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ORIGINAL ARTICLE

Effects of Different Fungicides on *Paecilomyces formosus*, the Causal Agent of Dieback and Canker Diseases of Pistachio

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K E Y W O R D S

Chemical control;
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A B S T R A C T

In this study, the effect of 13 fungicides on mycelial growth, spore germination and sporulation of *Paecilomyces formosus*, the main causal agent of dieback disease, were investigated *in vitro* and *in vivo*. Treatments included Profiler®, Rovral-TS®, Elit®, Oxychromes®, Cidley Top®, Cuprosit C®, Ortivatop®, Captan®, Acrobat MZ®, Benomyl®, Luna®, Folicur® and Falcon® at different concentrations of 500-3000 ppm. The results showed that the efficiency of different fungicides on fungal development, measured as mycelial growth, spore germination ranged from 36.8 to 100% and 21.7 to 100%, respectively. The inhibition of sporulation was between 0 to 4.3×10^6 spores mL^{-1}. *In vitro*, the highest reduction in mycelium growth and spore germination was observed for the fungicides Benomyl®, Luna®, Folicur®, and Falcon® at concentrations of 1000, 500, 1500, 2000 and Ortivatop®, Elite®, Falcon®, Benomyl®, Captan®, and Acrobat MZ® at concentrations of 750, 2500, 2000 1000, 3000, 3000 ppm, respectively. *In vivo*, the effect of selected fungicides was evaluated on inoculated shoots. The rate of inhibition of pathogen progression using immersion inoculation and the vertical method was 18.7-43.2% and 39.8-45.5%, respectively. The highest inhibition (45.5%) was observed with Luna® fungicide at 500 ppm using the vertical method and the lowest inhibition (18.7%) was related to Benomyl fungicide at 1000 ppm using the immersion method ($P\leq0.01$). No significant advantages were observed in fungicides applications in terms of DBP control compared to those control trees with no-spraying fungicides. In contrast, pruning showed comparative advantages in the management of DBP.

Introduction

Pistachio (*Pistacia vera* L.) is one of the most important horticultural products and one of the main non-oil exports of Iran (Nazoori *et al*., 2022). Iran is the second largest producer of pistachios in the world and has a high export volume (Alipour. 2018; Norozi *et al*., 2019; Sharifkhah *et al*., 2020), but the yield of pistachios is low in some areas due to salinity or week management (Behzadi Rad *et al.*, 2021) . Currently,

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the pistachio area harvested in Iran is 125544 hectares, the average yield is about 1075 kg ha^{-1} and the amount of production is 135000 tons (FAO, 2021).

Today, the dieback disease of pistachio (DBP) is one of the most important diseases with an increasing annual prevalence. Annual yield losses can reach up to 90% in poorly managed orchards (Mozaffari *et al*., 2005).

Dieback is a symptom characterized by extensive necrosis of twigs beginning at their tips and advancing toward their base (Agrios, 2005). DBP is dictated by the fungal pathogen but also by the presence and interaction of biotic and abiotic stresses in orchards. Wounds caused by harvest, pruning, drought, sunburn, strong winds as well as soil salinity, low quality of water, nutrient imbalance in soil and damage of pests and diseases are factors influencing dieback disease (Sami *et al*., 2005). Different fungal species have been reported from infected trees showing symptoms of dieback, but fungal species belonging to the genera *Paecilomyces* and *Nattrassia* have been implicated as fungal causal agents of DBP. Initially, *Paecilomyces variotii* was introduced as the causal agent based on morphological characteristics (Samson 1974; Aminaei and Ershad 1989; Alizadeh *et al*. 2000; Ershad 2009; Ghelchi *et al*., 2012). Later, polyphasic taxonomy based on morphological, physiological and molecular phylogenetic characteristics revealed *Paecilomyces formosus* (Sakag, May, Inoue and Tada) Houbraken & Samson as the causal agent of DBP in Iran (Heidarian *et al*., 2015a; Heidarian *et al*., 2015b). Gelichi *et al*., (2012) also confirmed the pathogenicity of several isolates of *Paecilomyces* on pistachio *in vitro* and *in situ* and introduced them as *Paecilomyces variotii*. In California and Italy, *Eutypa lata* (Pers.) Tul. & C. Tul and *Botryosphaeria ribis* Grossenbacher & Duggar, respectively, have been reported as the causal agent of DBP (Ashworth *et al*. 1985; Corraza *et al*., 1990).

