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IDENTIFICATION AND PATHOGENICITY OF *Botryosphaeria parva* ASSOCIATED WITH GRAPEVINE DECLINE IN KURDISTAN REGION – IRAQ

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Abstract

During a survey on fungi associated with decline symptoms on grapevine cultivars growing in Kurdistan region of Iraq, several isolates of *Botryosphaeria* species were encountered. All isolates were identified as *Botryosphaeria parva* Pennycook and Samuels. Pathogenicity test for isolate DKI 1 was performed on two cultivars, Taefi and Rashmew. Under greenhouse conditions, one-year grape rooted cuttings were inoculated with the pathogen isolate by two methods, injecting the spore suspension into the green shoots and by artificial inoculation of wounded shoots with mycelial mat. The highest canker length (15.0 mm) was produced after four months on the shoots of the Taife cultivar artificially inoculated with mycelial mat of the pathogen. Under field conditions, two methods of inoculation were adopted, wounding the green shoots and drilling a hole in the arms of mature vine, followed by inoculation with mycelial mat. The highest canker length (11.17 mm) was obtained after 5 months on wounded shoots of the Rashmew cultivar and with a significant difference from the Taefi cultivar. The pathogen caused a reduction in fresh and dry weight of green shoots and roots compared with the non-inoculated control. This is the first report on *B. parva* in Iraq.

Key words: *Botryosphaeria parva*, grapevine, Iraq

INTRODUCTION

Botryosphaeria spp., the causal agents of black dead arm, have also been associated with young grapevine showing decline symptoms (Larignon et al. 2001; Phillips, 2002; Van Niekerk et al. 2004, 2006). Recent have studies identified *Botryosphaeria* species as important grapevine pathogens capable to cause stem canker, wood streaking, cane bleaching, and bud necrosis in all major viticulture regions throughout the world (Van Niekerk et al. 2004; Taylor et al. 2005; Urbez-Torres et al. 2006). *Botryosphaeria* species have been isolated from grapevine from across the world. This study is the first registration of *Botryosphaeria* spp. in Iraq.

A symptom that is often associated with *Botryosphaeria* species is bud mortality, which leads directly to yield reduction. Bud mortality is often the result of young shoots being infected by the fungus early in the season (Phillips, 1998; Larignon et al. 2001). A symptom closely resembling the wedge-shaped necrosis is arch-shaped lesions leading to brown internal necrosis, which can be seen in cross-sectioned arms and trunks (Castillo-Pando et al. 2001).

Van Niekerk et al. (2004) conducted *in vitro* and *in vivo* pathogenicity studies of 21 isolates, representing eight different species of *Botryosphaeria*, on green shoots and mature canes. The results revealed that *B.australis, B.parva, B.ribis* and *B.stevensii* were among the most virulent species. All species were successfully re-isolated from the respective lesions and thus should be considered as potential pathogens of grapevine.

The aim of this work was to isolate, identify and determine the pathogenicity of *Botryosphaeria parva* on grapevine cultivars with different methods of inoculation under greenhouse and field conditions of Iraq.

MATERIALS AND METHODS

Isolation of the pathogen

Samples from different locations of Duhok governorate were collected. *Botryosphaeria* species were isolated from complete vine tissues, from cane (bark

and wood), bud, trunk or arm (bark and wood), leaves, and berries. Plant tissue was surface-sterilized by placing in 70% ethanol for 30 s, 1% NaOCl for 1 min and again in 70% ethanol for 30 s before drying under a laminar-flow hood as described by Van Niekerk et al. (2004). Small pieces of tissue were taken from the margin between necrotic and apparently healthy tissue and plated onto 2% Potato Dextrose Agar (PDA) with 0.25 mg/ml chloramphenicol. Hyphae growing out from the tissue pieces were subcultured onto fresh PDA plates and incubated at 25±2°C (Van Niekerk et al. 2004). Seven isolates from different locations were used for morphological characterization. In order to enhance sporulation, cultures were placed on 2% water agar containing autoclaved grapevine wood chips and incubated at 25°C under intermittent light (12 h) (Luque et al. 2005). Isolates were examined weekly for formation of pycnidia and conidia.

