve our understanding of conditions required for initial disease occurance and development of suitable pest management system for this pathogen. Ability of *P. citrophthora* to grow at comparatively high temperature (25-30 °C) can explain its appearance in Mediterranean Region of Turkey.

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