Bacterial dieback of pistachios with typical symptoms such as decline, dieback, vascular discoloration, and death of pistachio trees caused by *Xanthomonas* strains was first reported in Australia in 1992 (Edwards and Taylor, 1998). *Bacillus licheniformis* Weigmann was identified as one of the causal agents of bacterial pistachio dieback in Kerman province, Iran (Baradaran and Ghasemi, 2010).

During 1994-95 the disease intensity of DBP has been estimated between 0- 85% in Iranian pistachio growing areas (Ashkan *et al*., 1997). Infection often begins at the site of pruning or accidental wounds as small black spots on the surface of the bark of trunks, twigs, and branches. Infected parts are slightly sunken and settle into the healthy tissue. Cracks gradually develop downward the trunk and branches become withered and dry (Sami *et al*. 2005). So far, researchers have proposed various strategies to control the disease, including plant sanitary measures such as disinfection of pruning equipment's, collection and burning of infected pruned branches, strengthening the trees with appropriate cropping methods (regular irrigation, nutrition), use of resistant cultivars and pruning of branches 10-15 cm lower than infected areas (Sami *et al*. 2005). Additionally, application of pesticides is recommended. Studies showed that various fungicides can slow the rate of mycelial growth of different fungal species (Gelichi *et al*., 2012; Zarei *et al*., 2014). Also the effects of fertilizers containing calcium, zinc and potassium had significant effects on reducing the intensity of DBP evaluated positively (Mozaffari *et al*., 2005).

Due to the importance of DBP for Iranian pistachio growers, and the need for efficient control methods, the effect of 13 fungicides on *P. formosus* was examined through *in vitro* and *in vivo* studies.

Materials and Methods

Pathogen and fungicide treatments

The chemical grouping, modes of action, target site of mechanism and application concentrations of the 13 treatments are presented in Table 1.

A pure *Paecilomyces formosus* isolate (PRC-P1674) previously isolated from pistachio twig in

Kerman province, Iran, was obtained from the collection of the Pistachio Research Center. The isolates were identified based on the macro- micro-morphological and physiological features (Samson, 1974 and Samson *et al*., 2009).

Table 1. Fungicides and concentrations studied for inhibitory effect on *Paecilomyces formosus.*

No.	Treatment	Concentration (ppm)	Trade name	Company, Country	Movement in plant	FRAC* code	Mode of action (MOA)
1	Fluopicolide 4.45% + Fosetyl-Al 66.67% w/w	2000	Profiler ®	Bayer, Germany	Systemic and contact	B5 (43) + P 07 (33)	Cytoskeleton and motor protein+ host plant defense induction
2	Spiroxamine 25% + Tebuconazole16.7% + Triadimenol 4.3% EC	2000	Falcon ®	Bayer, Germany	Systemic	G2 (5) + G1 (3)	Sterol biosynthesis in membranes
3	Copper oxychoride 39.75% + Cymoxanil 4.2% WP	3000	Cuprosate®	Agria, India	Systemic and contact	M 01+ U(27)	Chemicals with multi-site activity+ Unknown mode of action
4	Fosetyl Aluminium 80% WDG	2500	Elite ®	Shandong Tiansheng Biotechnology, China	Systemic	B5 (43)	Host plant defense induction
5	Iprodione 35% + Carbendazim 17.5 WP	3000	Rovral TS ®		Contact	E3 (2)+B1 (1)	Signal transduction+ Cytoskeleton and motor protein
6	Benomyl 50% WP	1000	Benomyl®	China	Systemic	B1 (1)	Cytoskeleton and motor protein
7	Dimethomorph 9% + Mencozeb 60% WG	3000	Acrobat ® MZ	BASF, Germany	Systemic and contact	H5 (49) + M (03)	Cell wall biosynthesis+ Chemicals with multi-site activity
8	Azoxystrobin 20% + Difenocozole 12.5% SC	750	Ortiva Top®	Syngenta, Netherland	Systemic	C3 (11) + G1 (3)	Respiration+Sterol biosynthesis in membranes
9	Captan 50% WP	3000	Captan®	India	Contact	M 04	Chemicals with multi-site activity
10	Tebuconazole 38.7% F	500	Folicur®	Bayer, Germany	Systemic	G1 (3)	Sterol biosynthesis in membranes+
11	Cyflufenamid 1.5% + Difenoconazole 12.5% SL	3000	Cidely Top®	Syngenta, Netherland	Systemic	U 06 + G1 (3)	Unknown mode of action+ Sterol biosynthesis in membranes
12	Fluopyram 21.4% + Trifloxystrobin 21.4% SC	500	Luna® Sensation	Bayer, Germany	Systemic	C2 (7) + G1 (3)	Respiration+ Sterol biosynthesis in membranes
13	Copper Oxychloride 35% WP	3000	Oxychromes®	India	Contact	M 01	Chemicals with multi-site activity

