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Response of Some Walnut Genotypes (*Juglans regia* L.) to Anthracnose Attack (*Ophiognomonia leptostyla*)

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ABSTRACT

Anthracnose, caused by *Ophiognomonia leptostyl* Fr., is one of the most destructive walnut (*Juglans regia* L.) foliar diseases in the world. To facilitate development of cultivars in new growing areas, four recently released cultivars in Iran ('Alvand', 'Chaldoran', 'Caspian', and 'Persia'), a local promising genotype (C25), 'Hartley', 'Ronde de Montignac', and K72 were examined for their response to the anthracnose attack. Fully expanded leaflets of grafted plants were inoculated by fungal conidia in two consecutive years. The number and size of necrotic spots were recorded at 15-day intervals until 45 days after inoculation. According to the data of 30th day, the maximum number of necrotic lesions belonged to 'Alvand' while the largest spots developed on K72 and 'Chaldoran'. Cultivars were categorized into six susceptibility classes depending on Necrotic Leaflet Area (NLA). The average of NLA ranged from 0.61% ('Ronde de Montignac') to 99.94% (in K72). All tested cultivars, except 'Ronde de Montignac' and 'Hartley', were susceptible, but symptoms development and disease severity varied among the cultivars. 'Persia' exhibited a low level of infection and was relatively resistant. 'Caspian' with an average NLA of 20% was slightly susceptible. 'Alvand' and C25 grouped as susceptible and moderately susceptible, respectively. K72 and 'Chaldoran' showed the maximum level of infection. The amount of disease was intensified in 'Caspian', C25 and 'Alvand' between the days of 30-45 after inoculation, while 'Ronde de Montignac', 'Hartley' and 'Persia' appeared consistently more resistant even 45 days after inoculation. These three cultivars could be used in integrated management approaches to control anthracnose in walnut orchards.

Keywords: Disease resistance, Foliar diseases, *Juglans regia*, Integrated disease Management.

INTRODUCTION

The Persian walnut (*Juglans regia* L.) belongs to the generic section of *Dioscaryon* (Stanford *et al*., 2000) and is the most important *Juglans* species mainly grown in the west, northwest, central, and northeast areas of Iran. Although Iran is among the top three walnut producing countries in the world (FAOSTAT, 2020), unfortunately, production is still largely dependent on trees originating from seedlings. Therefore, the quality and quantity of the walnuts are not desirable. But in recent years, development of modern walnut orchards in Iran is being thrived and traditional orchards have been gradually replaced either by grafted or own-rooted trees. Development of top-working technology with the aim of renewing traditional orchards has also created a revolution in walnut industry of Iran (Hassani *et al*., 2020b). The walnut breeding programs in Iran started in 1983 through selection of native germplasm to develop new cultivars carrying suitable horticultural features (Hassani *et al*., 2020b). In addition, introduction of foreign commercial cultivars after compatibility tests, was another walnut breeding strategy in Iran. Determination of the resistance level of new cultivars to the main diseases such as anthracnose

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(*Ophiognomonia leptostyla)* and bacterial blight (*Xanthomona arboricola* pv. *Juglandis*) has been intensively involved in breeding programs. Introduction of cultivars 'Jamal' and 'Damavand' in 2010 was the first accomplishment of walnut breeding activities in Iran (Hassani *et al*., 2012a and b).

Black spot or anthracnose caused by the fungus *Ophiognomonia leptostyla* (Fr.) Sogonov (imperfect state *Marssoniella juglandis* (Lib.) Höhn) is one of the most destructive foliar diseases of walnut that was first reported in Iran in 1952 (Ershad, 2009). It is prevalent in all walnut growing regions. Disease incidence especially in cold and rainy springs of the northwest Iran is associated with yield losses of 60-80% in some years (Saremi and Amiri, 2010). The fungus mostly attacks leaves, petioles, young shoots and fruits after prolonged periods of wetness. Irregular circle brown and black lesions occur on plant organs. Necrotic spots coalesce to form larger necrotic areas that lead to premature defoliation and fruit drop. Primary lesions provide conidia that spread by wind and water splashing and cause secondary infections in fields. Two weeks latent period is demonstrated for the pathogen prior to conidia dissemination. Trees may become weak and then yield and nut quality may be affected (Cline and Neely, 1983; Reid, 1990; Teviotdale *et al*., 2002).

Host plant resistance is an effective component in integrated management of many diseases (Mehlenbacher, 1995). Use of cultivars resistant to anthracnose could be incorporated with other control methods such as cultural and chemical techniques to manage the disease (Berry, 1977). Several studies show variation in walnut cultivars for susceptibility to anthracnose (Berry, 1961; Black and Neely, 1978; Balaz *et al*., 1993; Maria *et al*., 1997; Woeste and Beineke, 2001; Annunzati *et al*., 2007; Belisario *et al*., 2008; Arnaudov and Gandev, 2009; Dastjerdi and Hassani, 2009; Arnaudov *et al*., 2014; Jelev and Marinov, 2018). Some of the reports have demonstrated that resistance of black walnut cultivars to anthracnose disease is a phenotypic trait that could be genetically transmitted (Reid, 1990). Although some black walnut cultivars (*J. nigra* L.) such as 'Thomas', 'Ohio' and 'Sparrow' show high level of resistance to anthracnose (Reid, 1990; Reid *et al*., 2004), none of Persian walnut cultivars have been reported to be entirely immune. Dastjerdi and Hassani (2009) reported that 'Jamal', a cultivar suggested for moderate-cold areas of Iran, has been reported to be low susceptible to anthracnose infection, while 'Damavand' exhibited a very low susceptibility, especially in semi-arid climate.

