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Effect of temperature and inoculum density on disease intensity of *Phytophthora parsiana*

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ABSTRACT- The effects of inoculum density and temperature on the disease intensity of *Phytophthora parsiana* on almond seedlings were investigated. Almond seeds (Rabie and Kaghazi cultivars) were placed in moist vermiculite at 4°C for 45 days. Germinated seeds were sown in a soil: sand mixture (2:1 v/v) and grown in greenhouse (18°C-25°C). One-month-old seedlings were transferred from the greenhouse to the growth chambers set at 15, 18, 20, 25,30 and 32°C. The seedlings were subsequently inoculated either with mycelium of *P. parsiana* grown for 4-6 weeks on vermiculite amended with hemp seed extract or with zoospore (10^3, 10^4, 10^5 and 10^6 ml^{-1}) by root dip method. The effect of temperature, inoculum density and their interaction on seedling mortality was measured. The results indicated that all three factors had significant effects on seedling mortality. While the highest disease incidence (100% mortality in almond seedlings) occurred at 30°C and 32°C, no mortality was observed at 15°C and18°C. Increasing temperature from 20°C to 30°C and inoculum rate from 10^3 to 10^6 zoospores ml^{-1} increased disease incidence significantly. Higher temperatures and inoculum densities also caused significant increases in the colonization level of the crown, main and lateral roots as well as reductions in the fresh and dry root weights of the seedlings.

INTRODUCTION

Some nonpapillate species of *Phytophthora*, such as *P. polonica* (Belbahri et al., 2006), *P. irrigata* (Hong et al., 2008), *P. parsiana* (Mostowfizadeh-Ghalamfarsa et al., 2008), and *P. hydropathica* (Hong et al., 2010) have been reported as high-temperature-tolerant species in recent years. These species may be distinguished from each other with a few morphological characteristics, growth temperature maxima and DNA sequence analysis (Hong et al., 2010; Mostowfizadeh-Ghalamfarsa et al., 2008).

The closest species to *P. parsiana* are *P. hydropathica* and *P. irrigata* (Hong et al., 2010). Among 11 isolates of *P. hydropathica*, seven isolates are clustered with two isolates of *P. parsiana*, but the other isolates were distinct from these two isolates of *P.parsiana* based on DNA fingerprinting technology. (Hong et al., 2010). In addition, *P. hydropathica* can grow at a higher temperature (maximum of 40°C) than *P. parsiana* and it has been isolated from irrigated nurseries during hot summer months. Recent studies showed that *P. parsiana* is one of the causal agents of pistachio gummosis in Iran (Hajebrahimi and Banihashemi, 2011). Since its first reports a decade ago (Banihashemi and Gheisi, 1993), this species has been widely isolated from pistachio orchards in Kerman, Rafsanjan and Yazd provinces (Rafiee and Banihashemi, 2012).

Temperature is the most important physical factor which favors the growth and pathogenicity of all *Phytophthora* species. The effect of temperature on pathogenicity of *Phytophthora* species has been studied extensively. Zentmyer (1981) reported a close relationship between infection of avocado seedlings with *P. cinnamomi* and soil temperature and showed that maximum infection occurred during the summer and fall when soil temperatures reached 24.5°C-25.5°C. In many cases, the severity of root rot symptoms caused by root diseases increases when temperature approaches 30°C, but a slower development of the symptoms has been documented at lower or higher temperatures (Dhingra and Sinclair 1985). Extreme temperatures prevent disease development and they may be the best indicator of periods of pathogen inactivity (Thomidis, 2003). The host plant vulnerability to *Phytophthora* species is greatly influenced by zoospore concentrations. It has been reported that high levels of disease development caused by some Pythiaceous fungi resulted from very low inoculum densities (Raftoyannis and Dick, 2002).

Results from previous studies indicate that *P. parsiana* can grow at relatively high temperatures with

an *in vitro* optimum temperature of 30°C. However, knowledge regarding the suitable temperatures and zoospore densities required for optimal infection of host plants of this pathogen under flooded conditions is lacking. The aim of this study was to analyze the effects of inoculum levels at various temperatures on intensity of root rot and wilt in almond which is a susceptible host of *P. parsiana*.