Isolated fungi were grown on PDA and MEA at 25°C in darkness or under NUV + fluorescent illumination with a 12-h photoperiod (Philips TLD18W/33) for 10-15 days until culture sporulated. *Botryosphaeria* isolates were identified based on characters of the anamorph in culture and on natural substrates (Phillips, 2002; Denman et al. 2000; Crous et al. 2006; Urbez-Torres et al. 2006).

Pathogenicity test in the greenhouse

Rooted cuttings of two *Vitis vinjfera* L. cultivars, 'Reshmew' and 'Taefi', were planted in pots containing 20 kg of sterilized sandy loam soil (3:1) in a greenhouse. When they were well established and the shoots bore more than five nodes, the fourth internode from the tip of each shoot was inoculated by *B. parva* (isolate DKI 1). At the time of inoculation, these internodes were green but no longer succulent (Philips, 1998). The green shoots were inoculated using two methods: 1) by wounding shoots (8 mm) with a sterilized sharp blade and a colonized agar plug, about 4 mm in diameter cut from the margin of a seven-day-old culture of *B. parva*, was placed in the wound and covered with parafilm (Van Niekerk et al. 2004); 2) by injecting the spore suspension (5×10^6 spores $\times$ ml^{-1}) to the green shoot. For the control treatment, the shoots were injured with no inoculation. The length of canker was measured after 2 and 4 months after inoculation. Fresh and dry weight of green shoots and roots were measured after 4 months of inoculation.

The experiment was arranged in a completely randomized design. There were three replicates and each replicate had three plants (Phillips, 1998).

Pathogenicity test in the field

For this trial, the same set of *B. parva* was used as in the greenhouse experiment. Inoculations were made in a vineyard on 15-year-old grapevine plants of two cultivars, Reshmew and Taefi, in Duhok governorate, Kurdistan region of Iraq, by applying a colonized agar plug cut from the margin of a 7-day-old culture to the wounds (8 mm) on the bark, and then the inoculated wounds were wrapped with Parafilm. Inoculations were also made in mature wood by pouring the arm (drilling a hole (4 mm wide and 15 mm deep) into the arm of the vine). A colonized agar plug cut from a 1-week-old culture of *B. parva* was placed in the wound. The wound was sealed and covered with Parafilm. The length of canker was assessed after 2.5 and 5 months (Van Niekerk et al. 2004). The layout of the trial was a randomized complete block design with three replicates; 3 shoots were inoculated in each replicate. Data was statistically analyzed using SAS/STAT (SAS/STAT, 1999).

RESULTS AND DISCUSSION

Phenotypical characterization

Based on morphological characters, all isolates of *Botryosphaeria* species were identified as *Botryosphaeria parva* Pennycook and Samuels (anamorph: *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A. J. L. Phllips (Fig. 1A-F). The colony growth reached a diameter of 90 mm on PDA and MEA after 10 days of incubation. Colonies on PDA formed abundant aerial mycelium that was initially white but turned dark-olivaceous after 5-6 days at 25 °C. The reverse side was almost black in older cultures. Pycnidia usually were aggregated and appeared after 20-25 days in cultures incubated under black light with a 12-hour photoperiod and appeared on the moist cane bark after 30 days under room temperature. Conidiophores were reduced to conidiogenous cells. Conidiogenous cells were hyaline, holoblastic forming conidia at their tips. Conidia were hyaline, guttulate, thin-walled, non-septate, smooth, fusiform to ellipsoidal with a subobtuse apex and truncate or rounded base, often with a minute basal frill. Conidia frequently become olivaceous or light brown and develop 1 or 2 septa with a darker middle cell. Measurements of conidial dimensions for our isolates, as shown in Table 1 were in line with those reported by Pennycook and Samuels (1985); Phillips (2002); Slippers et al. (2005), and Urbez-Torres et al. (2006).

Pathogenicity test in the greenhouse

The greenhouse pathogenicity test produced obvious canker on young shoots inoculated by the pathogen by two methods (Fig. 2A-B). The results presented as canker length in Table 2 show that *B. parva* produced canker on both cultivars, Taefi and Rashmew. The highest canker appeared on the cultivar Taefi; it reached 13.80 mm and 15.0 mm in length after two and four months of inoculation, respectively.