'Alvand', 'Chaldoran', 'Caspian', and 'Persia' are newly released walnut cultivars selected through the population of native germplasm in Iran. 'Caspian' and 'Persia' are characterized by late leafing and early-medium ripening habit. They are recommended for walnut growing areas with late spring frosts. According to the features of 'Alvand' and 'Chaldoran', these cultivars are suitable for regions with less risk of late spring (Hassani *et al*., 2020a and b). The objective of this study was to evaluate the response of eight walnut genotypes to *O. leptostyla*, of which four are newly released in Iran.

MATERIALS AND METHODS

Plant Materials

Four newly released walnut cultivars in Iran ('Alvand', 'Chaldoran', 'Caspian', 'Persia') and the local promising genotype C25 were tested for resistance to *O. leptostyla* under the greenhouse conditions. The well-known cultivars 'Hartley', 'Ronde de Montignac' (RDM) and K72 genotype were also included in the experiments. During February 2018, one-year-old open pollinated *J. regia* rootstocks were placed in 10-L plastic pots (30×25 cm) containing a mixture of sterile soil: sand: perlite: peat moss (1:1:1:2 V/V) plus 500 g of triple

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superphosphate, 100 g KNO_3, 50 g $FeSO_4$, 50 g $CuSO_4$, 30 g $MgSO_4$, 20 g $ZnSO_4$, 10 g $MnSO_4$ per cubic meter. The pots were then maintained in the greenhouse of Temperate Fruits Research Center, Karaj, Iran, at 27±1°C. At the end of February 2018, the scion of cultivars (one-year-old shoots) was collected from 7-year-old trees planted at Meshkinabad Horticultural Research Station, Karaj, and kept in cold storage (4±1°C) till grafting time. Once the rootstocks were established at the end of March, propagation of the cultivars was performed by modified chip budding. Each cultivar was propagated on 10 rootstocks of which 6 were assigned for the experiment. The grafted plants were placed in greenhouse with temperature of 27±2°C and relative humidity of 80-90% (Figure 1-a).

Fungal Isolates and Preparation of Inoculum

Two single spore cultures of *O. leptostyla* (Q57 and LA) used in this study were isolated from naturally infected walnut leaves in Gazvin and Lavasan orchards in Iran, during 2005-2007. More details of these two selected isolates were previously published (Dastjerdi *et al*., 2009). The stock cultures of isolates had been preserved on filter paper in Oatmeal Agar (OMA) medium at -20°C. Prior to using, stability of morphological characteristics, sporulation potential and retention of isolate pathogenicity were tested (Dastjerdi and Nadi, 2019). Inoculum was produced from one-month-old colonies grown on OMA at 21±2°C with a photoperiod of 18-hour light and 6-hour darkness to stimulate production of acervular conidiomata. The concentration of conidial suspension was then determined using a Hemocytometer (0.0025 mm^2) and adjusted to 1×10^5 spore mL^{-1} (Matteoni and Neely, 1979). Tween®20 (0.1%) was added to the fungal suspension as a surfactant. The spore suspension for each tested isolate was prepared individually, kept on ice during inoculation, and homogenized well before using.

Evaluation of Resistance

A completely randomized factorial design with six replicates was assigned to evaluate the relative resistance of walnut cultivars. Treatments were a combination of two factors including cultivars (8 levels) and fungal isolates (2 levels). The experiment was conducted on May 5, 2018 and repeated in April 27, 2019, using the method of Black and Neely (1978) with minor amendments. The cultivars were watered 1-2 hours prior to inoculation and the top three compound leaves per plant were labeled for repeated assessment over time. Cultivars were inoculated by atomizing the fungal conidia on upper and lower surface of mature and

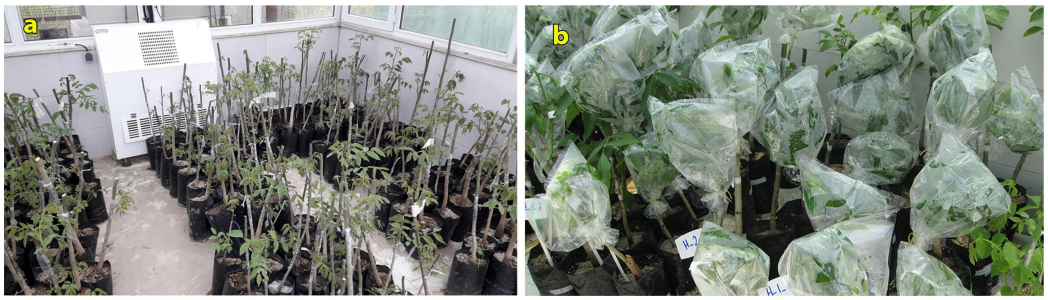

Figure 1. Plant materials growing in greenhouse in April, 2019 (a), and inoculated plants covered by plastic bags (b).

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fully expanded leaflets. Each plant was then covered with a clear plastic bag (Figure 1-b). Two trees per cultivar were added as control and sprayed with sterile water. Forty hours after incubation, the plastic bags were removed and the plants watered as necessary. The average temperature until removal of bags and following after that was 21 and 25°C, while the relative humidity was measured 91 and 71%, respectively.

Two or three days after inoculation, 1-2 leaf from each inoculated cultivar was collected at random. The leaves were placed in ethanol:acetic acid (1:1 V/V) and cleared in lacto-phenol. The samples were then stained in cotton blue. Germination, penetration and subsequent colonization of pathogen in plant cells was detected microscopically (Cline and Neely, 1983). Re-isolation of the fungus from the infected leaves was performed on PDA.