MATERIALS AND METHODS

Plant Growth

One-month-old local almond seedlings (Rabie and Kaghazi cultivars) were used for all experiments. Almond seeds were washed and surface sterilized in 0.5% sodium hypochlorite and incubated in moist vermiculite at 4°C for 45 days. Germinated seeds were transferred to 20 cm diameter pots containing steam sterilized soil and sand mixture (2:1v/v) and were incubated in a greenhouse with temperatures between 18°C and 25°C. One-month-old seedlings (35-40 cm in height) were transferred to growth chambers maintained at 15, 18, 20, 25, 30, and 32 °C with a 16-h photoperiod.

Inoculum Preparation

Mycelial inoculum of *P. parsiana* (isolate pH 21-3-08) was produced on vermiculite amended with hemp seed extract (Banihashemi and Fatehi, 1989) and was used to evaluate the effect of temperature on seedling infection. To prepare the inoculum , four plugs of young colony of *P. parsiana* (isolate pH 21-3-08) were inoculated into sterilized vermiculite amended with hemp seed extract. The growing medium consisted of 200ml of vermiculite amended with 120ml of hemp seed extract (extract of 60g of hemp seeds per 1l of distilled water) and incubated at room temperature in the dark for four weeks (Banihashemi and Fatehi, 1989). To produce zoospore, three colonized agar pieces (2×3 mm) from the edge of V8 agar were transferred to the Petri dishes containing 20 ml of sterile distilled water and placed under fluorescent illumination at room temperature. Zoospores were released within approximately 20 hours and 10^3, 10^4, 10^5, 10^6 zoospore ml^{-1}suspensions were prepared in sterile distilled water. Zoospores concentrations were checked under the microscope using a hemocytometer slide.

Inoculation

One-month-old almond seedlings were inoculated with mycelia inoculum prepared on vermiculite amended with hemp seed extract. Fifty ml of inocular was placed around the basal stem of each plant, and all pots were kept flooded overnight.

Plants inoculated with vermiculite amended with hempseed extract served as control. The drained water from each pot was collected 24 h after inoculation and an overnight flooding. One ml of drainage water was spread on PARPH medium and incubated at room temperature. After 48 h, the number of developing mycelia was recorded (Banihashemi, 2004). To study the effect of temperature and inoculum density and their interactions, almond seedlings were carefully removed from soil, and roots were washed with tap water. Roots were dipped in different concentrations of zoospores and incubated at room temperature for four hours and then transplanted in pots and moved to the growth chambers each set to a different temperature. Roots of the control plants were dipped in sterile water (Kuan and Erwin, 1980; Mitchell and Kannwischer, 1983).

Assessment of Plant and Pathogen Parameters

The percentage of mortality was recorded as the number of blighted seedlings over by the total number of seedlings inoculated multiplied by 100. The seedlings were uprooted 30 days post-inoculation and the soil around their roots was washed off. The main and lateral roots were cut into small pieces and placed on PARPH medium (Ferguson and Jeffers, 1999) without any further treatment. After two days, the proportion of the root system colonized was recorded. Plant height as well as fresh and dry weights of the roots were also determined and used as criteria for disease assessment.

Experimental Design and Statistical Analysis

The experiment was designed as a completely randomized design (CRD) with three replications to evaluate the effect of temperature on pathogenicity of *P. parsiana*. Another factorial experiment was carried out in pots laid out in a completely randomized design as the basic design (in three replications and five plants per replicate) to study the effect of temperature and inoculum density and their interaction on the incidence of the seedling blight. The analysis of variance was performed using procedure of SAS program (SAS institute Inc., 1999) to analyze the effect of temperature and/or inoculum density on pathogenicity of the pathogen and also plant parameters such as fresh and dry weights of root and stem of the seedlings as well as their heights. Data were checked for normality and homogeneity of variance before analysis. The means of the experimental factors and their interactions were compared using Duncan's Multiple Range Test at alpha=0.05 (Clewer, 2001).

Disease incidence in each experimental unit was calculated as the proportion of the blighted (dead or symptomatic) seedlings recorded in three day intervals for thirty days. Mean area under the disease progress curve (AUDPC) was calculated as a synoptic response variable which measures the total disease intensity over the length of the experiment via midpoint method using the following formula:

$$\mathrm{AUDPC} = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i) \quad (1)$$

Where et_i and y_i are the ith time and disease incidence observations, respectively, and n is the total number of assessment times. The effect of the main factors of the experiment and their interaction on the mean AUDPC

was analyzed and finally the means were compared using Duncan's Multiple Range Test at alpha=0.05 significance level (Clewer, 2001).