The results presented in Fig. 3 represent the interaction between pathogen and grapevine cultivars. The results showed obvious canker on young shoots of both cultivars was caused by the fungus. However, there were no obvious symptoms observed on the leaves of both cultivars. This result is in agreement with those of Larignon et al. (2001). Based on the speed of wood colonization, cv. Taefi was rated more susceptible to *B. parva* than cv. Rashmew. Philips (1998) showed that symptoms of *Botryosphaeria* spp. usually develop slowly, and severe symptoms become visible only in grapevines that are 8 or more years old. In inoculation of one-year-old Taefi rooted cutting, the canker length increased from 5.83 to 7.00 mm by the wounding method and from 3.00 to 8.57 mm by the injecting method after 2 and 4 month of infection by *B. parva,* respectively (Fig. 4).

The results in Table (3) show that *B. parva* decreased the fresh and dry weight of shoot and root in the Taefi cultivar, with no significant differences from the control treatment.

The results from cuttings obtained in this work showed that the most important symptom to evaluate *Botryosphaeria* pathogenicity was vascular discoloration, a typical symptom associated with plants infected by vascular fungi (Sands et al. 1997; Harrington et al. 2000), which may be associated with oxidation and translocation of some breakdown products of plant cells attacked by fungal enzymes (Agrios, 2005). However, four months may have been insufficient for symptom expression, as pointed out by Hallen et al. (2007).

Pathogenicity test in the field

The pathogenic fungus *B. parva* caused a dark brown canker on young artificially wounded shoots and black internal discoloration visible in the cross-sectioned mature arm of a 15-year-old grapevine inoculated by drilling a hole (Fig. 2 C-D). The pathogen was re-isolated from inoculated parts.

The results in Table (4) show that *B. parva* produced the highest canker length reaching 11.17 mm in the Rashmew cultivar, which was inoculated by wounding the shoots, after 5 months under inoculation. This treatment was significantly different from other treatments. Inoculation by drilling a hole in the mature arm gave the lowest value for the cultivars Taefi and Rashmew.

The interaction between the pathogenic fungus and inoculation methods (Fig. 5) indicated that wounding the green shoots with the mycelium of *B. parva* caused a significant increase in canker length which reached 11.12 mm after 5 months of inoculation.

This result is also in agreement with other studies in which *B. parva* is identified as an important grapevine pathogen causing severe lesions on green shoots and mature wood (Savocchia et al. 2007; Van Niekerk et al. 2004, 2006).

Table 1.
Conidial dimensions of *Botryosphaeria parva* from Kurdistan region, Iraq, isolated during this study and comparison with those reported from previous studies

Botryosphaeria parva in this study			*Botryosphaeria parva* from previous studies	
B. parva isolates	Conidial size (µm)	Mean ±SD (µm)	Conidial size (µm)	Source of data
DKI 1	(7.5-)12.5 - 17.5× 5 - 7.5	15 ± 0.4* × 5.6 ± 0.2	(11–)14–18 (–23) × 5–7(–10)	Pennycook and Samuels, 1985
DKI 2	(13-) 16 - 22 × 4 - 7	18 ± 1.7 × 5.2 ± 0.6		
DKI 3	(13-) 14 - 20 × 5 – 7	17 ± 2.9 × 5.8 ± 1		
DKI 4	(10-) 12.5 – 17 × 5 – 6	14.5 ± 2.9 × 5.4 ± 0.5	(12–)15–20 × (4–) 4.5–6(–7.5)	Phillips, 2002
DKI5	(10-) 11.5 – 18 × 4.5 – 5.5	13.7 ± 3.3 × 4.9 ± 0.3		
DKI 6	(11-) 12 – 13.5 × 7.5 -8	11.9 ± 0.5 × 7.4 ± 0.6		
DKI 7	(19-) 20 – 22 × 5 – 5.5	20.9 ± 0.8 × 5.3 ± 0.3	17 – 19 × 5 – 6	Slippers et al. 2005.
			(-10) 14.5 – 17 × (5) 7 – 9	Urbez-Torres, 2006

