

Research Article

Evaluation of systemic fungicides for the control of pistachio gummosis disease, caused by *Phytophthora* spp.

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Abstact

Pistachio, which is of great economic importance in Iran, is highly susceptible to crown and root rot disease, also known as gummosis. To evaluate the effectiveness of systemic fungicides in controlling this destructive disease, different concentrations of fosetyl-Al (Elite[®]), potassium phosphonate, Metalaxyl (Ridomil[®] Gold), and phosphorous acid were tested against the major causal agents of gummosis, including Phytophthora citrophthora, P. dreschleri, P. pistaciae, and P. cryptogea under in vitro and in situ conditions. The findings revealed a significant increase in inhibition of mycelial growth in concomitant with an increase in concentration of fungicides compared with control. At concentrations of 1120, 320, 80, and 40 µg/ml, Elite, potassium phosphonate, phosphorous acid, and Ridomil, respectively, completely inhibited mycelial growth. All tested fungicides exhibited a reduction in colonization levels on branches, with potassium phosphonate at 320 µg/ml and Ridomil at 20 µg/ml concentrations showing 73% and 100% reduction, respectively. The effectiveness of fungicides in decreasing fungal colonization in the inoculation experiments on pistachio seedlings varied. Specifically, Elite, phosphoric acid, potassium phosphonate, and ridomil exhibited the highest levels of reduction, respectively. However, no significant differences were observed among the fungicide compounds with phosphite structure in inhibiting crown colonization in pistachio seedlings. These findings provide valuable insights for further investigation into the management of crown and root rot in pistachio trees.

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Introduction

Crown and root rot, an important disease affecting pistachio trees, which leads to the death of numerous fertile and infertile trees annually. This disease is caused by various *Phytophthora* species, including P. citrophthora (Leonian), P. cryptogea (Pethybr. & Laff.), P. drechsleri (Tucker), P. melonis (Katsura), P. nicotianae (Breda de Haan), P. parsiana (Mostowf., Cooke & Banihash.), and P. pistaciae (Mirab.) (Mirabolfathy et al. 2001; Fani et al. 2005, 2019; Mostowfizadeh-Ghalamfarsa et al. 2008). Unfavorable conditions such as excessive irrigation, deep planting, monoculture, and inadequate management practices adopted by growers over the years have facilitated the development of Phytophthora species in orchards, resulting in substantial damage and mortality rates ranging from 2% to 11% (Mirabolfathy et al. 1990; Moradi & Masoumi 2011).

While the use of pesticides in agriculture has decreased due to their negative impact on the environment and non-target microorganisms, chemical control remains an effective approach for managing plant diseases worldwide. Phosphonates and their derivatives are commonly used fungicides for preventing diseases caused by Oomycetes in plants. The use of phosphonates in agriculture dates back to the 1930s when phosphonic acid salts were utilized as substitutes for phosphate fertilizers in Germany (Erwin & Ribeiro 1996). Fosetyl-Al, a phosphonate fungicide, converts into phosphorous acid and phosphonate salts after application, allowing for movement within the plant's symplastic and apoplastic systems (Cohen & Coffey 1986; McGrath 2004). Phosphonates have shown both direct effects and the activation of defense mechanisms against Oomycete pathogens in lentils, tobacco, and papaya (Smillie et al. 1989). Studies have demonstrated that the concentration of phytoalexins, lignins, ethylene, and phenylalanine ammonia lyase activity increases more rapidly in fosetyl-Al-treated stems of susceptible tobacco cultivars compared to untreated stems (Nemestothy & Guest 1990). Trunk injection of potassium phosphonate into avocado trees resulted in acropetal translocation of phosphonic acid, detectable in the

