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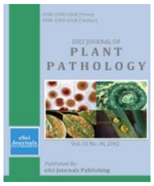

DEVELOPMENT AND SUPPRESSION OF GRAPEVINE BLACK FOOT CAUSED BY *ILYONECTRIA RADICICOLA*

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ABSTRACT

Present study investigated the development and suppression of grapevine black foot using a highly virulent strain of *Ilyonectria radicicola* during 2010-2012 after its widespread outbreak in Duhok - Iraq since 2008. Inoculated roots showed distinctive symptoms of sunken necrotic lesions with internal black streaking of rootstocks. Production of mycelial mass (in vitro) was higher at pH 5.0 resulting in 57% severity of foot rot compared to 46.16% at pH 7.0. In general, Kamali cv. was the most susceptible cultivar with 59.29% of stubby root growth affected compared to 53.32% and 40.83% on Rashmew and Taefi cvs. respectively. Wounding roots of a susceptible cultivar developed the conspicuous symptoms of black foot rot with a severity of 90%. Increasing the conidial inoculum was essential for severe infection development to more than 62%, whereas interaction between inoculation and wounding of roots increased lesion severity to 80.09%. However, acidic pH significantly enhanced disease progression on inoculated vine cuttings to 84.41% compared to 40% at neutral pH. Unfortunately, inoculum of *Ilyonectria radicicola* was not suppressed significantly even with fungicide application and continued its damage on Rashmew cv. Resulting in disease severity of 23.70% . More than 25% of the inoculated vines grown in amendments of *Trichoderma harzianum* and farmyard manures were infected compared to 14.80% and 20.73% of the non-inoculated. Exclusion of fungal inoculum improved the plant vigours as measured by dry weight and shoot growth, whereas significant stimulation of root growth were evidenced in the amended treatments particularly those with farmyard manures and fungicides.

Keywords: *Ilyonectria radicicola*, Soil pH, Root wounds, Soil amendments.

INTRODUCTION

Investigation of affected rootstock nurseries and older grapevines (2-8 years old) in Iraq revealed black discoloration and brown to dark streaks mainly at the basal end (Black foot) (Haleem, 2010; Haleem *et al.*, 2012) and the diseases was caused by *Ilyonectria radicicola* (Gerlach & L. Nilsson) P. Chaverri & C. Salgado (Anamorph: *Cylindrocarpon destructans*). The symptoms included a reduction in the root biomass (plant vigour) with small-sized trunks, shortened internodes, sparse foliage and small leaves with interveinal chlorosis and necrosis (Rego *et al.*, 2000; Halleen *et al.*, 2006). Typical black foot symptoms on rotted cuttings including sunken, necrotic root lesions, vascular black discoloration and necrosis developed within two months (Rego *et al.*, 2001) with reduction in plant vigor (Hallen *et al.*, 2004). Black foot pathogen infected grapevines which had been previously planted in open contaminated nurseries (Hallen *et al.*, 2003). grapevines which had been previously planted in open contaminated nurseries (Hallen *et al.*, 2003). *Ilyonectria* species can be segregated into four groups based on the presence or absence of micro-conidia and chlamydospores (Hallen *et al.*, 2006). The factors that become favoured development of disease by *I. radicicola* included host malnutrition, poor water drainage, soil compaction, and planting of vines in poorly prepared soil (Hallen *et al.*, 2004). *Ilyonectria* species are often part of disease complexes with other fungi or nematodes (Brayford, 1993). These pathogens include: *Phaeoacremonium* spp., *Phaeomoniella chlamydospora, Botryosphaeria* spp., *Phomopsis* spp., *Pythium* spp., and *Phytophthora* spp. (Oliveira *et al.*, 2004; Edwards and Pascoe, 2004).

The fungus is a slow growing and like other soil-borne pathogens, including *Fusarium* spp., and *Rhizoctonia solani*, can exist as highly virulent or weakly virulent strains on the host (Seifert *et al.*, 2003). There is little published information on the effects of abiotic factors

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such as soil pH, temperature, and soil moisture on *Cylindrocarpon* black foot as a result of soil amendments. The objective of this research was to examine some of the biotic and abiotic factors that can influence root infection by *Ilyonectria radicicola* such as root wounding, soil pH and application of soil amendments with farmyard manures, fungicides and biocontrol agent of *Trichoderma harzianum*.