* Data are means of forty conidia per isolate ± SD

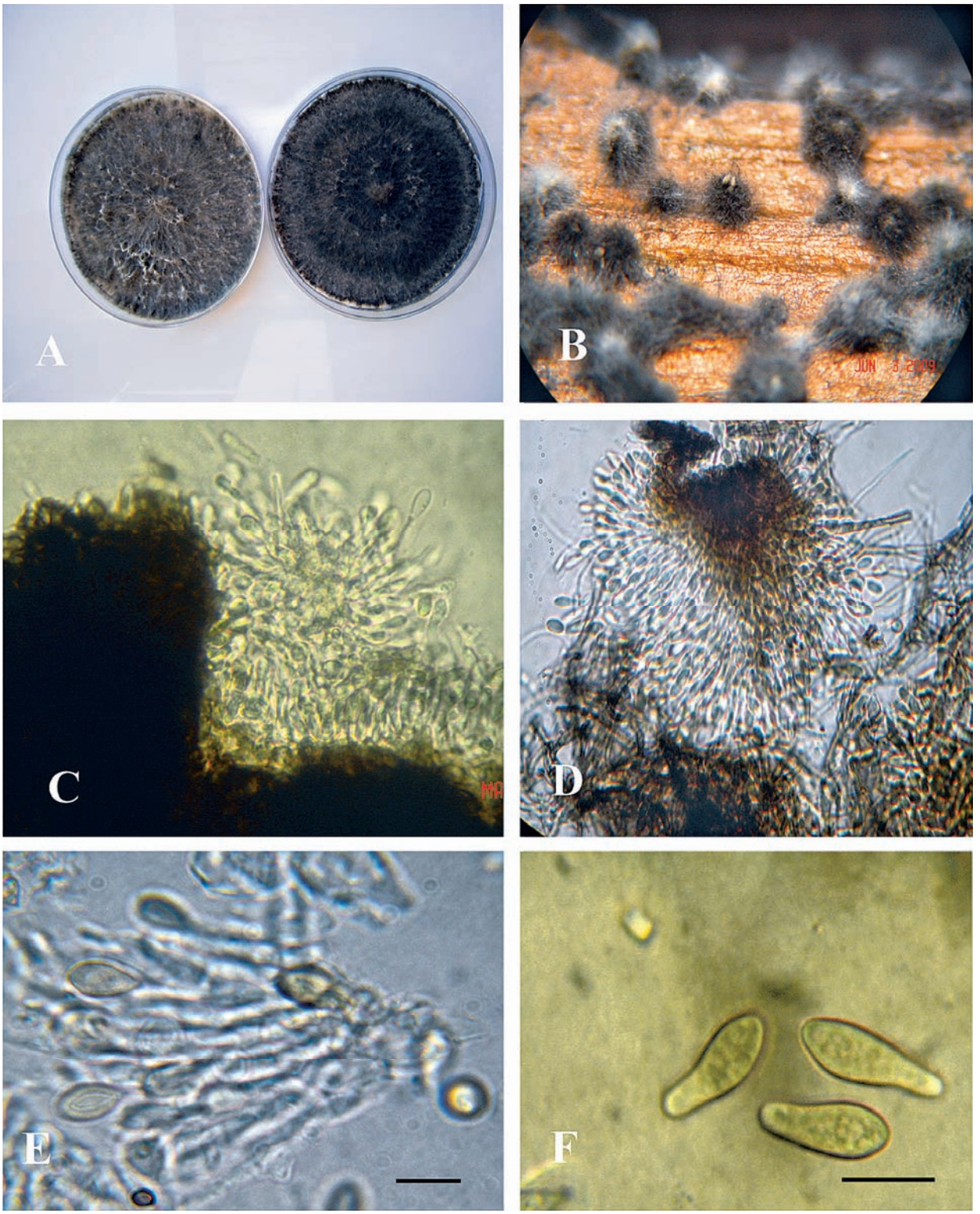

Fig. 1. A) Colony of *Botryosphaeria parva* on MEA-left, PDA-right, after 10 days of incubation at 25ºC. B) Pycnidia of *B. parva* on the surface of grapevine cane bark. C-D) Conidiogenous cells hyaline, holoblastic forming conidia at their tips discharged from mature pycnidium. E) Conidiogenous cells and immature conidia. F) Mature conidia. Scale bars: F= 20 µm, E = 10 µm.