RESULTS AND DISCUSSION

Effect of temperature on zoospore production and pathogenicity of *Phytophthora parsiana*

Based on the number of colonies on PARPH medium obtained from equal volumes of water drained from each pot, the maximum and minimum numbers of zoospores produced by the pathogen were recorded for the pots incubated at 30°C and 15°C, respectively (Fig. 1A). The experiment was repeated at relatively higher temperatures and the maximum and minimum zoospore counts were recorded at 32°C and 18°C, respectively. Six days after inoculation, three seedlings showed poor growth and disease symptoms such as wilting as well as crown and root rot at 30°C. The frequency of mortality over time was higher at 30°C and 32°C so as all the seedlings were infected by the pathogen 10 days post-inoculation (dpi). By the third week, 66% of the seedlings were recorded blighted at 25°C and only 22% of seedlings showed disease symptoms at 20°C. No disease symptoms were observed for inoculated seedlings at 15°C and 18°C (Fig. 1B). The pathogen was recovered only from symptomatic plants at 25°C-32°C.

Infection rates at different temperatures were calculated from the observed disease progress curves, and plotted based on logit-transformed disease incidence data. The rate parameters of these logit-transformed lines were compared by t-tests (Fig. 2A). The rate of the seedling blight at 32°C and 30°C was significantly higher than the rate recorded at 25°C. While no significant difference was found between disease progress rates at 32°C and 30°C, these rates were significantly higher than the rates observed at 18°C or 25°C (Fig. 2B).

Relative weights of fresh and dry roots as well as height of inoculated seedlings adjusted for their controls significantly reduced at 32°C, 30°C and 25°C. No significant difference was observed in fresh/dry root weights and seedling height at 18°C as compared to the control (Fig. 3).

The results strongly suggested that temperature had a great impact on the development of diseases caused by *Phytophthora parsiana*. The results are in agreement with the results obtained with other forms of *Phytophthora* species. For example, temperature was very important for disease progress and development of *Phytophthora* crown rot of peach trees caused by *P. cactorum* and *P. citrophthora* (Thomidis, 2003). The greatest production of sporangia by *P. citrophthora* and *P. parasitica* at 20°C and 30°C on seedlings.

Hüberli et al. (2012) reported that mean lesion area for infection of *Umbellularia californica* leaves in detached inoculations with *P.ramorum* zoospores was highest at 19°C and lowered significantly at higher temperatures.

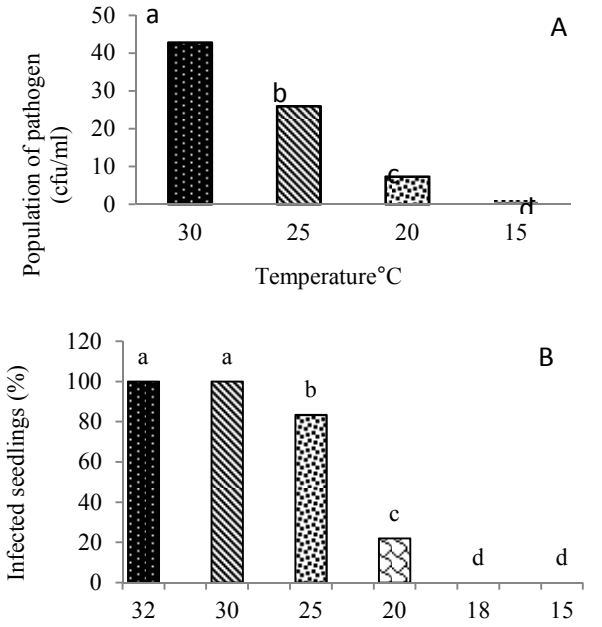

Fig. 1. A) The number of colony forming unit (cfu) of *Phytophthoraparsiana* recovered on PARPH medium from drained water of each pot at different temperatures; B) Infection percentage of seedlings inoculated by *Phytophthoraparsiana* at different temperatures. Columns with the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$)