J Appl Res Plant Prot -

leaves after 24 hours, and increased its concentration in the trunk and roots, indicating its movement through the phloem. Timing and dosage of injection, aligned with tree phenology, can enhance the effectiveness of fungicides by targeting specific organs in need of protection (Whiley et al. 1995). In a study conducted by Guest et al. (1994), different application methods of potassium phosphonate, including trunk injection, trunk paintings, and foliar spraying, were used to control stem canker or collar rot in avocado trees. The results indicated that the application of 15 grams of active ingredient potassium phosphonate per tree had the greatest efficacy in controlling the disease. The effectiveness of the treatment was influenced by factors such as tree size, disease density, and the timing of injection. Similarly, McMahon et al. (2010) reported that trunk injection of potassium phosphonate, at approximately 16 grams of active ingredient per tree, provided effective control of stem canker and *Phytophthora* pod rot caused by *P*. palmivora (E.J. Butler) E.J. Butler 1919. The longterm use of potassium phosphonate as a trunk injection proved to be a valuable option for disease management in avocado trees (Guest et al. 1994; Whiley et al. 1995). Metalaxyl is a phenyl-amide fungicide with chemical name, methyl N-(methoxyacetyl)-N-(2, 6-xylyl)-DL-alaninate which inhibits protein synthesis in fungal cells (Erwin & Ribeiro 1996). It can be absorbed by roots, stems, and leaves through various application methods to control Oomycete pathogens within plant tissues. Limited information is available regarding the effects of fungicides on Phytophthora species causing crown and root rot in pistachio trees. Therefore, this study aimed to investigate the impact of phosphite-based fungicides (potassium phosphonate, fosetyl-Al, phosphorous acid and Ridomil) on the growth of P. citrophthora, P. drechsleri, P. pistaciae, and P. cryptogea on culture media, as well as their effects on 2-year-old pistachio tree branches and 2-month-old pistachio seedlings (Sarakhs cultivar).



Materials and Methods

Fungal isolates

In this research, four isolates of *Phytophthora* spp. (*P. citrophthora*, *P. dreschleri*, *P. pistaciae* and *P. cryptogea*) deposited at Department of Technology and Production Management in Pistachio Research Center were used. The isolates were maintained in small tubes containing distilled sterile water and tubes containing corn meal agar (CMA, Merck, Germany) at 4°C (Boesewinkel 1976).

Fungicides

Four fungicides fosetyl-Al, including phosphorous acid 30%, Ridomil Gold and potassium phosphonate were used (Table 1). The considered doses were added in sterile water and poured into medium at 45-55 °C. To prepare potassium phosphonate, at first, 500g phosphorous acid (with 99% purity) and 500g potassium hydroxide (with 90% purity of Bayer Company, Germany) dissolved in 500 ml sterile water, separately. Then, phosphorous acid solution was slowly added to potassium hydroxide solution. According to heat production of reactions the essential cautious associated with chemicals were done.

Biometric in laboratory conditions

Mycelial growth: To investigate inhibitory effects of fungicides on mycelial growth of Phytophthora species, various doses of Elite, potassium phosphonate, phosphorous acid and Ridomil Gold in 100 ml CMA was prepared and dispensed into five petri dishes (Table1). Phytophthora citrophthora, P. dreschleri, P. megasperma and P. cryptogea species (each species one strain isolated from pistachio grown areas) were cultivated on CMA. After seven days, discs (five mm in diameter) were cut from the edge of young colonies and transfered to the center of petri dishes containing CMA amended with different doses of fungicides and kept in a dark at 25 °C for a week. Radial growth of different species was recorded using a ruler accurate to one mm. The efficacy of fungicides was calculated compared to control (without fungicide) as follow:

$$I = (C - R/C) \times 100$$

I: Inhibitory percentage (%), R: radial pathogen growth in reaction condition, C: radial pathogen growth in control plate

Table 1. Characteristics of fungicides and used doses in laboratory conditions.

