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Influence of culture media, temperature, pH and light regime on mycelial growth of *Ascochyta rabiei*

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Abstract

With a cosmopolite widespread worldwide, *Ascocyhta rabiei* may result in yield losses in chickpea fields up to 100% when the climate conditions were available for growth and development of the pathogen. In this study the effects of culture media, temperature, pH and light regimes on mycelial growth of nine isolates of *A. rabiei* isolated from different agroecological zones of Yozgat province (Turkey) were investigated. Malt Extract Agar was most suitable for mycelial growth of all nine isolates tested. Chickpea Seed Dextrose Agar, Oat Seed Dextrose Agar and ½ Potato Dextrose Agar were not suitable for mycelial growth of all isolates, whereas pycnidial production was observed usually on CSDA or OMA. *A. rabiei* isolates generally developed smooth, sparse and immersed mycelium in all of culture media. Mycelium was characterized with pale cream, light greenish grey or grey-black color. *A. rabiei* grew from 15 to 30 °C, with optimum growth at 20 °C and no growth at 35 °C. The temperature required for maximum daily mycelial growth (2.0 mm day^{-1}) was identified as 20.0°C Present isolates formed smaller-diameter colonies at acidic pH values as compared to basic pH values. The pH values of 6.0 and 7.0 promoted mycelial growth. Light significantly influenced radial mycelial growth of YBUAr7 isolate of *A. rabiei*. Prolonged light exposures promoted radial growth in culture media and the cultures incubated under continuous light exhibited greater radial growth rates than the cultures incubated under 12 h photoperiods and 24 h dark.

Key words: *Ascocyhta rabiei*, Environmental factors, Growth

INTRODUCTION

Chickpea (*Cicer arietinum* L.) occupies a significant position among the legume crops in the world. Turkey is the second most important chickpea producing country worldwide with an annual production of 0.63 million tons (t) from 0.51 million hectare (ha) land area with a mean yield of 1.2 t ha^{-1} (Faostat, 2019). *Ascochyta* species have lately come into prominence as major pathogens of family Poaceae and Legumunaceae plants worldwide (Kosiada, 2012). They are cosmopolitan and facultative saprotrophs, and quite widespread in various geographies (Pande et al., 2005).

Ascochyta rabiei (Pass.) Labr. is one of the most common pathogens of chickpea, causing Ascochyta blight disease, which affects all above−ground parts of the chickpea plants such as leaves, shoots, stems, flowers and pods (Duzdemir et al. 2014; Bayraktar et al., 2016). Ascochyta blight causes death to plant, reduces seed quality and causes yield losses up to 100% under suitable climatic conditions. (Haverson et al., 2011; Bahr et al., 2016). Literature reported that pycnidia and pseudothecia of *A. rabiei* produced abundant quantities of conidia and ascospores, which are dispersed during spring rains. The ascospores of this pathogen are responsible for primary infections while conidia serve to start severe blight epidemics (Trapero-Casas et al., 2012). On the other hand, environmental factors have a pronounced effect on diagnostic characters of fungi.

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In spite of identification of *A. rabiei*, useful information is out of date on its biology, including environmental requirements for growth. This information will be valuable to further mycological and pathological research on this fungus and disease, including development of measures for disease management (Sharma and Pandey, 2010; Benzohra et al., 2017). Therefore, the objective of this study was to provide information on effects of culture media and various environmental factors including temperature, pH, and light regime on mycelial growth of *A. rabiei* isolated from different agroecological zones of Yozgat province (Turkey) were investigated.

MATERIALS AND METHODS

Fungal Isolates

All the trails used nine isolates of *A. rabiei* which had been isolated from chickpea field in Yozgat province of Turkey (Table 1). For isolation of the pathogen, lesions on leaves (Figure 1a), pods (Figure 1b) and stems (Figure 1c) tissues were surface-sterilized by immersing into 1% sodium hypochlorite solution for 3 to 4 min and then washed three times by autoclaved distilled water. The samples were dried on filter papers by putting in a sterile bench. Then, four and five small pieces (0.5–10 mm^2) were placed onto potato dextrose agar (PDA) including 0.01% streptomycin. Plates were incubated at 25°C in 24 hours (h) dark and for 5–7 days. Pure cultures of *A. rabiei* were obtained by single spore isolation method. *Ascochyta* spp. were subsequently identified according to their cultural and morphological characteristics (Aveskamp et al., 2010).