MATERIALS AND METHODS

Isolation and pathogenicity of *Ilyonectria radicicola*: Vine roots with characteristic root rot symptoms that included dark brown sunken lesions and rotting of the primary root tip and lateral roots were collected from several plantations and young vineyards located in Badi (East of Duhok city) northern of Iraq from 2008-2009. Tissue sections from border between healthy and rotted tissues were surface sterilized by immersion in 1% NaOCl for 2min. followed by two rinses in sterile water, then blotted on sterile filter paper and transformed to Potato Dextrose Agar (PDA) with 0.25 mg/ml chloramphenicol. The culture plates were grown at 25 ± 2^0C for 14 days and the isolated pathogen was stored on PDA slants at 4^0C.

Healthy roots of three grapevine cultivars (Kamali , Rashmew and Tefi) were inoculated with a 0.5 cm^2 mycelia agar plugs, with four replicates for each cultivar. Roots were either wounded at the point of inoculation with a fine needle to a depth of 1.0 x 0.25 mm in diameter or left unwounded. Lesions development was assessed after 3 weeks.

Effect of pH on the mycelial mass production: Citrate and phosphate buffer solutions (0.01M) containing citric acid\Na- citrate and Na_2HPO_4 respectively, were made to achieve pH of 5.0 and 7.0. The buffer solution (100 ml) was poured in a 250 ml flask and the required amount of potato dextrose broth was added to be followed by autoclaving. The pH of the cooled broth was measured and readjusted with 1N HCl or NaOH. Each flask was inoculated with a 0.5 cm^2 mycelial agar plug from a 14 day old culture and incubated on a rotary shaker for 2 weeks. The mycelia from each flask was filtered, dried at 40^0C for 72 h, and weighted. There were four replicates for each pH.

Effect of pH and wounding on the root rot in the greenhouse: Taefi, Kamali and Rashmew old root cuttings were carefully dug up from agriculture college nursery with surrounding rhizosphere soil and transplanted into 15cm diameter pots which were covered with polyethylene bags and brought to the laboratory within 24 hrs. The roots were gently washed, and cutting with healthy roots were planted under greenhouse condition in plastic pots (25 cm diameter) containing field soil that was modified by adding 1 N HCl or NaoH to adjust the pH to 5.0 or 7.0. The pH was monitored daily and adjusted as needed.

Wounds were made on roots prior to inoculation by piercing them 8 to 10 times with a fine needle to a depth of 1.0 x 0.25 mm in diameter. Inoculations were conducted by adding the required amount of spore suspension to 900 ml distilled water to achieve 5x108 spores per ml in each container. Spore suspension was prepared from one month old culture grown on PDA at 25°C, by flooding the agar surface with 10 ml of sterile distilled water (SDW) and scraping with a spatula. The resulting spore suspension was filtered through two layers of cheesecloth. Spore concentration was calculated with haemocytometer, and then adjusted by dilution with SDW to $5x10^8$ spores per ml. Wounded and unwounded roots were randomly assigned to each of three replicate containers (six cuttings for each cultivar). The control included six no wounds with non-inoculated cuttings at each pH (5.0 and 7.0). Thus, the treatments were: 1- Control (no wounds with non-inoculated); 2- Wounded roots with no inoculation; 3- Inoculation of non-wounded roots;4- Inoculation of wounded roots.

Roots were rated for disease incidence and severity after three week by a continuous scale of 1 to 6: 1=no visible lesions, 2= brown lesion up to 0.9 mm in diameter, 3=dark brown of 1 to 4.0 mm, 4= black lesion of 4 to 7.0 mm, 5=black lesion > 7.0 mm and 6= fully rotted roots .Disease severity index (DSI) was then calculated (Michenny1923) using the following formula : $\%DSI=\sum d/ dmax \times n$ (DS is the disease severity; d is the disease rating on each plant; d max is the maximum disease rating possible and n is the total number of plant examined in each replicate).