Common name of	Commercial name	Doses purred substance (µg/ml)	Produced by Company
fungicides			
Fosetyl-Al	Elite [®] 80% (WDG)	0, 14, 28, 140, 280, 560, 1120, 2800, 3000	Shandong Dacheng, China
Phosphorous acid (30%)	-	0, 5, 10, 20, 40, 80, 160	Merk, Germany
Metalaxyl	Ridomil Gold® SL 48%	0, 0.01, 0.05, 0.1, 1, 5, 10, 20, 40	Syngenta, Netherland
Potassium phosphonate	-	0, 5, 10, 20, 40, 80, 160, 320	Prepared in Pistachio Research Center

Reducing the level of Phytophthora species colonization on branches: First, two-year old branches of the Sarakhs variety with similar diameters were collected and transported to the laboratory. The branches were then sterilized using 70% ethanol and immersed in different concentrations of Elite, Potassium phosphonate, Phosphorous acid, and Ridomil Gold, which were 1120, 320, 80, and 40 µg/ml, respectively. This immersion process lasted for 3 hours, after which the branches were rinsed with sterile distilled water.

Next, small incisions measuring 0.5-1 mm in depth and 5-10 mm in length were made on the bark of the branches using a sterile scalpel. An agar block containing a three-day-old culture of Phytophthora was used for inoculation. During this period, the longitudinal growth rate of the different Phytophthora species on the branches was measured using a ruler with millimeter accuracy. The inhibition rate of each species, compared to the control without any fungicides, was calculated to determine the effectiveness of the tested fungicides



26

in preventing the growth of the *Phytophthora* species on the branches as follow.

$$I = \left(\frac{L1 - L2}{L1}\right) \times 100$$

I: Inhibition (%), L_1 : length growth with only pathogen inoculation, L_2 : length growth with fungicide,

Biometric in greenhouse condition: Two-month-old pistachio seedlings of the Sarakhs variety were treated to different concentrations of Elite, potassium phosphonate, phosphorous acid, and Ridomil Gold as soil drench. The selected concentrations were 1120, 320, 80, and 40 µg/ml, respectively, with 200 ml of the Elite, potassium phosphonate, phosphorous acid, and Ridomil Gold solution applied to as a soil drench in four replications to each pot. To prepare the seedlings for treatment, the crown area was disinfected with ethyl alcohol 70% and then washed with sterile distilled water. For the inoculation of Phytophthora species, incisions measuring 1 mm in depth and 5-10 mm in length were made in the bark of the seedlings using a scalpel. Actively growing mycelium plugs were placed over the incisions. To maintain a suitable environment for growth, the inoculated area was covered with wet sterile cotton and sealed with laboratory parafilm (Parafilm, USA). In control treatments, seedlings were immersed in sterile distilled water without the presence of pathogens (negative control) or inoculated with Phytophthora species (positive control). The seedlings were then placed under greenhouse conditions for two weeks. The progress of the pathogen was measured using a millimeter scale ruler. Crown colonization inhibition was calculated as follow.

$$I = \left(\frac{L1 - L2}{L1}\right) \times 100$$

C: crown colonization inhibition (%), L₁: length growth with only pathogen inoculation, L₂: length growth with fungicide

Statistical analysis

Collected data were analyzed in completely randomized design and Duncan's multiplerange test was used for compare means at the 5% level. Data was analyzed with the SPSS version 16 statistical analysis system

Results

Mycelial growth inhibition

The results indicated that the growth of Phytophthora spp. was inhibited by various concentrations of fungicides, Elite with varying effectiveness (Table 2). As the concentrations of the fungicides increased, the inhibition of mycelial growth also increased. Complete inhibition (100%) of mycelial growth was observed at concentrations of 1120, 320, 80, and 40 µg/ml for Elite, potassium phosphonate, phosphorous acid, and Ridomil Gold fungicides, respectively. The highest levels of inhibition were 97%, 84%, 90%, and 96% for Elite, potassium phosphonate, phosphorous acid, and Ridomil Gold, respectively. Moreover, no inhibition of mycelial growth was observed at concentrations below 140, 40, 5, and 0.01µg/ml for Elite, potassium phosphonate, phosphorous acid, and Ridomil Gold fungicides, respectively. The sensitivity of Phytophthora species to the fungicides used followed the order of P. citrophthora, P. drechsleri, P. megasperma, and P. cryptogea, with decreasing sensitivity. Colonization of pathogen on branches

Figure 1 present the results of study on twig colonization of two-year-old seedlings the Sarakhs cultivar treated with different concentrations of fungicides. The lowest inhibition rate belonged to potassium phosphanate at 320 μ g/ml with a range of 73 to 87% in the studied species. The highest level of inhibition was also observed in the concentration of 20 micrograms per liter of Ridomil with 100% inhibition.