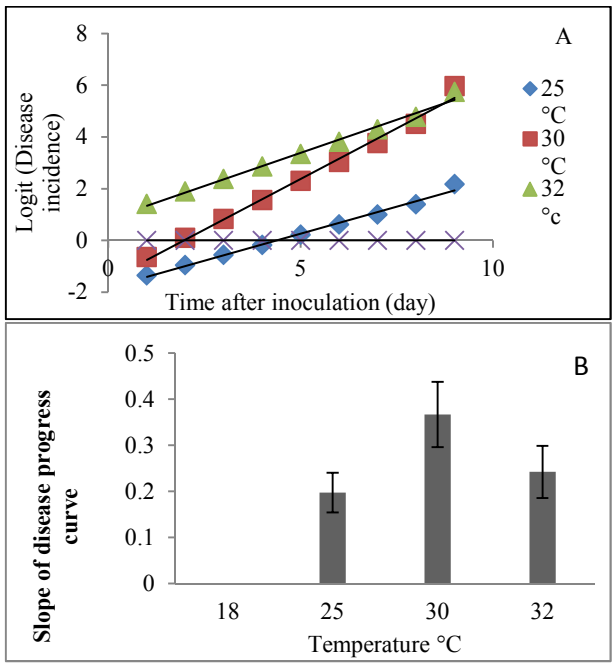

Fig. 2. A) Linear logistic model of disease progress curve. Vertical axis:, *logit (x)=ln (x/1-x),* Horizontal axis: days post-inoculation (dpi). B) Slope of disease progress curve of infected seedlings with *Phytophthoraparsiana* at different temperatures. Bars: SEM (Standard error of mean).

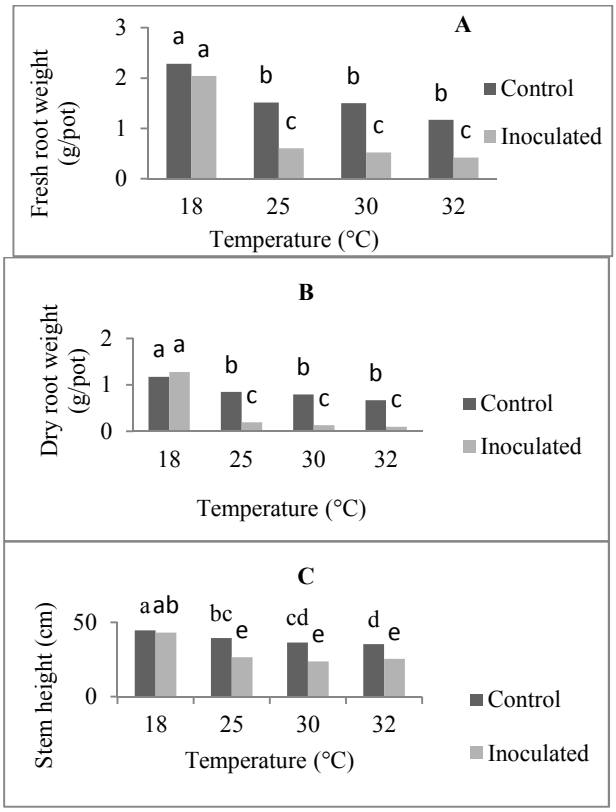

Fig. 3. The effect of temperature on fresh (A) and dry root (B) weight and stem height (C) of seedlings inoculated with *Phytophthora parsiana*. Columns with the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$)

Results of the present study indicated that the rate of seedling infection by *P. parsiana* was optimized in the range of 30-32°C. Based on the observed disease progress curves, seedling mortality progressed more rapidly at higher temperatures. Our findings showed that the pathogen could produce the highest number of zoospores at the highest temperature (30°C). No disease symptoms were observed in the seedlings inoculated and incubated at the lowest temperatures (15°C and 18°C) over the course of the experiment. These findings are in agreement with those of Harris and Tobutt, (1978) who showed that disease symptoms of apple seedlings infected by *P. cactorum* were more severe at a higher temperature (25°C compared to 18°C). In this study, the severity of disease increased with greater inoculum densities. It was reported that approximately 10^6 zoospores ml^{-1} of *P. palmivora* was required for 90% mortality of papaya seedlings (Ramirez and Mitchel, 1975; Gooding and Lucas 1959, 1959; Kliejun as and Ko, 1974).