Table 1. The origin and geographical location of *A. rabiei* isolates used in entire experiments

Isolate	Isolated Plant Part of Chickpea	Latitude (North)	Longitude (East)	Altitude
YBUAr1	Leaves	39°07'20.93''	35°12'26.36''	1158
YBUAr2	Pods	39°15'20.34''	35°15'54.16''	1115
YBUAr3	Pods	39°25'39.74''	35°21'32.66''	1237
YBUAr4	Leaves	39°31'47.72''	35°20'06.25''	1067
YBUAr5	Stems	39°40'27.62''	35°13'53.45''	1047
YBUAr6	Leaves	39°49'03.13''	35°07'10.49''	1104
YBUAr7	Stems	39°51'31.33''	34°56'26.50''	1191
YBUAr8	Pods	39°43'57.87''	34°43'15.45''	1061
YBUAr9	Stems	39°39'26.15''	34°32'50.41''	983

Effects of Culture Media on Mycelial Growth

Growth characteristic of *Ascochyta* spp. were investigated on five different media (Table 2). Potato Dextrose Agar (½PDA and PDA) and Malt Extract Agar (MEA) were prepared in accordance with the specifications provided in product label. For prepare Chickpea Seed Dextrose Agar (CSDA); 800 ml de-ionized water was added into the single-grain chickpea seed (40g L^{-1}) and boiled for 30 minutes. After that the single-grain chickpeas were removed by straining through four layers of cheesecloth, and 15g of agar and 20g of glucose were supplemented into the filtrate. Total volume was adjusted to 1L before re-autoclaving, and subsequently autoclaved at 121°C at 15 psi for 20 min. Oat Seed Dextrose Agar (OSDA) was prepared by adding 60g of single-grain oat seed to a container including 800 mL distilled water that was boiled for 30 minutes. Then, the liquid was filtered and supplemented with 15 g of agar and 20g of glucose. Total volume was adjusted to 1L before re-autoclaving, and subsequently autoclaved as described above. The media were poured into 9 cm diameter plastic plates.

To carry out the experiment, a 4-mm-diameter fungal plug from a week-old colony was placed in the center of plates containing 20 mL of medium. Isolates of *A. rabiei* were incubated on all of media at 25±1°C and the dark for 7 d. Colony diameters were measured 7 d after inoculation, two measurements were averaged. The complete randomized design with four replicates was used for this trail and four replicates of each isolate (one replicate = one petri dish) were used for each culture medium.

Effects of Temperature on Mycelial Growth

In order to find out the effects of different temperatures on radial mycelial growth, 4-mm-diameter mycelial discs obtained from a week-old colony of *A. rabiei* isolates were placed into the plates with 20 mL PDA. Plates were incubated at 5°C intervals between 5°C and 35°C at dark for 7 days. Colony diameters were measured as described above. Regression curves were drawn for each isolate on a graph plotting radial growth vs temperature values. Optimum temperature (°C) for radial growth, maximum daily radial growth and area under the growth curve (AUC) were obtained from these graphs.

Effects of Different pH Values on Mycelial Growth

The pH value of PDA medium was adjusted to 7 different values (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) with the use of pasteurized NaOH (1.0M) or HCl (1.0M). Plates were inoculated and incubated at 25±1°C and dark for 7 days. Colony diameters were measured to consider the effects of pH on mycelial growth rates.

Effect of Light on Mycelial Growth

The *A. rabiei* isolates were inoculated into to the culture media as described above. Plates were then incubated at

22±1°C under 3 different light regimes (continuous light for 24 h; 12/12 light/dark photoperiods, continuous dark for 24 h). Plates were incubated for 14 days in all light regimes. Colony diameters were measured at 7 days after inoculation. The same incubator was used throughout the entire trials.

Statistical analysis

Experimental data were subjected to analysis of variance (ANOVA) with the use of SPSS v.25 software and Least Significant Difference (LSD) test was used to compare treatment means at 5% significance level.