*Classification of fungicides by the Fungicide Resistance Action Committee (Anonymous 2021)

In vitro assays

Fungal growth inhibition studies

Colony growth inhibition was assessed on potato dextrose agar (PDA) amended with different concentrations of fungicides (treatment). Each treatment was evaluated in three replications.

Briefly, PDA was prepared according to the manufacturer's instructions, sterilized in an autoclave at 121 °C and 1.2 atmospheres pressure for 15 minutes, and cooled to below 50°C on a magnetic stirrer. Recommended concentrations of fungicides were added aseptically and liquid PDA + fungicide were poured into petri dishes. Fungicide concentrations are listed in Table 1. For growth inhibition studies, *P. formosus* was cultivated and a 4 day-old mycelium block with a diameter of 4 mm was transferred to the center of each PDA+ fungicide plate. Petri dishes were incubated at 25 ± 1 °C for 7 days and the diameter of growing colonies was measured and recorded daily. Mycelial growth inhibition of isolates was calculated based on the following formula (Ghelichi *et al*., 2012).

$$\mathrm{GI} = \frac{\mathrm{Ta} - \mathrm{Tb}}{Ta} \times 100$$

GI: Inhibition of mycelial growth (%)
Ta: Colony diameter in control treatment
Tb: Colony diameter in fungicide treatment

Fungal sporulation inhibition studies

Inhibition of sporulation by *P. formosus* was assessed on potato dextrose agar (PDA) amended with different concentrations of fungicides, as described above. Each treatment was evaluated in three replications. *Paecilomyces formosus* was grown on PDA amended with different fungicides and their concentrations. After 7 days, three 4mm fungal agar blocks from each petri dish were transferred to a test tube containing 20 ml of sterile distilled water and shaken at 150 rpm for 10 minutes. Spore concentrations were enumerated using a Neubauer haemocytometer chamber (Mauni *et al*., 2007).

Spore germination studies

After 7 days of incubation on PDA + fungicides, a 4mm mycelial agar block of *P. formosus* was placed in sterile distilled water and shaken at 150 rpm for 10 minutes. After dilution to 10^3 spore/ml, 100 µl of the obtained suspension was spread on the surface of Petri dishes containing PDA + fungicides. After 24 hours, using a lens, the germinated spores were counted at 10× magnification and the percentage of germ spores was calculated. Each treatment was evaluated in three replications (Dhingra and Sinclair, 1995).

Immersion of branches in fungicide solution

Healthy annual branches (cultivar Fandoghi) with a length of 30 to 40 cm and a diameter of 1 to 2 cm from the orchards of Zarand were collected. The surface of the branches was sterilized using 70% ethanol. The candidate fungicides according to the *in vitro* experiments were prepared. The branches were immersed in fungicide suspension with various concentrations for 4 hours. Then, 3mm incisions were made with a sterile scalpel and a 4 mm mycelial block of *P. formosus* was placed under the bark and covered with a sterile cotton swab and parafilm. To obtain the required moisture, the cotton swab was moistened using a sterile syringe. Fungal-free culture medium was used as a negative (no-inoculation) control. The experiments were performed in five replications and the inoculated branches were incubated at 28°C ±1. The longitudinal development was measured using a ruler after 2 weeks. The pathogen isolated from the margins of healthy and infected (Dhingra and Sinclair 1995).

Vertical method (inserting the end of the branch into the fungicide mixture)

With the base facing downward, branches were

placed in different concentrations of fungicides for 4 hours. Then, branches were inoculated with *P. formosus* as described above and incubated at 28°C ±1 for 2 weeks (Dhingra and Sinclair 1995). The treatments were compared using Duncan's multiple range tests at the level of 0.01.