Effect of soil amendments on the disease development in the greenhouse: Farmyard manures at 12.5 t h^{-1}, fungicides of Metalaxyl 2g plus 1.5 g Benlate, or a biocontrol agent *Trichderma harzianum* (20 KI) was added to each pot (25 cm diameter) except for the control. The treatments were replicated 3 times in complete randomized design using 3 pots for each replicate.

One year old dormant rooted cutting of Rashmew cultivar was planted in pots (25 cm in diameter) containing 20 kg autoclaved sandy loam soil plus peat moss (3:1) in the greenhouse at the Faculty of Agriculture and Forestry.

Fungal inoculum was prepared as spore suspension at 1 × 10^6 spores per ml. Before inoculation, the roots were trimmed and disinfested by immersion in 1.5 % NaOCl for 2 min., and washed twice with distilled water. Plants were inoculated before planting by slightly pruning of roots before dipping its roots for 30 min. in the conidial suspension. None inoculated plants serving as the control treatment. After 5 months of inoculation, cuttings were uprooted and washed free of soil. Root symptoms of each individual plant were based on Alaniz *et al*. (2007) depending on the grade of black discoloration and mass reduction of roots using scale of 0 to 5:

0	healthy with no lesions
1	slight discoloration with 1 to 10 % root mass reduction
2	slight discoloration with 11 to 25 % root mass reduction
3	moderate discoloration with 26 to 50 % root mass reduction
4	severe discoloration with >50% root mass reduction
5	dead plant

Inhibition of plant vigor as a result of a pathogen depended on shoots length, plant dry weight and the reduction in root mass. The later was estimated using the following formula:-

$$\%\text{Reduction} = \frac{\text{Whr} - \text{Wdr}}{\text{Whr}} \times 100$$

Where:

Wh_r = Weight of healthy root

Wd_r = Weight of diseased root

The data were analyzed using Statistic Analysis System program and means (SAS Institute Inc., Gary, NC, USA, 1999). Data were subjected to analysis of variance (ANOVA). Means of the treatments were compared by Duncan's multiple range test of the 5% level.

RESULTS AND DISCUSSION

Ilyonectria radicicola was the dominant pathogen isolated frequently by 41-75% on PDA from naturally infected vine roots collected randomly during 2008-2009 in Duhok vineyards, northern Iraq (Haleem, 2010). Inoculated roots of Kamali, Rashmew, and Taefi cultivars showed sunken necrotic lesions, black discoloration and dark streaks in the wood of rootstocks (Fig. 1). These symptoms were also reported previously (Scheck *et al*., 1998 and Rego *et al*., 2001).

Figure 1: Symptoms of *Ilyonectria radicicola* on the Rashmew cv.: Discolored pith of the inoculated roots under greenhouse conditions. (right). Health root pith (left).

Effect of pH on the mycelial mass production: Mycelial mass production in buffered potato dextrose broth was significantly higher at pH 5.0 (5 g) than at pH 7.0 (2.9g).

Effect of pH and wounding on the root rot of grapevine cultivars: Lesions appeared on wounded roots at pH 5.0 and 7.0 as early as 5 days after inoculation compared with 11 and 14 days for unwounded roots at the same pH. Furthermore, soil pH 5 resulted in severe foot rot 57 % compared to 46.16 % at pH 7.

Effect of wounding on the susceptibility of vine cultivars to foot rot: Kamali cv. was the most susceptible cultivars, since the severity of foot rot was 59.29 % compared to 53.32% for Rashmew cv. and 40.83 % for Taefi cv. This may be due to abundance of natural wounded shoots that facilitated the pathogen penetration; similar findings were obtained by Rahman and punja (2005). Wounding has been shown to enhance diseases caused by many other soil borne pathogens such as *Cephalosporium graminearum* (Specht et al., 1990), and *Chalara elegans* (Punja *et al*., 1992). Therefore, wounding was essential for the infection to develop from conidial suspensions and increasing the conidia in the inoculation droplet increased the severity of lesions to more than 62% in wounded roots (Fig. 2). These results suggest that the availability of nutrients from root exudates of wounded tissues is necessary for infection (Sweetingham, 1983) and this process is mostly accompanied with a higher pectinase activity level for highly virulent isolate of *Ilyonectria radicicola* (Wh and Zhong, 1999).