RESULTS AND DISCUSSION

Identification of the Pathogen

Isolated nine fungi were identified as *A. rabiei* based on cultural and morphological characteristics. The mycelium of YBUAr3, YBUAr4, YBUAr5 and YBUAr7 isolates were characterized with concentric circles starting from a center in pale cream, grey-black color and regularly discoloring toward to outer edge of the colony (Figure 1d, e, f). Other isolates have developed submerged mycelium (Figure 1g, h). Plenty of pycnidia were observed on aerial mycelium. Conidia were cylindrical or ellipsoid, one-septate and colorless (Figure 1n) and had an average size of 8–15 × 3–5 µm. These cultural and morphological characteristics were quite similar with the characteristics of isolates defined as *A. rabiei* in earlier research (Aveskamp et al., 2010; Bahr et al., 2016; Baite et al., 2016).

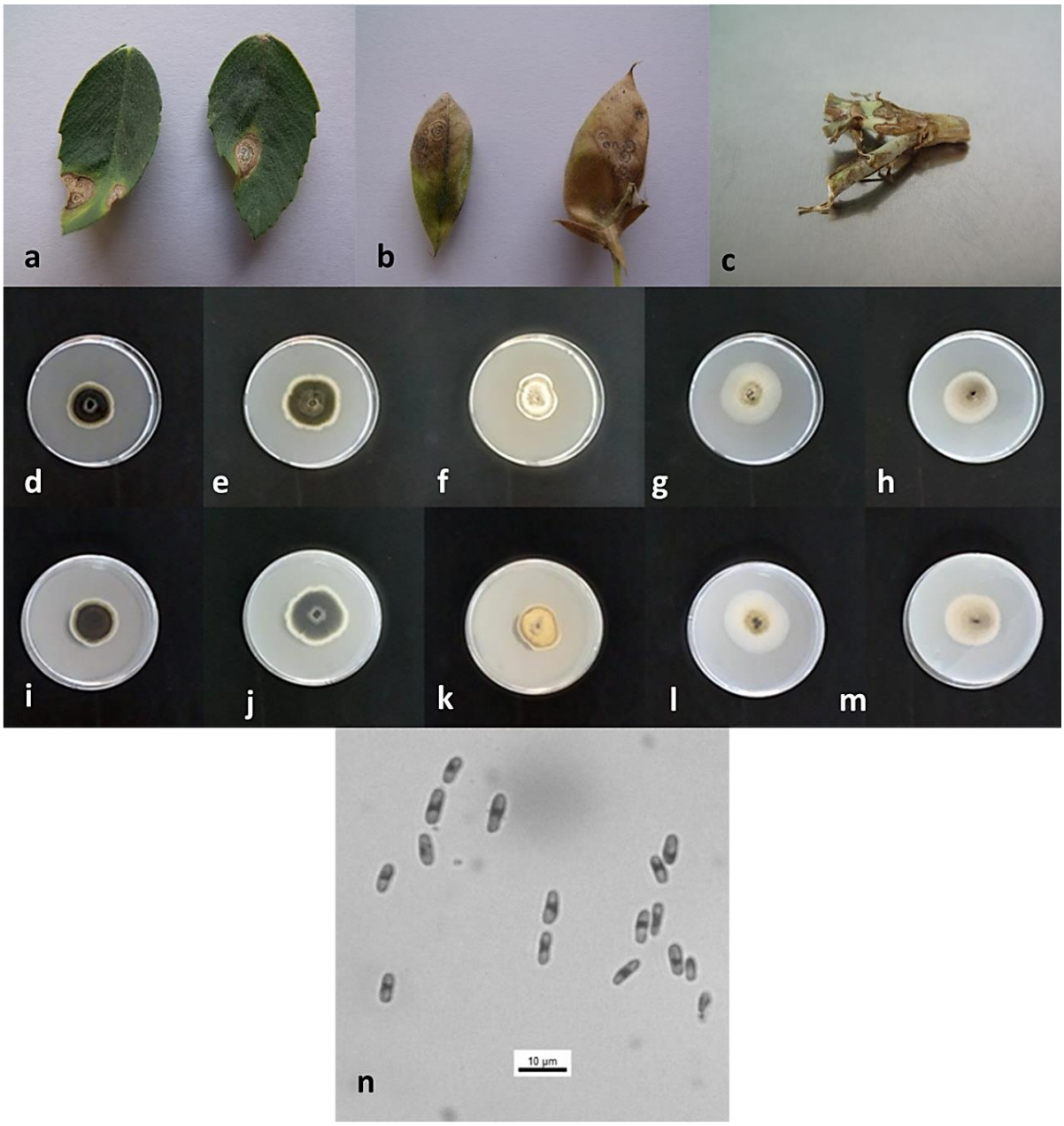

Figure 1. Disease symptoms of Ascochyta blight on chickpea leaf (a), pod (b) and stem (c). Colony patterns of *A. rabiei* YBUAr7 on ½ PDA (d, i), PDA (e, j), MEA (f, k), CDA (g, l) and OMA (h, m) at 25 °C for 14 days after incubation. Front (d, e, f, g, h) and reverse (i, j, k, l, m) view of petri dishes. Micromorphology of *A. rabiei* YBUAr7: direct microscopic observation of conidia (n). Scale bars 10 µm

Effects of culture media on mycelial growth

A. rabiei isolates exhibited significant differences ($P < 0.05$) and gathered under five groups based on mycelial growths (Table 2). The greatest mycelial growth was observed in YBUAr5 on MEA, followed by YBUAr3 and YBUAr4 isolates. The YBUAr8, YBUAr6 and YBUAr2 isolates were placed into the same statistical group and had the lowest colony diameter. Similar studies were also reported by Kaiser (1973), Basandrai et al. (2005) and Baite et al. (2016). It is possible that variation in *A. rabiei* isolates was a pre-variation since this variation was reported as not related to geographical origin in several of previous studies (Pande et al., 2005). Since *A. rabiei* is a heterothallic species, it is more common to see mutations in this species and variation in cultural and morphological characteristics of the isolates (Trapero-Casas and Kaiser, 2012; Attar et al., 2020). Mycelial growth of isolates was significantly influenced by culture media ($P < 0.05$). Isolate colonies developed smooth, sparse and immersed mycelium on artificial media (Figure 1). Mycelium color was initially pale cream, then turned into greyish white or dark greenish and creamy white (Baite et al., 2016). Besides, majority of the isolates formed greyish white colony. The greatest radial mycelial growth was observed on MEA and it was followed by PDA medium. Isolates were placed into the same statistical group in OMA, CDA and ½ PDA media. Contrary to present findings, Kaiser (1973) reported that CDA or OMA media had greater effects on maximum mycelial growth of *A. rabiei* isolates than MEA or PDA media. Additionally, Baite et al. (2016) reported that CDA medium better promoted mycelial growth of *A. rabiei* isolates. Differences between the present findings and findings of the above-given studies were mainly attributed to differences in composition of synthetic or semi-synthetic culture media. A significant interaction was observed between media and isolates in terms of radial mycelial growth ($P < 0.05$).

Table 2. Effects of culture media on mycelial growth of nine isolates of *Ascochyta rabiei* at 25°C for 7 days in the darkness

Media[a]	Radial Growth (mm)									Media Main Effect
	Isolates									
	YBU Ar1	YBU Ar2	YBU Ar3	YBU Ar4	YBU Ar5	YBU Ar6	YBU Ar7	YBU Ar8	YBU Ar9	
½PDA	15.0	14.3	16.0	16.6	16.5	14.4	15.6	14.0	14.6	15.2 c[b]
PDA	17.4	15.4	17.6	18.3	16.6	15.6	16.4	16.3	17.6	16.8 b
CDA	14.3	14.8	19.4	14.9	18.6	14.6	13.4	11.9	16.9	15.4 c
MEA	16.8	17.8	19.1	18.6	21.8	15.4	18.3	16.6	17.9	18.0 a
OMA	17.0	13.4	16.5	16.1	18.9	15.1	16.3	12.4	15.6	15.7 c
Isolates Main Effect	16.1 d	15.1 e	17.7 b	16.9 c	18.5 a	15.0 e	16.0 d	14.2 e	16.5 cd	