Interaction of Temperature and Inoculum Density on Disease Severity

Temperature, zoospore density and their interaction significantly affected disease severity and growth parameters. Maximum and minimum disease severity measured as the colonization percentage occurred at 30°C and 20°C, respectively. No infection was observed at 18°C. The lowest root weight and the highest amount of colonization of main and lateral roots and crowns were observed at 30°C. The highest and lowest colonization percentages were observed at 10^6 and 10^3 zoospores ml^1, respectively (Fig. 4).

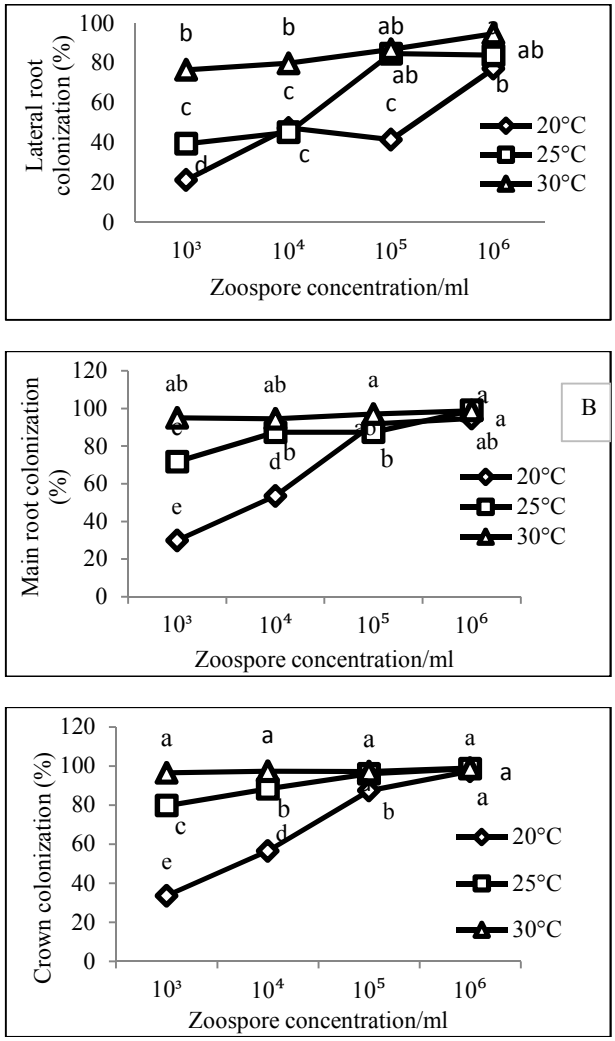

Fig. 4. A) The effect of temperature and inoculums density on main, lateral (B) root and crown (C) colonization of almond seedlings by *Phytophthora parsiana*. Numbers with the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Correlation of temperature and inoculum rate on disease severity was significant at 1% probability value. The lowest disease severity and colonization percentage of roots and crown was observed at 10^3 ml^{-1} zoospore density and 20°C(80-100% colonization of roots and crown was observed at 30°C and 10^6 and 10^3 zoospores ml^{-1}, whereas 20% colonization was observed at 20°C and 10^3 ml^{-1} zoospore). The highest percentage of mortality was observed at 30°C and 10^6 ml^{-1}zoospores. The highest disease severity was observed on seedlings inoculated with 10^5 and 10^6ml^{-1}zoospores irrespective of the temperature. Although the difference in disease severity on seedlings incubated at 20, 25 and 30°C was negligible at high zoospore concentrations (10^5 and 10^6

ml^{-1}), at 10^5 zoospore ml^{-1}lower disease severities were recorded at 25 and 30°C (Fig. 4, 5).