In vivo assays

Based on the results of the *in vitro* studies, the efficacy of Cuprosate® (treatment 3), Folicur® (treatment 10) and Falcon® (treatment 2) in disease control was also evaluated under *in vivo* conditions. The experiment was performed in a commercial pistachio orchard located in Zarand planted with 35 year-olds 'Fandoghi' trees. Experiments were conducted in a randomized block design incorporating six treatments with three single-tree replications per treatment. Experimental treatments included: intensive pruning with and without horticultural glue; light pruning with foliar spray with Oxychromes® (3 g L^{-1}), Folicure® (0.5 g L^{-1}) and Falcon® (2 ml L^{-1}). A buffer tree line was used between treatments to prevent spray drift. The spraying was done with a 20 liter backpack (rechargeable) sprayer in March and May 2019 and 2020. Different parameters including length of branch, number of buds, length and diameter of canker were measured before spraying up to 5 months after the last spraying and were recorded using a Calibrated ruler. In each tree, 10 branches from different directions and canopy heights were randomly selected for disease assessment.

Data analysis

Data was analyzed with the SPSS version 16 statistical analysis system and submitted for analysis of variance according to a randomized block design. Means were separated using Duncan's multiple range tests at the level of 0.01.

Results

In vitro studies

Fungicide efficacy is presented in Table 2. The results show significant differences among fungicides regarding inhibition of fungal sporulation, spore germination, mycelial growth and twig colonization on *P. formosus*.

Table 2. Results of analysis of variance (ANOVA) of fungicide efficiency for the dependent variables considered

Variable	Variation resources	Sum Squares	DF	Mean Square
Inhibition of Sporulation	Fungicide	36902.1	12	3075.1*
	Error	1838.1	26	70.6
	Total	38740.3	38	
Inhibition of Spore germination	Fungicide	14306.4	12	1192.2*
	Error	2996.8	26	115.2
	Total	17303.3	38	
Inhibition of Mycelial growth	Fungicide	3741.9	12	311.8*
	Error	243.2	26	9.3
	Total	3985.1	38	
Twig inoculations (immersion of branches)	Fungicide	7.3	5	1.4*
	Error	2.9	24	0.12
	Total	10.2	29	
Twig Inoculations (vertical method)	Fungicide	8.4	5	1.6*
	Error	2.3	24	0.1
	Total	10.8	29	

* Values are significant at p≤ 0.01; DF: degrees of freedom;

Colony growth inhibition

Results show that all fungicides significantly reduced mycelial growth of *P. formosus* from 58 to 91% (Table 3). Among the fungicides, Falcon® (treatment 2) inhibited mycelial growth the most followed by Folicur®, Luna®, Acrobat MZ® and Benomyl®, respectively. The lowest inhibitory effects of mycelial growth were observed in Profiler® (treatment 1). The differences between treatment 1 and the other treatments were statistically significant.

Fungal sporulation inhibition studies

Folicur® and Cidely Top® completely (100%) inhibited spore germination of *P. formosus*. While Falcon® had the highest effects on mycelial growth, low effects were observed (54%) on the spore germination. Luna® and Folicur® showed the highest effects on both assays.

Table 3. Effect of fungicides inhibition on mycelial growth and spore germination *in vitro* on *Paecilomyces formosus* based on Duncan multiple tests.

Fungicide	Mycelium growth inhibition %		Spore germination inhibition %	
Profiler	58.2	D	66.7	BCD
Rural TS	68.3	C	47.2	DE
Elite	69.5	C	54.5	DE
Oxychromes	72.4	C	83.5	AB
Cidely Top	82.1	B	100	A
Cuprosate C	81.1	B	60.8	CDE
Ortiva Top	81.3	B	59.0	CDE
Acrobat MZ	87.8	A	75.4	BC
Benomyl	86.0	AB	43.6	E
Luna	90.7	A	98.7	A
Folicur	90.7	A	100	A
Falcon	91.3	A	54.0	DE
Captan	81.6	B	66.4	BCD

Spore germination studies

All fungicides at the stated concentration reduced spore production by *P. formosus* to different degrees ranging from 17.5 to 100 %. Benomyl® and Falcon® were the only fungicides that completely inhibited spore production of *P. formosus* in culture media. Inhibition by other fungicides ranged from 4.0×10^5 to 3.3×10^6, compared to 1.3×10^7 in the control (Fig. 1).