[a] PDA = Potato Dextrose Agar; CDA = Chickpea Dextrose Agar; MEA = Malt Extract Agar; OMA = Oat Meal Agar

[b] Values are the means of pooled data from the experiment (four replicate plates of each medium for each isolate). For the main effect of media and isolate ($p<0.05$), means with the same letters are not significantly different; LSD = 0.65 and 0.49, respectively

Effects of temperature on mycelial growth

The *A. rabiei* isolates isolated from different chickpea cultivation locations of Yozgat province (Table 1) exhibited different growth rate at temperatures between 5 - 30°C. *A. rabiei* isolates exhibited radial mycelial growth on PDA medium at 10°C, 15°C and 20°C temperatures, but did not at 35°C temperature. The isolates formed colonies with limited diameter as to complete the growth at 5°C, 25°C and 30°C (Table 3). The differences in optimal temperatures of the isolates were not found to be significant ($P > 0.05$; Table 3). While the maximum growth rates of YBUAr7 and YBUAr3 isolates were significantly different from the YBUAr6 isolate ($P < 0.05$), maximum growth rates of the other isolates exhibited insignificant differences with a wide range of overlapping (Table 3). In all cases, regression coefficients were significant ($P < 0.05$) and coefficient of determination (R^2) varied between 0.779 - 0.882. Present isolates were generally gathered under two groups: Maximum growth rate of YBUAr3, YBUAr6 and YBUAr8 isolates was < 2 mm gün^{-1}; the rest had a maximum growth rate of > 2 mm gün^{-1}. When the area under mycelial growth curve (AUC) was calculated, it was observed that YBUAr7 isolate exhibited significantly different growth rates from the YBUAr2, YBUAr3, YBUAr6 and YBUAr8 isolates ($P < 0.05$) and the remaining three isolates were grouped in intermediate classes.

Similar with the present findings, Bahr et al. (2016) reported that *A. rabiei* isolates exhibited quite a slow mycelial growth rate after 25°C and mycelial growth was totally terminated after 28°C. On the other hand, Ozkilinc et al. (2010) reported that common-origin *A. rabiei* isolates exhibited the same growth rates at 15°C and 25°C and recorded faster growth at 25ºC than at 15ºC on PDA. However, in present study, optimal mycelial growth temperature of nine *A. rabiei* isolates was identified as about 20°C on PDA (Table 3). Differences in findings of the studies could be explained by variations in origins of *A. rabiei* isolates and growing techniques adopted for host plants. Kosiada (2012) reported that *Ascochyta* species or isolates might have different growth rates at different temperatures, for instance, *A. zeicola* isolated from maize (a hot season plant) exhibited optimal growth at 24.2°C, but *A. stipae* (syn. *A. antarctica*) and *A. ducis-aprutii* species isolated from the plants growing in Antarctica exhibited optimal growth at 15°C.

Effects of pH on mycelial growth

The *A. rabiei* isolates exhibited similar reactions against the changes in media pH, but exhibited different mycelial growth rates in each pH value (Table 4). Isolates formed smaller-diameter colonies at acidic pH values than the basic pH values. Significantly different mycelial growth rates were observed at pH 6.0 and pH 7.0 from the other pH values ($P < 0.05$), YBUAr4 and YBUAr7 isolates exhibited significantly different growth rates than the others ($P < 0.05$). There was a significant interaction between pH and isolates in terms of radial mycelial growth ($P < 0.05$) and media pH influenced radial mycelial growth rates. Similar with the present findings, Abubakar et al.

(2013) indicated that pH influenced growth rate of fungi and reported optimal growth at pH of between 3 – 8 (pH of 5 when the other requirements were fully met). It was indicated in previous studies that fungi exhibited the greatest dry micelle biomass at pH of 5.5 - 6.5 (Saha et al. 2008; Deshmukh et al. 2012).