Data Collection and Statistical Analysis

Data was recorded at 15-day intervals (15, 30 and 45 days after inoculation), by measurement of fungal growth in five leaflets of top three leaves of each tree. In each leaf, leaflets were recorded from top to bottom, left to right. The leaflet length and width, number of necrotic spots and spot diameter were noted. The lesions mostly were irregular circle in shape. Therefore, by computing leaflet area and infection area, the percentage of Necrotic Leaflet Area (NLA) for each isolate/cultivar combination was determined. Before analysis, the square root transformation was used with the data when necessary to standardize the variances, but the actual values for the means are reported. Data were analyzed using Analysis Of Variance (ANOVA) implemented within the GLM procedure of SAS software. In combined analysis, "genotype" and "isolate" were specified as the fixed effects and "year" as the random factor. Means were separated by Least Significant Difference (LSD) at P= 0.05. Due to the appearance of mature fruit bodies (acervuli) and starting of natural infection cycles 36-42 days after inoculation in some cultivars, the second recorded data (data from the 30th day after inoculation) were used for determination of susceptibility classes. Cultivars were classified in six different susceptibility groups depending on percent of NLA as follows:

Class 0: Resistant- NLA of 0-1%

Class I: Moderately resistant- NLA of 1-10%

Class II: Slightly susceptible- NLA of 10-30%

Class III: Susceptible- NLA of 30-50%

Class IV: Moderately susceptible- NLA of 50-70%

Class V: Highly susceptible- NLA of 70-100%.

RESULTS

The differentiation between resistant and susceptible genotypes to anthracnose was achieved by inoculating mature and fully expanded leaves in the greenhouse. Sixty hours after inoculation, conidial swelling and germination were detected in stained leaf tissues (Figure 2). The necrotic lesions were revealed as small round brown spots 4-5 days after inoculation mainly under and 1-2 days later on upper part of the infected leaves (Figure 3). The symptoms appeared first on 'Caspian' and 'Chaldoran' cultivars inoculated with isolate Q57 of *O. leptostyla*. The genotype K72 showed the symptoms later than other examined cultivars. The spots gradually developed, sometimes joined together to form larger brown necrotic areas. The control plants that were sprayed with sterile water developed no necrotic lesion on leaves until occurrence of natural conidial dissemination in the greenhouse by acervuli. In 2018, the appearance of black acervuli was observed first on the leaves of 'Alvand', 39 days after inoculation. In 2019, the acervuli were produced on C25 and 'Alvand' cultivars 36 and 42 days after inoculation, respectively. Leaf dropping before acervuli development occurred in

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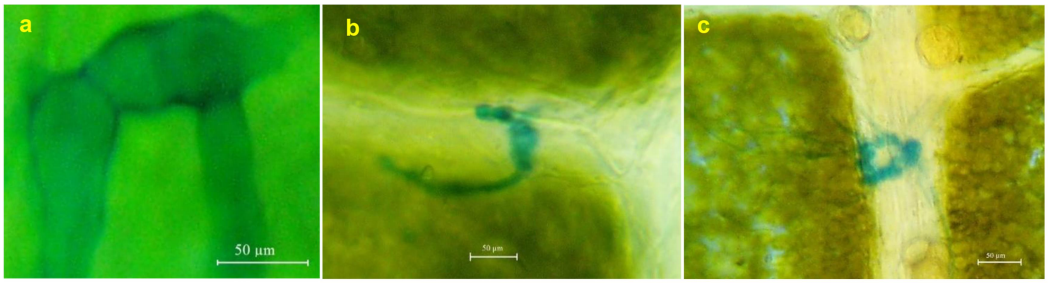

Figure 2. Spore swelling (a), and germination (b) 60 hours after inoculation; hyphal branches and colonization of walnut leaves 108 hours after inoculation (c) with *Ophiognomonia leptostyla*. Scale bars: a, b and c= 50 µm.

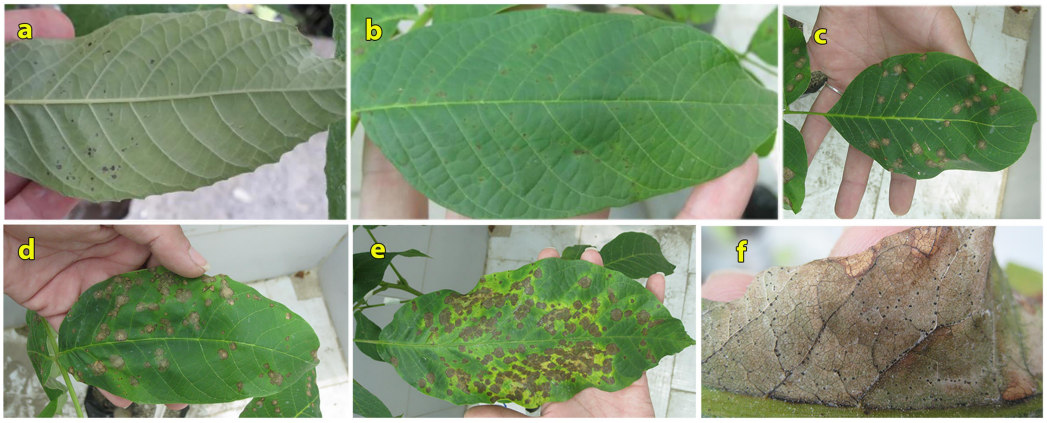

Figure 3. First symptoms of infection in lower (a) and upper (b) surface of the leaves six days after inoculation; development of spots 15 (c), 30 (d) and 45 (e) days after inoculation; appearance of acervuli on leaves of C25 genotype, 36 days after inoculation (f).

some trees of 'Chaldoran' and K72. Due to the high level of infection and leaf dropping of some cultivars as well as starting of secondary disease cycles in the greenhouse, data recording was stopped 45 days after inoculation and the data of 30th day was used for classification of cultivars susceptibility. Presence of the pathogen in walnut leaves was confirmed by fungal isolation from the necrotic leaflets and then observation of bicellular bacilli shape conidia. The fungus was not detected in leaf tissues of control plants.