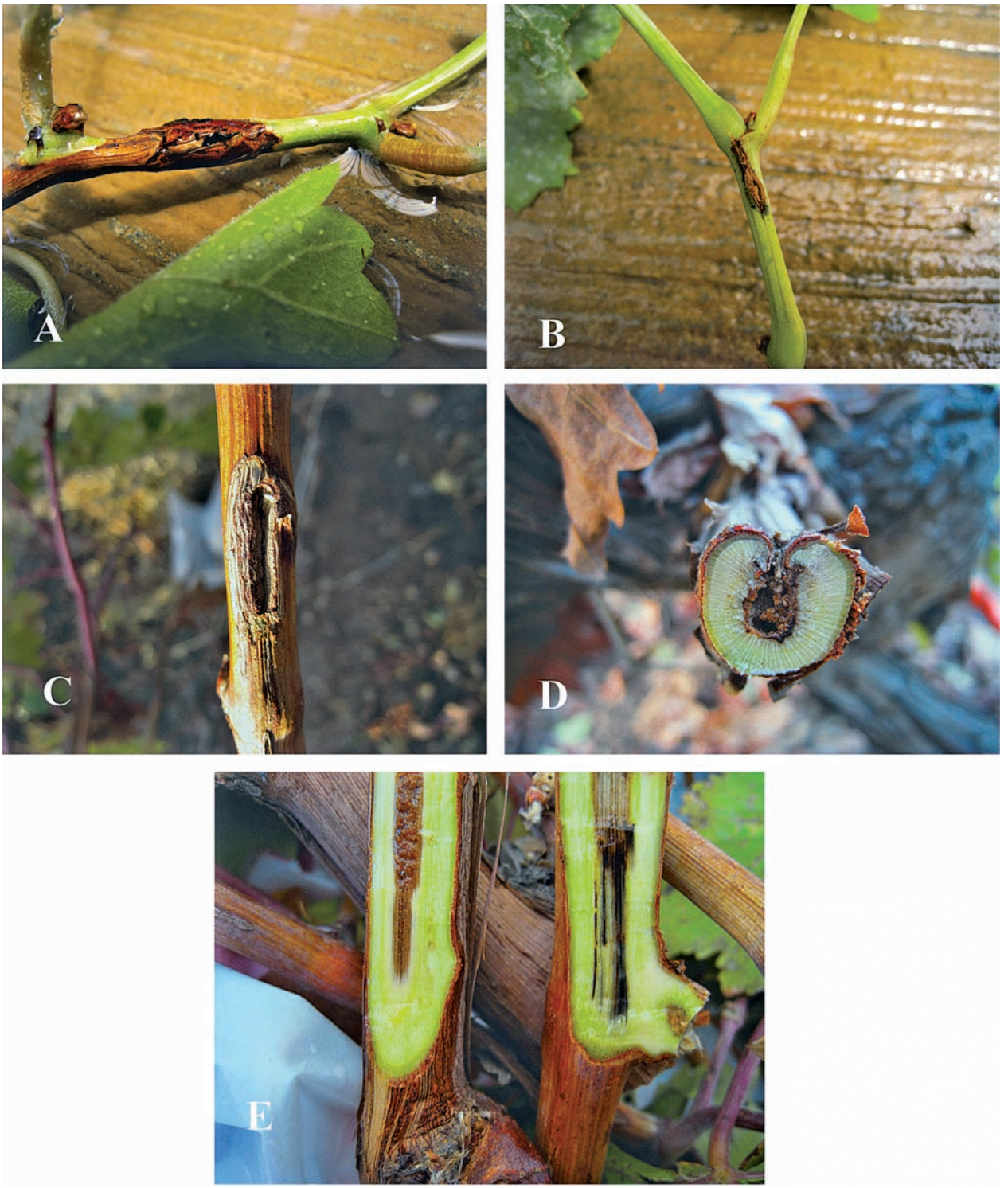

Fig. 2. Symptoms associated with B. parva on infected grapevine: A- Dark brown, discoloration canker on young shoot inoculated by wounding, and B) Dark brown, discoloration canker on young shoot inoculated by the injecting of spore suspension, under greenhouse condition; C) Dark brown canker on young shoots wounded artificially under field conditions; D) Black internal discoloration visible, in cross-sectioned arms inoculated by drilling a hole; E) Black streaking in the longitudinal section of a young shoot inoculated by wounding of a 15-year-old Rashmew plant under field conditions.