Table 3. Temperature – growth relationship for *Ascochyta rabiei* isolates[1]

Isolates	Adjusted Model[2]				Temperature (°C)[3]	Growth (mm gün^{-1})[4]	AUC[5]
	R^2	a	b	c			
YBUAr1	0,807	-0,010	0,399	-1,786	20,30 a[5]	2,25 ab	39,30 ab
YBUAr2	0,779	-0,010	0,403	-2,038	20,88 a	2,02 abc	36,55 b
YBUAr3	0,842	-0,008	0,327	-1,273	19,38 a	1,78 c	37,43 b
YBUAr4	0,852	-0,010	0,390	-1,809	20,55 a	2,10 abc	38,66 ab
YBUAr5	0,838	-0,009	0,338	-1,268	20,43 a	2,09 abc	39,49 ab
YBUAr6	0,817	-0,008	0,313	-1,260	20,50 a	1,80 c	34,50 b
YBUAr7	0,860	-0,011	0,438	-2,048	20,55 a	2,31 a	44,01 a
YBUAr8	0,882	-0,008	0,321	-1,163	19,68 a	1,88 bc	36,96 b
YBUAr9	0,873	-0,009	0,367	-1,474	20,65 a	2,18 abc	40,63 ab

[1] Average of four replicates. Means with the same letter are not significantly different (P = 0.05)

[2] Mycelial growth on PDA at 5 to 35ºC was adjusted to a second-degree polynomial model: $Y = aT^2 + bT + c$, in which Y = mycelial growth (mm day-1); a, b, and c are the regression coefficients; and R^2 = coefficient of determination

[3] Model estimated optimal temperature

[4] Model estimated maximum growth rate

[5] Area under mycelial growth curve (AUMGC)

Effects of media and light intensity on mycelial growth

Light significantly influenced radial mycelial growth rate of YBUAr7 isolate of *A. rabiei* in ½PDA, PDA, MEA, CDA and OMA media (Figure 2). Prolonged light exposures promoted radial growth in culture media, except for CDA and the cultures incubated under continuous light exhibited greater radial growth rates than the cultures incubated under 12 h photoperiods and 24 h dark. The 24 h light regime yielded significantly different outcomes from the other light regimes ($P < 0.05$). A significant interactions was observed between the media and light regime in terms of radial mycelial growth ($P < 0.05$). Such a difference in light x media interaction could be explained by combined effect of light exposure duration and culture media on radial mycelial growth rates of *A. rabiei*. Similar with the present findings, Kaiser (1973) reported that *A. rabiei* isolates incubated on PDA medium at 24 h light exhibited greater growth rates than the isolates incubated at 24 h dark. The *A. pisi* isolates, close relative of *A. rabiei*, also exhibited greater mycelial growth rates at 24 h light regime than the other two light regimes (Terbeche et al., 2015).

Table 4. Effect of pH on mycelial growth of isolates of *A. rabiei* on PDA at 25°C for 7 days in darkness

Isolates	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
YBUAr1	13.3 de[*]	16.0 ab	16.3 e	17.0 a	16.4 e	18.7 a	18.7 b
YBUAr2	16.6 ab	16.0 ab	19.1 abc	15.8 b	20.6 ab	15.5 c	17.7 bcd
YBUAr3	14.4 cde	17.2 a	18.5 bcd	15.8 b	20.5 abc	16.8 b	16.8 d
YBUAr4	17.0 a	15.0 bcd	20.6 a	14.5 d	20.3 abc	18.5 a	21.3 a
YBUAr5	15.6 abc	16.7 ab	19.8 abc	17.2 a	19.5 bc	16.3 bc	17.0 cd
YBUAr6	12.9 e	15.3 bc	19.4 abc	14.7 cd	18.8 cd	16.0 bc	15.7 e
YBUAr7	16.6 ab	16.0 ab	20.4 ab	15.8 b	21.5 a	16.3 bc	18.0 bc
YBUAr8	14.9 bcde	13.3 d	17.9 cde	13.1 e	17.6 de	12.5 d	15.2 e
YBUAr9	15.0 abcd	14.2 cd	17.0 de	15.7 bc	16.8 e	12.0 d	12.5 f
LSD (%5)	2.0	1.8	1.9	1.1	1.8	1.1	1.0

[*] Values are mean of four replicates for each isolate. Means in each column, followed by the same letters are not significantly different according to Fisher's LSD (0.05) test