The descriptive statistics of evaluated traits for susceptibility of walnut cultivars to anthracnose disease are summarized in Table 1. According to the results, the average number and size of necrotic lesions among eight tested cultivars were 21.8 and 9.6 mm, respectively. The percentage of NLA ranged from 0 to 100 while its mean was measured 37.8%. Disease incidence was detected on 67.4% of the inoculated leaflets. The maximum number of affected leaflets was determined on 'Caspian' and 'Persia' (86.9 and 84.3%, respectively), while only 36.5% of inoculated leaflets of 'RDM' exhibited visible symptoms of disease.

Due to no infection of the control plants, the final analysis was applied after exclusion of the data from the controls. The combined variance analysis for data recorded 30 days after inoculation is in Table 2. The results showed significant differences in the number and size of spots as well as the percentage of necrotic leaflet area among eight walnut studied cultivars ($P< 0.0001$). None of the measured values were affected by year or

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Table 1. Descriptive statistics of evaluated features for susceptibility of walnut cultivars to anthracnose disease.

Evaluated features	Min	Max	Mean	Variance	Std. deviation
Number of spots/leaflet	0.00	90.00	21.8 ± 7.22	416.60	20.41
Disease incidence (%)	36.54	86.94	67.4 ± 6.32	319.48	17.87
Spot diameter (mm)/leaflet	0.00	47.30	9.6 ± 3.42	93.65	9.68
Leaflet area (mm^2)	947.23	9989.33	4307.8 ± 639.70	3273269.24	1809.22
Leaflet infected area (mm^2)	0.00	32093.63	3318.6 ± 2067.00	34190722.55	5847.28
Necrotic leaflet area (%)	0.00	100.00	37.8 ± 14.31	1637.84	40.47

Table 2. Variance analysis for effect of year, cultivar, fungal isolate and their interaction on measured traits, 30 days after inoculation with *Ophiognomonia leptostyla* in greenhouse.

Source of variance	df	Mean squares			
		Number of spots/Leaflet	Spot diameter/ Leaflet (mm)	Infection area (mm^2)	Necrotic leaflet area (%)
Year	1	15.07 ^{ns}	1.84 ^{ns}	4367.79 ^{ns}	3.64 ^{ns}
Rep (year)	10	0.32	0.23	195.52	1.78
Cultivar	7	113.62 **	47.50 **	38122.15 **	327.81 **
Fungal isolate	1	1.40 ^{ns}	6.09 ^{ns}	4884.77 ^{ns}	30.58 ^{ns}
Year × cultivar	7	0.86 ^{ns}	0.97 **	1142.56 **	6.83 **
Year × isolate	1	23.67 **	0.05 ^{ns}	1665.93 **	3.39 ^{ns}
Cultivar × isolate	7	2.23 **	1.66 **	1770.58 **	20.13 **
Year × cultivar × isolate	7	2.28 **	1.00 **	999.53 **	4.78 **
Error	150	0.49	0.27	190.37	1.30
Cv (%)		17.16	19.13	34.82	23.58

^{ns}: Not significant, ** and *: Significant at 1 and 5% probability, respectively.

isolate. In general, the average number and size of necrotic spots were more in 2019 (24.83, 10.50 mm) compared to 2018 (18.68, 8.68 mm). NLA also did not show significant differences between the years (F = 0.42, P = 0.56). Two tested isolates were not statistically different for the number and size of lesions (P = 0.84 and P = 0.06, respectively); however, mean of spots produced by Q57 was more extensive than those by LA (10.72 and 8.46 mm, respectively). The average of NLA produced by both fungal isolates was not statistically different (42.7% for Q57 compared to 33.0% for LA).

The interaction between year and cultivar was significant for spot diameter (F = 0.56, P = 0.0015), infection area (F = 6.00, P < 0.0001), and NLA (F = 5.25, P < 0.0001) (Table 2). The largest lesions as well as maximum of NLA belonged to K72 and 'Chaldoran', regardless of the two experimental years. Similarly, 'RDM' and 'Hartley' displayed the smallest necrotic spots in both years. 'Alvand' and C25 also exhibited a medium amount of infection for both years (Table 3). The effect of cultivar × isolate on all measured traits was significant at the probability level of 1% (Table 2). The largest necrotic spots were reported on K72, 'Chaldoran' followed by C25 with both fungal isolates. C25 exhibited 82.78% infection when tested with Q57; however, the amount of disease was only 24.09% with isolate LA. Nevertheless, when tested with different isolates of *O. leptostyla,* all cultivars were grouped within the same susceptibility class, with the exception of those for C25 and 'Alvand'. These two cultivars were more affected by Q57 than LA (Table 4).

Disease severity was affected by interaction year × cultivar × isolate (Table 2). In both years, larger lesions appeared on

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Table 3. Interaction between year and cultivar on spot number, spot diameter, infection area, and necrotic leaflet area, 30 days after inoculation with *Ophiognomonia leptostyla* in greenhouse. [a]

Cultivar	Year	Number of spots/ Leaflet	Spot diameter mm/Leaflet	Infection area (mm^2)	Necrotic leaflet area (%)
Caspian	2018	16.29 fg	5.85 f	443.39 f	28.74 e
Persia	2018	14.40 g	4.45 fgh	252.40 f	3.94 f
C25	2018	23.58 de	10.34 e	2134.49 ef	57.67 c
Alvand	2018	53.67 b	5.16 fg	1277.00 ef	37.38 de
Hartley	2018	1.54 h	1.77 gh	8.40 f	0.24 f
K72	2018	22.47 def	25.40 b	12518.04 b	99.88 a
Chaldoran	2018	16.46 efg	15.11 d	3092.52 de	80.89 b
RDM [b]	2018	1.02 h	1.38 h	4.38 f	0.10 f
Caspian	2019	24.11 cd	4.49 fgh	490.8 1ef	12.07 f
Persia	2019	15.67 fg	4.16 fgh	457.57 ef	10.64 f
C25	2019	30.91 c	13.39 de	4831.00 d	49.20 cd
Alvand	2019	72.88 a	5.10 fg	1481.00 ef	32.42 e
Hartley	2019	2.34 h	2.39 fgh	16.09 f	0.33 f
K72	2019	24.04 cd	30.35 a	16842.58 a	100.00 a
Chaldoran	2019	26.34 cd	21.06 c	9227.62 c	90.58 ab
RDM	2019	2.33 h	3.10 fgh	19.32 f	1.12 f

[a] (a-h) Means followed by at least one letter in common are not significantly different at the 0.05 level of confidence using Least Significant Difference (LSD). [b] RDM= Ronde De Montignac.