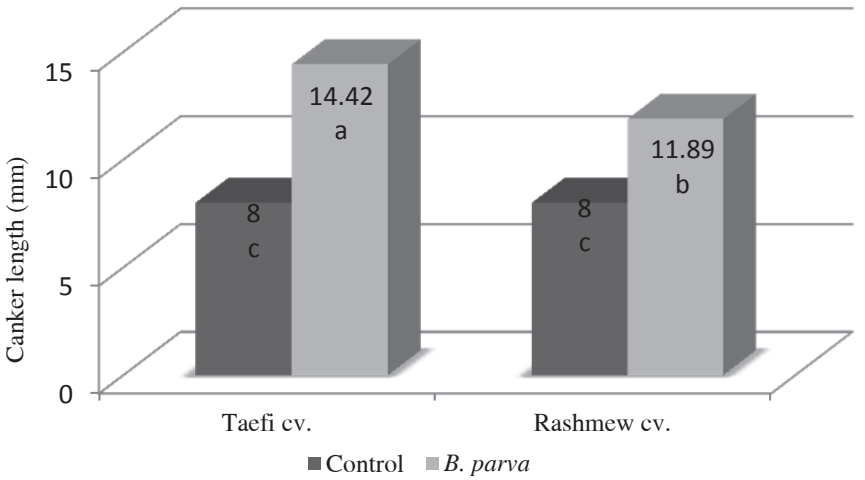

Fig. 3. Effect of pathogenic fungus and grapevine cultivars on canker length under greenhouse conditions.

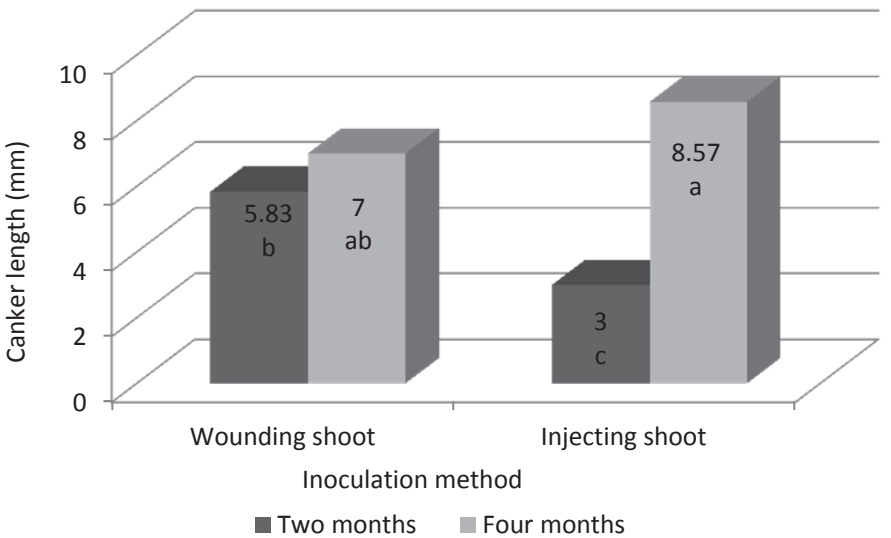

Fig. 4. Effect of inoculation method on the canker length (mm) after 2 and 4 months of inoculation