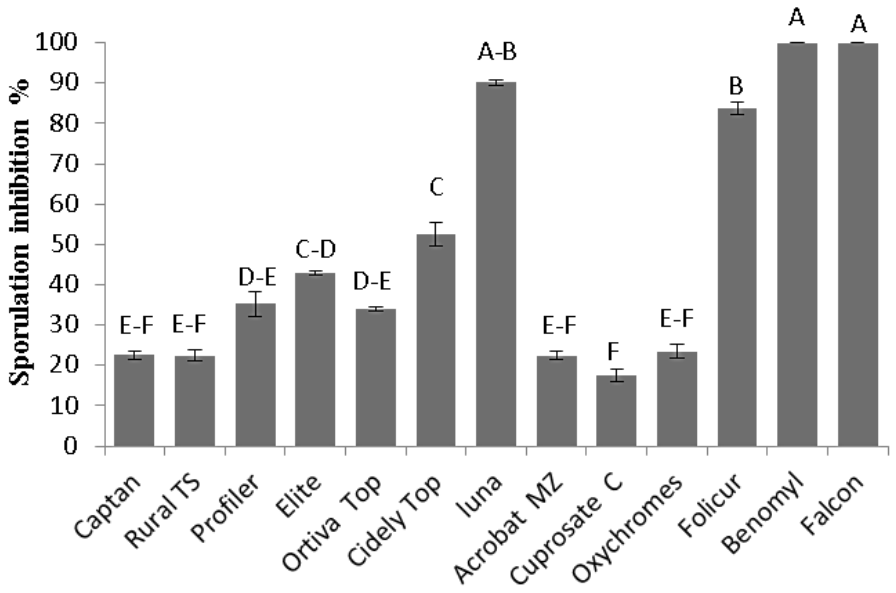

Fig. 1. Reduction in sporulation of *Paecilomyces formosus*, the causal agent of pistachio dieback, as a result of fungicide treatment. Means (n = 3) followed by the same letter are not significantly different based on Duncan's multiple range tests (P = 0.05).

Twig colonization

Based on performance in PDA assays, five fungicides were evaluated for their ability to inhibit twig colonization by *P. formosus* through immersion and vertical method. The tested fungicides reduced *P. formosus* colonization ranging from 39.1 to 54.2% (immersion method) and 49.3 to 57.7% (vertical method) (Fig. 2). There was no significant difference between the two methods in terms of colonization. The highest inhibition rates were observed with Oxychromes® and Benomyl® for immersion and vertical methods, respectively.

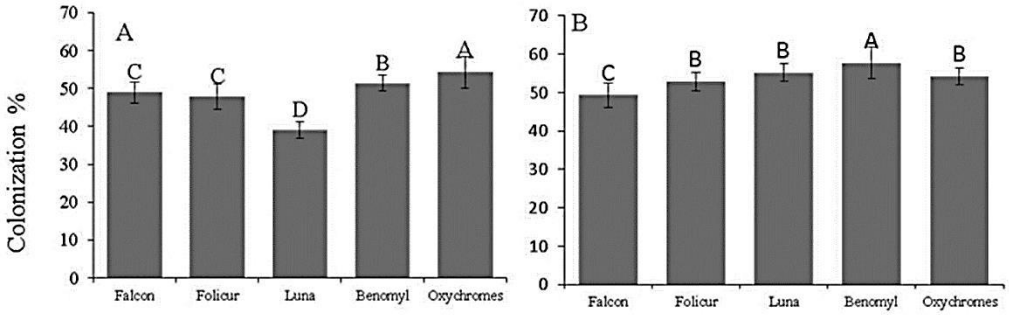

Fig. 2. The efficacy of fungicides on the *P. formosus* twig colonization using immersion (A) and vertical (B). Means (n = 3) followed by the same letter are not significantly different based on Duncan's multiple range tests (P = 0.05).

In vivo studies

The analysis and results of fungicide application in the orchards are shown in Tables 4 and 5. There were no significant differences between pruning and fungicide application or their combinations in terms of shoot growth and canker length. With 6.4cm, the highest amounts of canker progression were found in control trees (Table 5). The highest disease development in treated trees was seen in the intensive pruning without horticultural glue treatment (5.8cm). The lowest development was found in the intensive pruning plus horticultural glue treatment (3.7cm) (Table 5). All fungicide-treated trees were significantly healthier than the control. No significant differences to the control were observed on trees that

received fungicides and pruning in terms of shoot growth and canker length.