Table 4. Interaction between cultivar and fungal isolate on spot number, spot diameter, infection area, and necrotic leaflet area, 30 days after inoculation with *Ophiognomonia leptostyla* in greenhouse.

Cultivar	Isolate	Number of spots/Leaflet	Spot diameter mm/Leaflet	Infection area (mm^2)	Necrotic leaflet area (%)
Caspian	Q57	13.92 d	4.70 cde	269.09 d	16.09 def
Persia	Q57	14.64 d	4.68 cde	303.33 d	5.00 fg
C25	Q57	24.12 bc	16.84 b	5817.79 c	82.78 b
Alvand	Q57	64.78 a	5.98 cd	1832.16 d	50.20 c
Hartley	Q57	1.85 e	3.15 def	20.34 d	0.50 g
K72	Q57	27.72 b	29.26 a	18462.18 a	100.00 a
Chaldoran	Q57	18.30 cd	19.32 b	5909.05 c	86.14 b
RDM [b]	Q57	1.33 e	1.81 ef	6.07 d	0.51 g
Caspian	LA	26.49 b	5.63 cd	665.11 d	24.71 d
Persia	LA	15.43 d	3.92 cdef	406.64 d	9.58 efg
C25	LA	30.36 b	6.89 c	1147.70 d	24.09 d
Alvand	LA	61.76 a	4.23 cdef	926.16 d	19.61 de
Hartley	LA	2.03 e	1.01 f	4.15 d	0.07 g
K72	LA	18.79 cd	26.48 a	10898.44 b	99.88 a
Chaldoran	LA	24.50 bc	16.85 b	6411.10 c	85.32 b
RDM	LA	2.02 e	2.67 def	18.00 d	0.71 g

[a] (a-g) Means followed by at least one letter in common are not significantly different at the 0.05 level of confidence using Least Significant Difference (LSD). [b] RDM= Ronde De Montignac.

isolate Q57. For each cultivar in each year, two tested isolates did not reveal to be statistically varied for the amount of disease, with the exception of those for C25 and 'Alvand' in both years. The highest amount of infection occurred on K72 and 'Chaldoran' in each combination of year-isolate. Comparatively, the minimum of disease severity was observed on 'RDM', 'Hartley' and 'Persia' (Figure 4).

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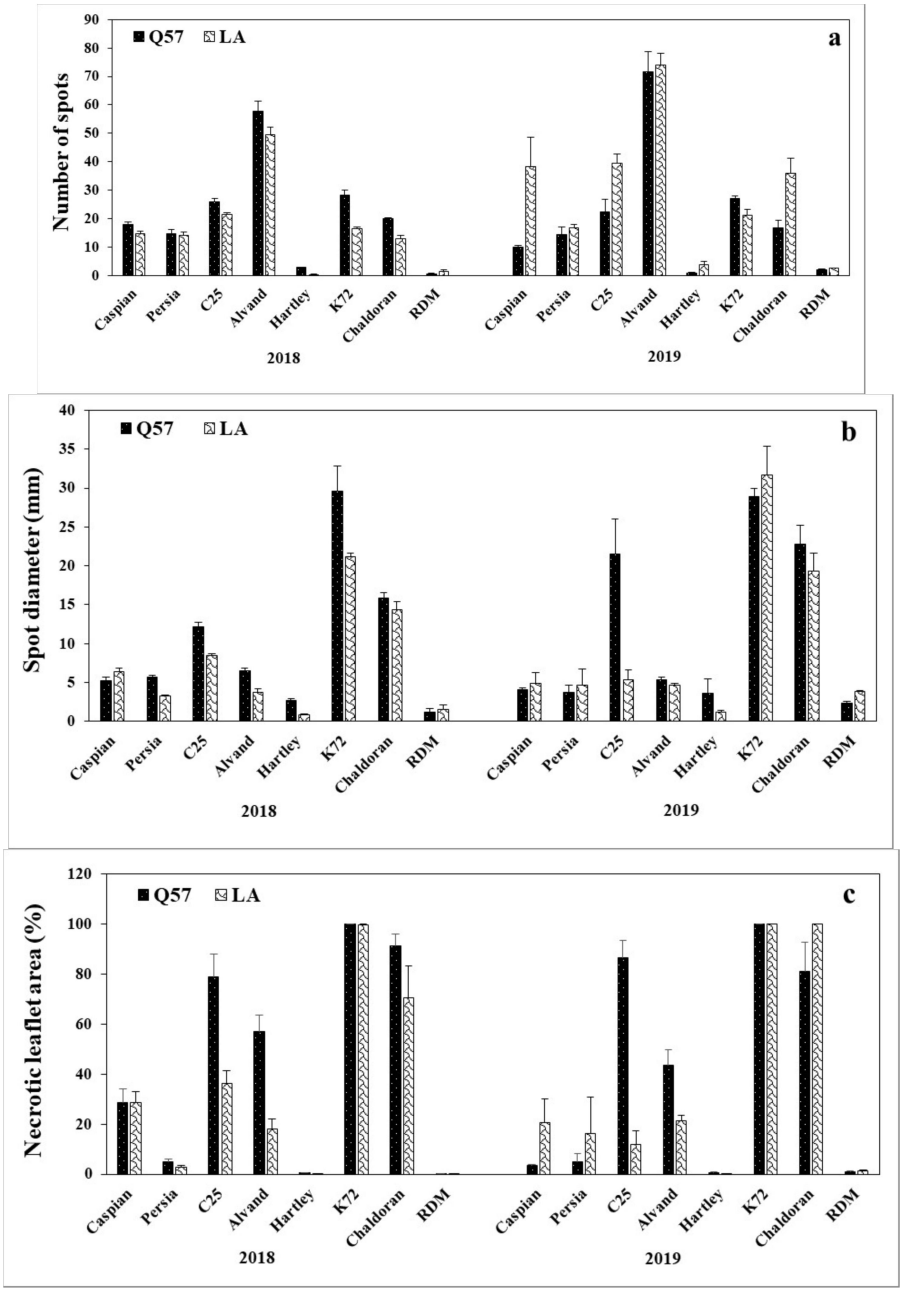