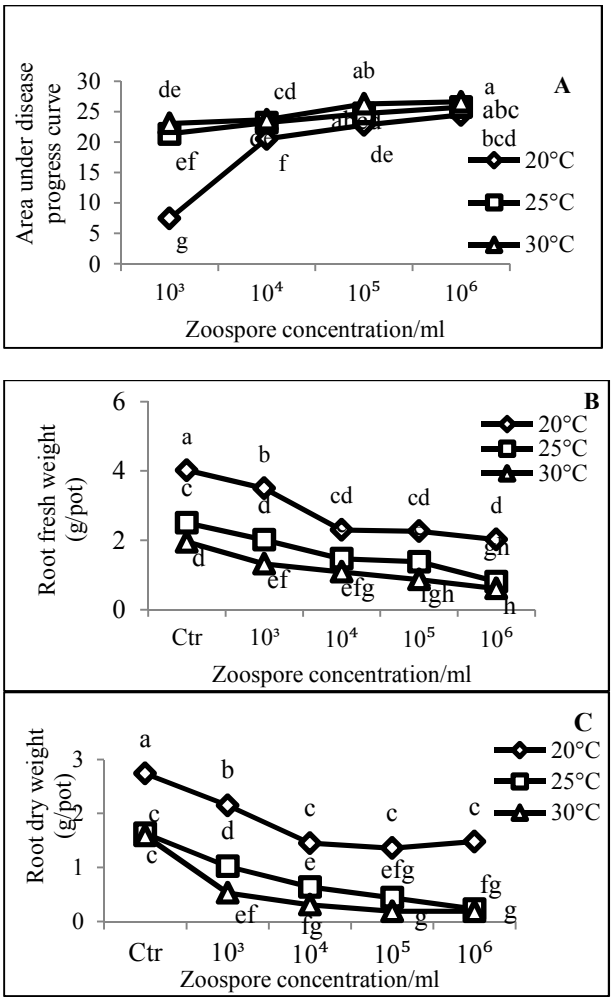

Fig. 5. A) Correlation of temperature and inoculum density on disease severity of *Phytophthora parsiana* on almond seedlings; B) The effect of temperature and inoculums density on fresh root weight (B) and dry root weight (C) of almond seedlings infected by *Phytophthoraparsiana*. Numbers with the same letter are not significantly different according to Duncan multiple range test ($p \leq 0.05$).

These observations are consistent with Raftoyannis & Dick (2002) who found that with increasing temperature and inoculum concentration of pythiaceous fungi, such as *Pythium aphanidermatum* and *Phytophthora nicotianae*, the length of plant roots decreased. They also reported similar results for the effects of inoculum density and temperature on disease severity caused by pythiaceous fungi on host species such as alfalfa, maize, sorghum, and sugar beet. In their research, the highest disease severity was recorded for 10^6 zoospores ml^{-1} at 30°C while 10^3 zoospores ml^{-1} at 20°C did not significantly affect disease severity. Ramirez and Mitchel (1975) reported that five chlamydospores per gram of soil of *P. palmivora,* led to infection of 50% papaya seedlings whereas doubling the inoculum density to 10 chlamydospores per gram of soil caused 95% infection. According to Gooding and Lucans (1959), 10^5 zoospores ml^{-1} of *P. parasitica* var. *Nicotiana* resulted in 100% mortality of tobacco seedlings and 10^3 zoospores ml^{-1} brought about the minimum mortality of plants.

Using the results of this study and the information on almond susceptibility to *P. parsiana* (Rafiee & Banihashemi, 2013), we suggest that *P. parsiana* is a threat to woody plants especially in years with hot summer (Banihashemi et al., 2009). It appears that other new high temperature species of *Phytophthora* such as *P. hydropathica*, *P. irrigata* and *P.niederhauserii* (Perez-sierra et al., 2010; Kurbetli & Değirmenc, 2011; Abad et al., 2014) are very close to *P. parsiana* isolates. However, more researches are required to verify the relationships between these new species of *Phytophthora* and their pathogenicity on almond trees. The most important finding which emerged from this study was that *P. parsiana,* which is a high temperature tolerant species, needs higher temperatures (30°C-32°C) for successful pathogenicity on almond seedlings. The importance of this finding will be doubled when we consider this fact that surface air maximum and minimum temperatures have increased in all regions of Iran at nearly equal rates of 0.4-0.5 and 0.2-0.3 (°C/decade), respectively (Figure 6).