The effect of the two fungicides, Elite and phosphorous acid, on the inhibition of colonization in skin tissue differed significantly among the studied species. However, there was no significant difference between Elite and phosphorous acid in terms of their inhibition of colonization on branches, despite the different doses of the two fungicides. Comparison of colonization on pistachio branches among the various species in the presence of the four fungicides did not reveal any significant differences. However, Ridomil Gold showed the highest efficiency, resulting in 100% inhibition.

Table 2. Comparison of inhibition of mycelial growth of *Phytophthora* spp. caused by different concentration of fungicides.

Fungicide dose (µg/ml)	Mycelial growth inhibition (%)				
	P. citrophthora	P. drechsleri	P. megasperma	P. cryptogea	
Elite	-				
140	61.1 ^{cA}	65.2 ^{cA}	46.7 ^{cA}	43.2 ^{cA}	
280	79.4 ^{bA}	76.1 ^{bA}	64.6 ^{bB}	60.2 ^{bB}	
560	97.3 ^{aA}	95.2 ^{aA}	83.8 ^{aB}	76.7 ^{aB}	
Potassium phosphonate					
40	22.4 ^{cA}	18.4 ^{cAB}	15.7 ^{cAB}	10.1 ^{cB}	
80	46.5 ^{bA}	41.5 ^{bB}	38.2 ^{bC}	30.9 ^{bD}	
160	84.3 ^{aA}	80.0 ^{aA}	77.3 ^{aA}	72.7 ^{aA}	
Phosphorous acid					
5	24.5 ^{cA}	23.2 ^{cA}	21.1 ^{cA}	17.7 ^{cA}	
10	39.3 ^{cA}	37.5 ^{cA}	32.6 ^{cA}	30.0 ^{cA}	
20	63.8 ^{bA}	60.7 ^{bA}	53.0 ^{bA}	49.5 ^{bA}	
40	90.4 ^{aA}	85.7 ^{aB}	78.5 ^{aC}	76.3 ^{aD}	
Ridomil Gold					
0.01	6.7 ^{dA}	5.7 ^{eA}	7.5 ^{eA}	7.1 ^{eA}	
0.05	23.2 ^{cA}	17.5 ^{dA}	17.9 ^{eA}	14.3 eA	
0.1	53.6 ^{bA}	31.4 ^{cB}	28.6 ^{cB}	24.6 ^{cB}	
1	63.6 ^{bA}	46.6 ^{bAB}	39.3 ^{св}	35.7 ^{cB}	
5	87.5 ^{aA}	85.0 ^{aA}	72.9 ^{bB}	64.3 ^{bB}	
10	93.2 ^{aA}	93.6 ^{aA}	84.3 ^{aB}	82.1 ^{aB}	
20	96.4 ^{aA}	96.1 ^{aA}	94.3 ^{aAB}	91.8 ^{aB}	

Mean followed with the same letters in each row (fungicide concentrations, capital letters) and column (*Phytophthora* species and fungicides, small letters) are not significantly different according to Duncan's multiple-range test at 5% level.

Colonization level of pathogen on pistachio seedling crown

Similar to the findings regarding the impact of fungicides on the inhibition of fungal colonization on pistachio branches, all the fungicides used exhibited varying levels of reduction in colonization on the crown of pistachio seedlings (as shown in Figure 2). Ridomil Gold demonstrated the lowest inhibitory effect on fungal colonization in the crown, with a range of variation between 83-92%. The inhibitory effects of the fungicides on pathogen colonization in the crown of pistachio seedlings by Phytophthora species decreased in the following order: Elite, phosphorous acid, potassium phosphonate, and Ridomil Gold. Moreover, there were no significant differences among the fungicides with a phosphite base in terms of their inhibition on the crown of pistachio seedlings against the four Phytophthora spp. Similarly, no significant difference was observed in the inhibition of colonization on the crown of pistachio seedlings by various fungicides when exposed to P. cryptogea. When comparing the various fungicides in terms of their inhibition on crown colonization of pistachio seedlings, no significant difference was observed among the three species, P. citrophthora, P. drechsleri, and P. megasperma, as well as P. cryptogea compared to P. megasperma. The maximum inhibitory effect of the fungicides on crown colonization was associated with P. cryptogea, with a variation range of 91% to 95%, while the minimum inhibitory effect was associated with P. citrophthora, with a variation range of 83% to 91%.