Figure 4. Interaction year×cultivar×fungal isolate on spot number (a), spot diameter (b), and necrotic leaflet area (c), 30 days after inoculation with *Ophiognomonia leptostyla* in greenhouse. On X axis: RDM= Ronde De Montignac. Mean values represent the average of 90 observations. Three leaves, five leaflets per leaf, replicated six times. Vertical bars indicate±standard error of means.

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Study of disease development in the greenhouse was carried out at 15-day intervals (Figure 5). In both years, with the appearance of leaf symptoms, the number of necrotic spots was gradually increased until 30 days after inoculation (Figure 5-a); but then changes in the number of spots was statistically significant only for C25 and 'Hartley' (in both years), 'Alvand' (in 2018) and K72 (in 2019). The spots were gradually enlarged at intervals between data recording (Figure 5-b). For both years, the largest spots belonged to K72, 'Alvand' and 'Chaldoran', which were developed 45 days after inoculation. In all genotypes, disease severity increased during the days after inoculation (Figure 5-c); however, the disease progress rate for 'Caspian', C25 and 'Alvand' increasingly developed after the 30th day, when the first acervuli appeared in the greenhouse. The amount of disease for 'Hartley' and 'Persia' significantly increased between the last two intervals of data recording ($p \leq 0.05$). Differences among intervals for infection of 'RDM' plants were not statistically significant in both years.

The eight walnut cultivars studied were categorized in different susceptibility classes according to the percentage of NLA. Although all cultivars were infected by *O. leptostyla*, 'RDM' and 'Hartley' exhibited very low level of infection and, therefore, were considered as resistant cultivars. 'Persia' and 'Caspian' were placed in moderately resistance and slightly susceptible infection classes, respectively. Genotypes K72 and 'Chaldoran' showed to be damaged the most by the pathogen, whereas 'Alvand' and C25 exhibited to be susceptible and moderately susceptible 30 days after inoculation, respectively. With appearing of acervuli and dissemination of secondary inoculum, 'Caspian', C25 and 'Alvand' were more affected by the pathogen. Some of the main characteristics of the studied walnut cultivars are summarized in Table 5.

There was a positive correlation between number of spots and percentage of NLA ($r= 0.46$, $P< 0.0001$). Furthermore, highly significant and positive correlation was determined between spot diameter and the percent of NLA ($r= 0.72$, $P< 0.0001$).

DISCUSSION

Due to the resistance to anthracnose associated with juvenile walnut leaves, fully expanded leaves of grafted *J. regia* were inoculated with *O. leptostyla* isolates. Development of necrotic spots and also formation of acervulus caused by causal agent of anthracnose are reduced in immature leaves of walnut trees (Cline and Neely, 1984; Belisario *et al*., 2001). Disease

Table 5. Some of the main phenotypic traits for the studied walnut cultivars and their susceptibility to anthracnose disease.

Cultivar	Leafing date[a]	Fruit bearing habit[a]	Mean leaflet area (cm^2)	Anthracnose susceptibility[b]
Caspian	6 April	Lateral	27.12	SS
Persia	3 April	Lateral	53.83	MR
C25	3 March	Lateral	56.28	MS
Alvand	23 March	Lateral	42.21	S
Hartley	15 April	Terminal-Intermediate	41.83	R
K72	14 March	Terminal	50.69	HS
Chaldoran	22 March	Lateral	43.51	HS
RDM[c]	26 April	Terminal	29.18	R

[a] Results are the average of experimental data collected during 2015-2018 (Hassani *et al*., 2020a). [b] SS: Slightly Susceptible; S: Susceptible; MR: Moderately Resistant; MS: Moderately Susceptible; R: Resistant, HS: Highly Susceptible. [c] RDM= Ronde de Montignac.