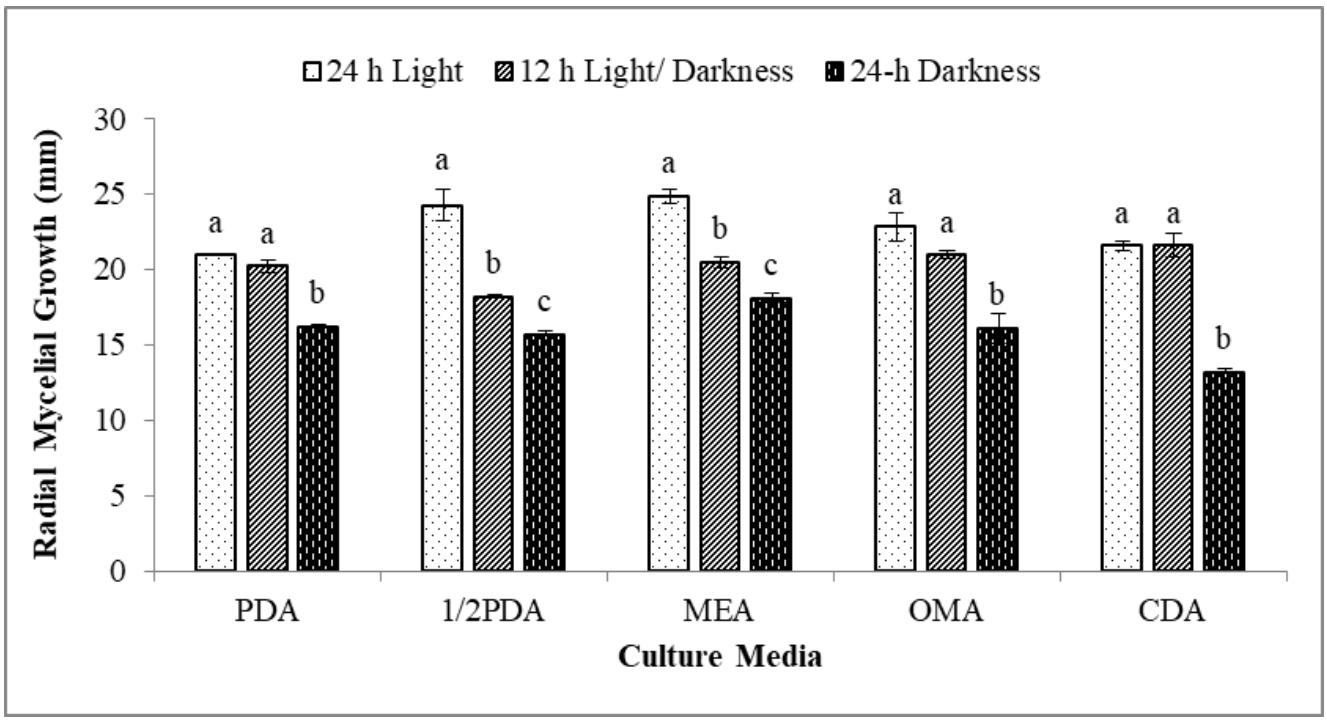

Figure 2. Radial mycelial growth of *A. rabiei* (YBUAr7) on five media after seven days incubation period at 22 °C under three light regimes. Bars represent means of four replicate plates of each medium for each light regime. Bars tops with the same letter are not significantly different.

CONCLUSION

Present findings revealed that culture media, temperature, pH and light regimes significantly influenced mycelial growth rates of *A. rabiei*. Optimal mycelial growth of *A. rabiei* was observed on MEA medium at pH of 6-7, average 20°C temperature and 24 h light regime. Present findings may provide contributions to further studies investigating anthracnose disease and behavior of *A. rabiei* in nature. Such studies may improve comprehension of the conditions required for the outbreak of the disease, progress of the disease in time and mitigation of the outbreak and may aid in development of disease control strategies. Besides, it is quite hard to fully estimate the effects of environmental factors on the pathogen and interactions of these factors under field conditions. Therefore, further research is recommended for validation of laboratory tests about *A. rabiei*-induced disease development in chickpea under field conditions.

AUTHOR CONTRIBUTIONS

The author solely conducted the entire study and wrote the manuscript.

CONFLICT AND INTEREST

The author declares no conflict of interest.

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