Table 4. Analysis of variance of the effect of pruning and fungicides on die-back of pistachio trees

Parameter	Variation resources	Sum Squares	DF	Mean Square
Shot growth Before experiments	Treatment	19.369	5	3.874^{ns}
	Replication	81.113	9	9.013
	Error	215.367	45	4.786
	Corrected Total	315.849	59	
Shot growth after experiments	Treatment	110.951	5	22.190^{ns}
	Replication	199.572	9	22.175
	Error	639.208	45	14.205
	Corrected Total	949.730	59	
Canker length before experiments	Treatment	124.375	5	24.875^{ns}
	Replication	268.567	9	29.841
	Error	1060.095	45	23.558
	Corrected Total	1453.037	59	
Canker length after experiments	Treatment	71.300	5	14.260^{ns}
	Replication	71.307	9	7.923
	Error	195.003	45	4.333
	Corrected Total	337.610	59	
Canker development	Treatment	71.300	14.260	220.969*
	Replication	158.507	17.612	2.429
	Error	264.203	5.871	3.000
	Corrected Total	494.010		

Table 5. Twig colonization and shoot growth before and after pruning and fungicides application.

Treatment	Shoot growth		Canker length		Canker Development*
	Before	After	Before	After	
Intensive Pruning	9.2	13.0	7.9	13.8	5.8 AB
Intensive Pruning + horticultural glue	9.0	13.4	11.9	15.6	3.7 B
Light Pruning + Oxychromes®	9.8	11.0	7.7	12.0	4.3 AB
Light Pruning + Folicure®	8.5	9.8	8.0	11.8	3.8 B
Light Pruning + Falcon®	8.2	10.8	8.2	12.0	3.8 B
Control	8.3	10.4	8.3	14.8	6.4 A

* Data recorded using a calibrated ruler (CM). Means followed by the same letter are not significantly different at $P < 0.05$, by Duncan's Multiple Range Test.

Discussion

Control of DBP as one of the most important diseases is a critical issue in improving yield production. Here, the efficacy of 13 fungicides on *P. formosus* was assessed under *in vitro* or *in vivo* conditions. All tested fungicides were able to inhibit the development of *P. formosus* by inhibiting mycelial growth, spore germination, and/or spore production *in vitro*. In twig experiments, Oxychlromes® and Benomyl® successfully inhibited fungal colonization in immersion and vertical fungicides application, respectively.

Fungicides whose mode of action was inhibitory of sterol biosynthesis in membranes were more effective in control of mycelium growth, sporulation, spore germination as well as canker progression either on agar medium or in pistachio twig assays. Overall, Luna®, Falcon®, Sidley Top® and Folicur® have been identified as the most effective fungicides in most

conducted assays. The range of inhibition of mycelium growth and sporulation of pathogenic fungi in these treatments was 82.1% to 91.3% and 54.7% to 100%, respectively. Among them, the fungicides Luna, Falcon and Follicor reduced the colonization of *P. formosus* on the branch from 39.1 to 49.3% (immersion method) and 49.3 to 53.2% (vertical method). Benomyl® as a classic MBC fungicide (Methyl Benzimidazole Carbamates) inhibiting cytoskeleton and motor protein mechanisms was also effective in inhibiting mycelial growth and progression of canker on branches in the immersion method, but due to environmental hazards and application restrictions in different areas is not recommended for use in orchards.

Based on the *in vivo* experiments, no comparative advantages were observed in fungicide application in terms of shoot growth and DBP control compared to those control trees with no-spraying fungicides. In contrast, pruning showed comparative advantages in the management of DBP in infected orchards. The use of horticulture glue in infected trees after pruning should be considered to improve shoot growth after pruning. This indicates a change in our attitude towards this disease to understand the critical factors affecting the disease and improve control strategy. There are several scenarios that have been proposed for occurrences of DBP, as a complex disease or disorder. The DBP can be caused by pathogenic organisms, although non-pathogenic factors such as frost and mechanical damages, toxicity or deficiency of elements, unfavorable weather, high winds, high yield, water stress and damage of root systems caused by cultural practices, or root-feeding nematodes, probably play a major role in the incidence of diseases in orchards and its severity on infected trees. Management of abiotic stresses can greatly help with disease management. To manage DBP, proper pruning, pest and disease management, early harvesting of pistachio, irrigation, application of macro-micro-nutrients and improving soil structure and texture are critical.

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Conflict of interest

The authors declare no conflict of interest.

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