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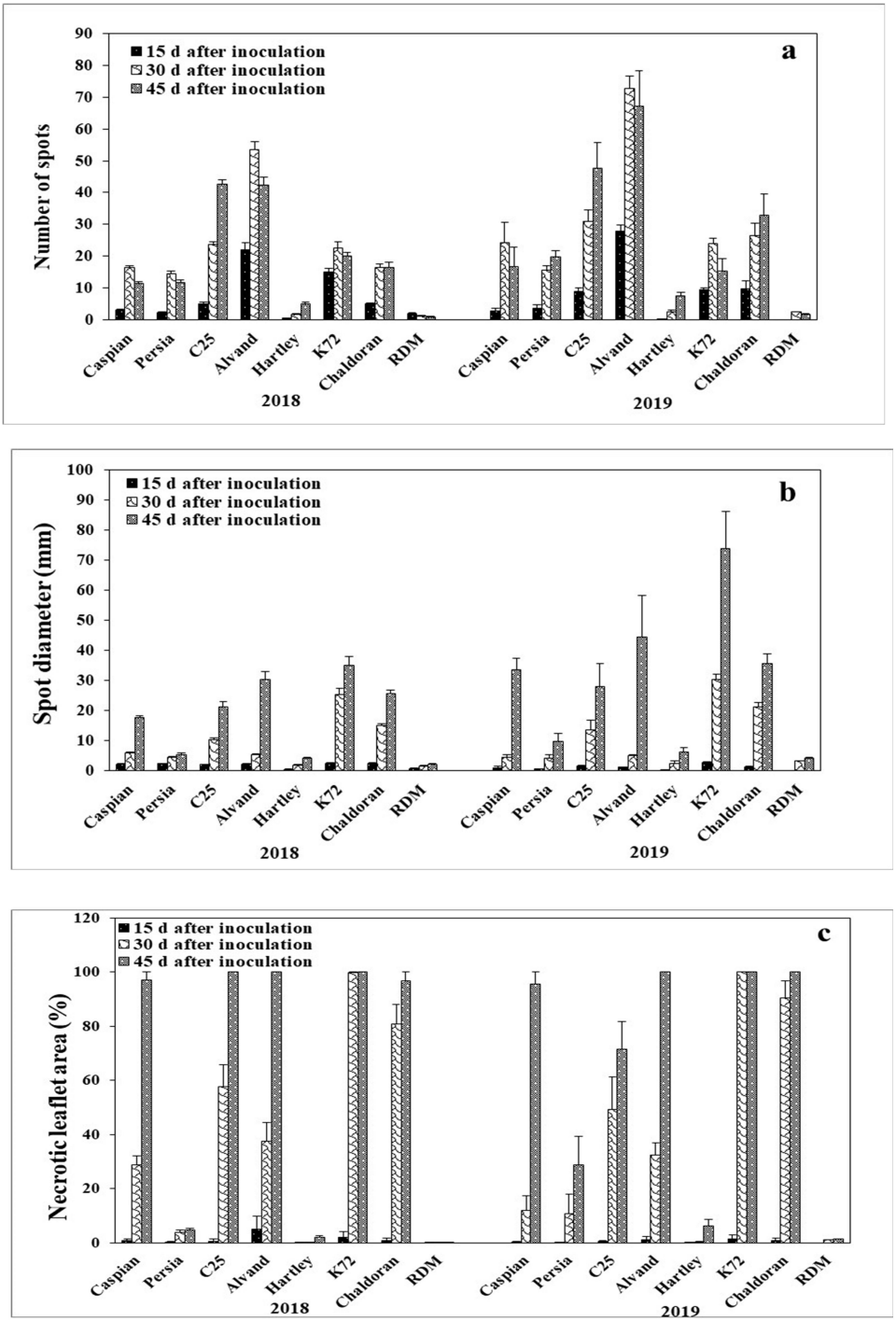

Figure 5. Changes of number of spots (a), spot diameter (b), and necrotic leaflet area (c) among walnut cultivars, 15, 30 and 45 days after inoculation with *Ophiognomonia leptostyla* in greenhouse during 2018 and 2019. On X axis: RDM= Ronde De Montignac. Mean values represent the average of 90 observations. Three leaves, five leaflets per leaf, replicated six times. Vertical bars indicate±standard error of means.

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incidence was reported on 61.7 and 73.1% of inoculated leaflets in 2018 and 2019, respectively. The successful establishment and detection of the fungus in leaf tissues suggested a repeatable method for assessing the susceptibility of future cultivars generated by walnut breeding programs.

Variance analysis showed that susceptibility of walnut cultivars to *O. leptostyla* was highly dependent on cultivars. Number of spots, amount of the fungal growth, and disease severity varied significantly among eight tested cultivars. Two isolates (Q57 & LA) studied did not show any significant differences in number and size of lesions. Meanwhile, the differences between isolates were not statistically significant for amount of leaf infection (F= 9.01, P= 0.20). Our previous studies had also indicated identical pathogenicity for isolates Q57 and LA when tested on 3-month-old seedlings of *J. regia* (Dastjerdi and Nadi, 2019). Interaction between isolate and cultivar show that the effect of isolate and cultivar is not independent. Cultivars were classified into six different susceptibility groups. Mean of NLA for K72 and 'Chaldoran', 30 days after inoculation, was 99.94 and 85.73%, respectively. They were considered as highly susceptible cultivars. The local genotype of K72 has been previously confirmed to be very susceptible to anthracnose (Dastjerdi and Hassani, 2009). Disease appearance happened very late in this genotype. Cline and Neely (1983) reported that the growth of subcuticular hyphae, produced in response to cellular toxins or to the composition of cell wall, could be a mechanical barrier to immediate penetration of fungi. High susceptibility of K72 and 'Chaldoran' suggests that these genotypes could not be suitable for the areas with favorable conditions to anthracnose infection. 'Alvand' and C25 exhibited to be susceptible and moderately susceptible to the infection, respectively. Although the average number and size of spots for cultivars 'Caspian' and 'Persia' were almost similar, due to the difference in leaflet area (27.12 and 53.83 cm^2, respectively) (Table 5), the mean of NLA was more for 'Caspian' (20.4%) compared to 'Persia' (7.3%). Therefore, based on the data 30 days after inoculation, these cultivars were grouped in two different classes of slightly susceptible and moderately resistant, respectively. The lowest necrotic leaflet area belonged to 'RDM' and 'Hartley'. Our previous studies had also shown the similar results for these two cultivars (Dastjerdi and Hassani, 2009). Maria *et al.* (1997) reported no infection on 'Hartley' in field evaluations, while Arnaudov *et al* (2014) showed a high infestation index on the leaves of this cultivar.