Thus, inoculation of wounded roots was increased severity of lesions to 80.0 9 %. Similar results were obtained by Manka (1981) in studies of pectolytic enzymes in *Fusarium* sp. isolates pathogenic to corn seedlings.

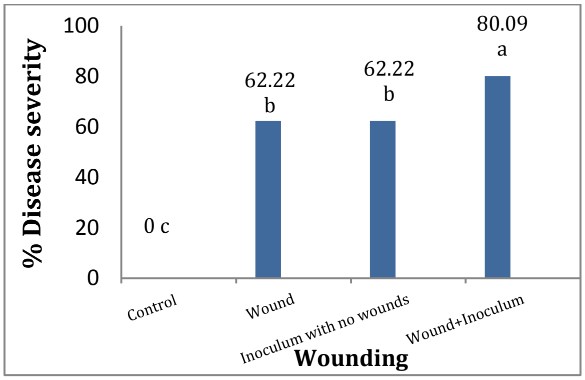

Figure 2: Effect of wounding on foot rot severity.

In spite of initial lesions appearing at almost the same time on wounded roots, irrespective of pH, lesion expansion was much slower at pH 7.0. Although the highly virulent isolate used in our experiment was capable of producing lesions on unwounded roots, wounding enhanced disease severity of this isolate. On old root cuttings, lesions severity developed to 84.41 % on inoculated vines when grown at pH 5.0, this may be due to combination of physical injuries and each of favorable soil pH5 and virulence of a pathogen that accelerated the disease progress. Wounding and inoculation of roots at both of pH 5.0 and 7.0 were also produced severe lesions of 79.97 % and 80.21 respectively (Table 1). However, Acidic pH was significantly enhanced the mean disease development to 57% compared with neutral pH (46.16%). Club root of crucifers was reduced by liming the soil to above pH 7.0 and at pH 7.8 , the disease was completely inhibited (Fletcher *et al.*, 1982). Murray et al. (1992) reported that the infected stems due to *Cephalosporium* stripe decreased significantly when $Ca(OH)_2$ was added to increase soil pH from 5.1 – 5.3 to 6.0 . Our findings suggest that soil pH may influence development of vine root rot because its significantly reduced at pH 7.0.

Table 1: Effect of pH and wounding on disease severity index due to Ilyonectria radicicola under greenhouse conditions.

pH	Wounding	% Dis. severity
5	Control	0* f
	Wound	60.0 d
	Inoculum	84.41 a
	Wound + Inoculum	79.97 b
Mean		57.0 a
7	Control	0 f
	Wound	64.45 c
	Inoculum	40.0 e
	Wound + Inoculum	80.21 b
Mean		46.16 b

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

Rashmew cv. was significantly influenced by inocula of *Ilyonectria radicicola* inserted in the wounded roots, since disease severity was greater 89.96 % compared with 76.96 % and 73.35 % of the same treatments for Kamali and Taefi cvs. respectively (Table 2) .

However, wounding roots of Kamali cv. enhanced the foot rot conspicuously, since its severity reached to 90 %. Such minor wounds could readily occur during the growth of vine roots by winter freezing and spring thawing, nematode feeding, tools of field practices or by soil- inhabiting insects. In this aspect, Slootweg (1956) reported that some isolates of *Cylindrocarpon radicicola* when inoculated vine roots produced no lesions, whereas the fungus inoculated together with *Pratylenchus penetrans* caused extensive lesions. It's likely that other organisms or any wounding agent accompanying weakly virulent *Ilyonectria radicicola* can facilitate its penetration. A histopathological study of the infection process by a highly virulent isolate revealed that direct penetration of the root epidermis as well as ramification of the mycelium in the cortical cells took place.

Therefore, its possible that virulent isolates infect the roots first and secondary colonization by weakly virulent isolates could follow (Rahman and Punja, 2005).

Effect of soil amendments on the disease development: The Severity of root mass reduction of inoculated Rashmew cv. reached to 23.7 % after five months despite the use of fungicides of benlate + metalaxyl. This damage developed to 25.9 % and 26.7% of the inoculated vines grown in the soil amended with *T. harzianum* and farmyard manures, respectively (Table 3).