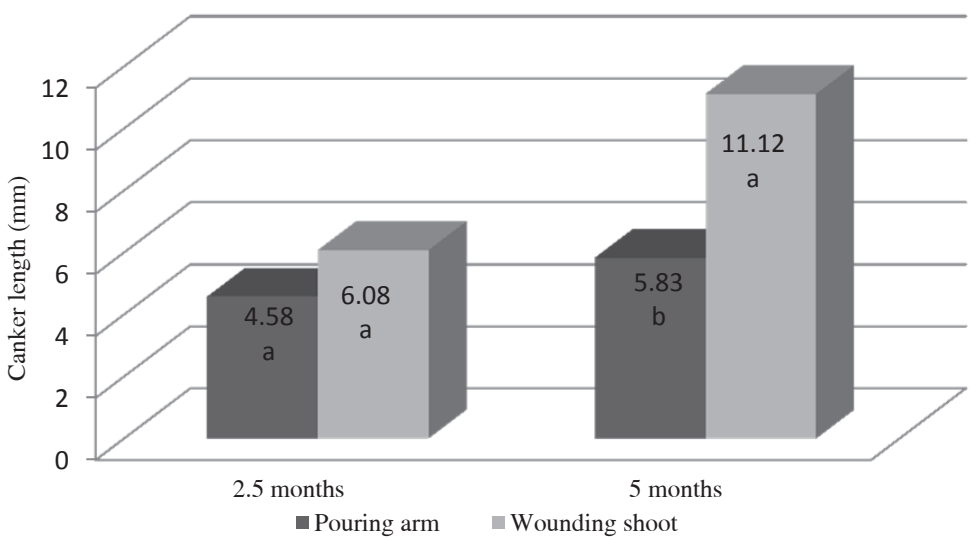

Fig. 5. Effect of inoculation method on disease development under field conditions after 2.5 and 5 months.

Table 2.
Effect of pathogenic fungus and grapevine cultivars on canker length (mm) after 2 and 4 months of inoculation under greenhouse conditions

Treatment	cv. Taefi		cv. Rashmew	
	2 months	4 months	2 months	4 months
Control	8.00 * d	8.00 d	8.00 d	8.00 d
Inoculated with *Botryosphaeria parva*	13.83 ab	15.00 a	10.75 c	13.03 b

*Means followed by different letters are significantly different based on Duncan's Multiple Range test (P=0.05)

Table 3.
Effect of isolated fungus on shoots and root of 'Taefi' and 'Rashmew' cultivars

Treatment		Shoots		Root	
		fresh weight (g)	dry weight (g)	fresh weight (g)	Dry weight (g)
Control	cv. Taefi	97.04* a	29.50 b	33.03 ab	19.35 ab
	cv. Rashmew	92.82 a	41.63a	44.83 a	24.85 a
Inoculated with *B. parva*	cv. Taefi	75.98 a	29.16 b	23.85 b	15.03 b
	cv. Rashmew	93.77 a	41.08 a	41.79 ab	24.66 a

* Means followed by different letters are significantly different based on Duncan's Multiple Range test (P=0.05).

Table 4.
Effect of inoculation method on canker length (mm) of *Botryosphaeria parva* in the grapevine cultivar after inoculation under field conditions

Cultivars	Inoculation method after 2.5 months		Inoculation method after 5 months	
	Wounding shoot	Drilling mature arm	Wounding shoot	Drilling mature arm
Taefi	5.27 *ab	4.50 b	11.06* a	4.67 b
Rashmew	6.90 a	4.67 ab	11.17 a	7.00 ab

* Means followed by different letters in each period are significantly different based on Duncan's Multiple Range test (P=0.05).

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Identyfikacja i patogeniczność grzybów *Botryosphaeria parva* związanych z zamieraniem winorośli w regionie Kurdystanu w Iraku

Streszczenie

W trakcie prowadzenia badań nad grzybami związanymi z symptomami zamierania gatunków winorośli rosnących w regionie Kurdystan w Iraku natrafiono na kilka izolatów gatunków z rodzaju *Botryosphaeria*. Wszystkie izolaty oznaczono jako *Botryosphaeria parva* Pennycook i Samuels. Na dwóch odmianach, 'Taefi' i 'Rashmew', wykonano test patogeniczności dla izolatu DKI 1. W warunkach szklarniowych jednoroczne ukorzenione sadzonki winorośli inokulowano izolatem patogena przy użyciu dwóch metod: wstrzykując zawiesinę zarodników grzyba w zielone pędy oraz poprzez sztuczną inokulację naciętych pędów za pomocą grzybni. Nekroza o największej długości (15,0 mm) powstała po czterech miesiącach na pędach odmiany Taife sztucznie inokulowanych grzybnią patogena. W warunkach polowych przyjęto dwie metody inokulacji: nacięcie zielonych pędów oraz wywiercenie dziurki w gałęziach dojrzałej winorośli, po czym dokonano inokulacji grzybnią. Nekrozę o największej długości (11,17 mm) uzyskano po 5 miesiącach na naciętych pędach odmiany Rashmew, przy czym różnica w stosunku do odmiany Taefi była istotna. Patogen spowodował zmniejszenie świeżej i suchej masy pędów zielonych i korzeni w porównaniu z nieokulowaną kontrolą. Jest to pierwsze doniesienie na temat grzyba *B. parva* w Iraku.