Disease development and merging of necrotic spots between the days of 30-45 after inoculation is the reason for reduced number of spots in some cultivars (Figure 5-a). For all cultivars, rapid growth of lesions as well as increase in disease severity were measured during the intervals of data recording. Only 30-32 days after inoculation, before acervuli appearance, highly susceptible cultivars showed the maximum level of infection (Figure 5-c); but in both years, size of lesions and also the amount of disease in 'Caspian', C25, and 'Alvand' was considerably intensified during the last 15-day interval. In general, conidial production during several infection cycles has been identified to be an important factor for intensification of anthracnose disease in walnut trees (Cline and Neely, 1983). In this study, increase in disease after the 30^{th} day could be related to the leaflets bearing fertile acervuli. These leaves provide continuing inoculum foci for secondary infections (Belisario *et al*., 2001). In 'Caspian', C25, and 'Alvand', the first disease cycle would probably predispose the plant to be more susceptible to secondary cycles of infection compared to other cultivars. It is concluded that, if cultivars with different levels of susceptibility are stressed by secondary inoculum of pathogen, they may be more susceptible to infection under favorable weather conditions. Unfortunately, we did not estimate acervuli

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population that could probably enable an assessment of the propensity of host cultivars to support secondary inoculum production. Although the size of necrotic spots increased for 'RDM', 'Hartley' and 'Persia' between the intervals of data recording, these three cultivars appeared to be more consistently resistant over both years, even 45 days after inoculation (Figures 5-b and -c).

Number of reproductive structures and also rate of lesion growth has been considered as common measurements for estimating of plant resistant reactions (Cline and Neely, 1984). Strong correlation between NLA and size of necrotic lesions (r= 0.72) show that pathogen capability for developing in plant tissues could be a more important factor than spot number to overcome host resistance. It means that number of necrotic spots defines the number of successful infections, but it is different from pathogen colonization potential. In this case, although the number of spots observed in 'Alvand' and C25 was more than those of 'Chaldoran' and K72, more extension of lesions in K72 and 'Chaldoran' led to success of the fungus to infect almost all leaf tissues only 30 days after inoculation (Figure 5). In polycyclic diseases, successful primary cycles result in reduced healthy plant tissues. Therefore, the plant could be more susceptible when exposed to further infection through secondary inoculum. That is the reason for the increase in the susceptibility of C25 and 'Alvand' at interval between 30 and 45 days.

In natural environmental conditions, the cultivars of early leafing with terminal fruit bearing are more susceptible compared to those of lateral type and late development (Arnaudov *et al*., 2014). It is expected that leafing date of 'Caspian', 'Alvand' and 'Chaldoran' (Table 5) in field conditions lead to their escape of disease and improvement of their resistance level.

The genotypes with low or moderate susceptibility play an important role in integrated management approaches to control walnut anthracnose (Hassan and Ahmad, 2017). Determination of anthracnose resistance level in new walnut cultivars, regardless of use in new plantations with the aim of disease management, would help us in walnut breeding programs to achieve new hybrids with complete resistance. None of the newly released walnut cultivars tested in this study were completely resistant to the infection; however, 'Persia', as a late-leafing-date cultivar (Hassani *et al*., 2020a), exhibited little infection to *O. leptostyla* in the greenhouse. Nevertheless, until achieving resistant plant resources, chemical and cultural control methods must be integrated into an overall strategy for disease management in areas favorable for walnut anthracnose infection.

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عکس‌العمل تعدادی از ارقام گردو(*Juglans regia* L.) در برابر حمله بیماری آنتراکنوز (*Ophiognomonia leptostyla*)

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چکیده

آنتراکنوز یکی از مهم‌ترین بیماری‌های برگی گردو (*Juglans regia* L.) در دنیا است که توسط قارچ *Ophiognomonia leptostyla* ایجاد می‌شود. به منظور تسهیل در توسعه کاشت گردو در باغات جدید، واکنش چهار رقم گردو که به تازگی در ایران معرفی شده‌اند (کاسپین، پرشیا، الوند و چالدران)، ژنوتیپ امیدبخش بومی C25، ارقام هارتلی و رونددمونتیگناک و ژنوتیپ بومی K72 در برابر بیماری آنتراکنوز مورد

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بررسی قرار گرفت. نهال‌های پیوندی گردو که برگ‌های مرکب آنها به خوبی رشد کرده بودند، در دو سال پیاپی با استفاده از سوسپانسیون کنیدی قارچ مایه‌زنی شدند. تعداد و اندازه زخم‌های نکروز ایجادشده، در ۳ نوبت به فواصل ۱۵ روز، یادداشت‌برداری شدند. براساس نتایج به‌دست‌آمده در روز سی‌ام، بیشترین تعداد لکه متعلق به رقم الوند بود. همچنین بزرگترین لکه‌ها بر روی ژنوتیپ‌های K72 و چالدران مشاهده شد. ارقام مورد مطالعه براساس درصد سطح آلوده برگچه‌ها (NLA) در ۶ گروه مختلف طبقه‌بندی شدند. میانگین NLA در دامنه‌ای از ۰/۶۱ (در روندمونتیگناک) تا ۹۹/۹۴ درصد (در K72) متغیر بود. کلیه‌ی ارقام مورد آزمایش (به جز روندمونتیگناک و هارتلی) در برابر بیماری حساس بودند، اما درجه حساسیت و شدت بیماری در آنها متفاوت بود. پرشیا به دلیل آلودگی اندک در گروه نسبتاً مقاوم قرار گرفت. کاسپین با متوسط ۲۰ درصد آلودگی برگچه‌ها، در گروه حساسیت کم طبقه‌بندی شد. ارقام الوند و C25 به ترتیب در گروه‌های حساس و نسبتاً حساس قرار گرفتند. بیشترین سطح آلودگی در چالدران و K72 رخ داد. میزان آلودگی در کاسپین، C25 و الوند به تدریج در فاصله روزهای ۴۵-۳۰ ام بعد از مایه‌زنی، افزایش یافت؛ در حالی که کلاس حساسیت در ارقام روندمونتیگناک، هارتلی و پرشیا حتی ۴۵ روز پس از مایه‌زنی، تغییر نکرد. به این ترتیب، ارقام مذکور می‌توانند در مدیریت تلفیقی بیماری مورد استفاده قرار گیرند.

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