Effect of soil amendments on the disease development: The severity of root mass reduction of inoculated Rashmew cv. reached to 23.7 % after five months despite the use of fungicides of benlate + metalaxyl. This damage developed to 25.9 % and 26.7% of the inoculated vines grown in soil amended with *T. harzianum* and farmyard manures, respectively (Table 3). The results confirm the pathogenicity of *Ilyonectria radicicola* to vine roots as evidenced by the development of necrotic lesions with different sizes and depths on artificially inoculated roots as well as naturally (non-inoculated) ones as a result of the application of soil amendments. Severity of root infection developed to only 14.8 and 19.3 % of the non-inoculated vines grown in soil amendments of *T. harzianum* or fungicides, respectively. Indeed no chemicals are currently available to control this pathogen (Rahman and Punja , 2005), but Zeizold et al. (1998) evaluated the toxicity of a range of fungicides against mycelial growth of *Ilyonectria radicicola* in vitro as well as on root rot of some vines. Inoculated plants showed a significant reduction of their root biomass (26.67%) and severe loss of root tips , rootlets, and lateral roots though when grown in soil amendments of fungicides or *T. harzianum* (22.1 %) whereas, this loss of root growth was reduced to 17.47% in the farmyard amendment compared to 30.33 % in non-inoculated plants of the same treatment.

Table 2: Effect of wounding on the susceptibility of grapevine cultivars to foot rot.

Cultivars	Wounding	% Disease severity
Kamali	Control (non-wound)	0^* g
	Wound	90.0 a
	Inoculum	70.0 c
	Wound+ Inoculum	76.96 b
Rashmew	Control (non-wound)	0 g
	Wound	63.33 d
	Inoculum	60.0 de
	Wound+ Inoculum	89.96 a
Taefi	Control (non-wound)	0 g
	Wound	33.34 f
	Inoculum	56.62 e
	Wound+ Inoculum	73.35 c

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

Table 3: Effect of soil amendments on the disease development.

Amendments	Inoculum	Dis. Severity %	Reduction of root biomass	Shoot length (cm)	Plant dry weight (gm)
Farmyard manures	+	26.67^* a	17.47 d	25.33 b	36.74 b
Farmyard manures	-	20.73 ab	30.33 c	28.77 b	38.0 b
T. harzianum	+	25.90 ab	22.10 cd	25.1 b	37.80 b
T. harzianum	-	14.80 e	43.23 a	28.47 b	38.97 b
Fungicides	+	23.70 abc	26.67 cd	28.90 b	38.77 b
Fungicides	-	19.27 cde	29.57 c	31.90 ab	38.37 b
Control (non- amended)	+	23.70 abc	31.67 bc	29.97 b	29.83 c
Control (non- amended)	-	17.80 de	40.33 ab	40.10 a	47.67 a

* Means followed by different letter within the same block are significantly different based on Duncan's Multiple Range test (P=0.05).

The result of our study revealed the role of fungal inoculum and wounding to enhance disease development. Other soil – borne pathogens such as *Cephalosporium graminium, Phytophthora capsici* it's likely that other organisms accompanying *Ilyonectria radicicola* can facilitate its entrance and infection of the vine roots (Adorada *et al*., 2000). Diagnostic symptoms of small – sized shoots, shortened internodes and sparse foliage resulting in obvious reduction in shoots growth the role of fungal inoculum since the healthy shoots extended to 40 cm with dry weight of 47.67 g compared to 29.97 cm and 29.83 g for diseased ones. These symptoms frequently leading to death of affected plants (Hallen et al., 2006 and Alaniz *et al*., 2007).

In general, there was no significant differences in the plants vigor (shoot growth or dry weight) when grown in soil amendments. The virulence of a pathogen might be attributed to a marked pectolytic enzyme production (Lyr and Kluge , 1968) , and the brown sunken lesions caused by *Ilyonectria radicicola* may be the outcome of degradation of host phenolic compounds by fungal pectinase (Rahman and Punja, 2005) .

We conclude that managing soil factors such as pH and minimizing wounding due to agricultural practices , nematodes , insects and extremes of weather may prove to be partially effective in disease management and the avoidance of pathogenic propagules of *Ilyonectria radicicola* by soil amendments of fungicides , farmyard manures , and antagonistic fungus of *T. harzianum* could lead to the suppression of vine foot rot.

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