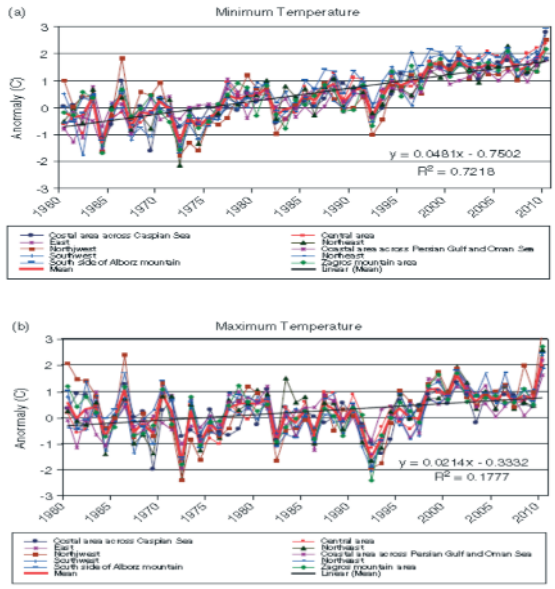

Fig. 6. Regional annual minimum (a) and maximum temperatures (b) anomalies for the period of 1960-2010, expressed as departures from 1961 to 1990 average for different regions of Iran. The straight black lines are least squares trends of the country average for 1960-2010 (adapted from "Effects of adjustment for non-climatic discontinuities on determination of temperature trends and variability over Iran" Rahimzadeh F., NassajiZavareh, M., 2014, *International Journal of Climatology*, 34 p. 2094, Copyright 2013 by the Royal Meteorological Society).

It appears that as the air temperatures rise across the country, selection pressure will change in favor of the temperature tolerant species of *Phytophthora* like *P.*

parsiana. This trend will be a major challenge for management of disease caused by these plant pathogens.

CONCLUSIONS

The effect of temperature and its interaction with the rate of inoculum showed that increasing temperature from 20 to 30 °C and inoculum rate from 10^3 to 10^6 zoospores per ml will significantly increase disease incidence and disease severity of *P. parsiana*. No disease was observed at 15-18°C and 80-100% infected seedlings at 25-32°C. Disease symptoms were correlated with high inoculum concentration, and 100% infection on almond seedlings was observed at $10^6 ml^{-1}$ and 30°C. The most obvious finding which emerged from this study was the fact that *P. parsiana* needs a high temperature for successful pathogenicity on its host. According to the results of this study and because almond is the most susceptible host for *P.parsiana*, it can be suggested that in some regions particularly in years with hot spring and summer, *P. parsiana* will be a threat to some plants such as almond and pistachio.

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اثردماو تراکم اینوکلوم بر شدت بیماری زایی *Phytophthora parsiana*

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چکیده- در این مطالعه، اثرات غلظت های مختلف مایه و دماهای مختلف، بر شدت بیماری زایی گونه *Phytophthora parsiana* روی بادام مورد بررسی قرار گرفت. برای این منظور بذور بادام (ارقام ربیع و کاغذی)در ورمی کولیت مرطوب در دمای۴°C به مدت ۴۵ روز نگهداری شد. بذور جوانه زده در گلدان های حاوی خاک ماسه به نسبت ۱:۲ کشت داده شد و در گلخانه در دمای ۲۵ °C-۱۸ تا رشد کامل نگهداری شدند. یک ماه پس از رشد، نهال ها به اتاقک های رشد با دماهای ۱۵، ۱۸، ۲۰، ۲۵، ۳۰ و ۳۲ °C منتقل و گلدان ها به دو روش، با ریختن عصاره شاهدانه– ورمی کولیت حاوی بلوک های کشت بیمارگر در پای طوقه و یا با غلظت های مختلف زئوسپور شامل 10^3، 10^4، 10^5 و 10^6 اسپور در میلی لیتر به روشroot dip مایه زنی شدند. نتایج نشان داد افزایش دما و تراکم مایه و برهمکنش آنها به طور معنی داری بر مرگ و میر دانهال ها تاثیر دارد . دامنه دماییC°۳۲-۳۰ سبب مرگ و میر ۱۰۰ درصدی دانهال های بادام شد، در حالیکه در دامنه دمایی ºC ۲۰-۱۵، هیچگونه مرگ و میر دانهال های بادام مشاهده نگردید. با افزایش دما از ۲۰ به C°۳۰ و افزایش غلظت زئوسپور از 10^3 به 10^6 در میلی لیتر، وقوع بیماری به طور معنی‌داری افزایش یافت. دماهای بالا و مقدار مایه بالاتر 10^6در میلی لیتر سبب کاهش معنی دار وزن تر و خشک ریشه و نیز درصد آلودگی ریشه‌های فرعی، اصلی و طوقه گردید.