Figure 1. The effect of different fungicides on branch colonization caused by *Phytophthora* spp. on two years Sarakhs cultivar. Means followed by the same letter are not significantly different at p < 0.05, by Duncan's Multiple Range, Error bars indicates standard deviation



Figure 2. Inhibitory effect of different fungicides on crown colonization caused by *Phytophthora* spp. in Sarakhs cultivar. Means followed by the same letter are not significantly different at p < 0.05, by Duncan's Multiple Range Test. Error bars indicates standard deviation.

Discussion

Crown and root rot are highly detrimental diseases in pistachio orchards which are normally caused by various *Phytophthora* spp., and can result in significant economic losses. To effectively manage diseases and prevent the growth of pathogenic agents in plant tissues, it is necessary to understand the pathogenicity mechanisms, optimal concentrations of fungicides, timing of application, and specific plant species involved.

The current study demonstrated that all the fungicides used in this study had a noteworthy inhibitory effect on the mycelial growth of *Phytophthora* spp. (with a significance level of $p \leq p$ 0.05), and their effectiveness increased as the dosage was increased. Among the fungicides, Ridomil Gold exhibited the lowest required dose for complete inhibition of mycelial growth, at 40µg/ml. Furthermore, there were no significant differences observed between the concentrations of 10 µg/ml and 20 µg/ml of Ridomil Gold in terms of their inhibition of mycelial growth, indicating a high level of toxicity of Ridomil Gold against Phytophthora species. Previous studies have reported that the required dose for 50% inhibition of mycelial growth with Ridomil ranges from 1-3.5 µg/ml (Rekanović et al. 2012; Benson & Grand 2000; Mbaka et al. 2009).

Phosphorous acid exhibited the highest level of inhibition of mycelial growth compared to fosetyl-Al and potassium phosphonate, at a concentration of 80µg/ml. Previous studies have shown that phosphites, such as phosphorous acid, can effectively reduce Oomycete pathogens through both direct and indirect modes of action (Lobato et al. 2010). The concentrations of phosphate ion and phosphite in the culture media play a significant role in inhibiting mycelial growth of Phytophthora species (Griffith et al. 1989). The interaction between phosphite and phosphate is likely to greatly influence efficacy in in vitro assays. This could be due to phosphate that competitively inhibits the uptake and transport of phosphites by Phytophthora spp., and this interaction may vary among different species (Barchietto et al. 1989; Griffith et al. 1989; Varadarajan et al. 2002).

In contrast, increasing the concentration of phosphate ion in the culture media had little to no effect on the antifungal activity of phosphite (H₃PO₃), while it reduced the inhibition of mycelial growth in the presence of fosetyl-Na. Among *Phytophthora* species, *P. infestans* is the only species known to be insensitive to phosphorous acid (Fenn and Coffey 1984), and fosetyl-Al has shown limited effectiveness in managing potato late blight (Schwinn 1983). However, our results indicate the susceptibility of *Phytophthora* spp., the causative agents of crown and root rot in pistachio, to phosphite-based compounds.

Similarly, to the results obtained in in vitro experiments, all the fungicides demonstrated a reduction in fungal colonization in the tissue of branches and the crown of pistachio seedlings. Among them, Ridomil Gold exhibited the highest significant reduction and prevention of tissue colonization on branches. This suggests that Ridomil Gold is able to penetrate the tissue and inhibit the development of Phytophthora spp. According to Staub et al. (1978), the acropetal transport of Ridomil Gold after spray applications primarily occurs through uptake into green stem parts, followed by apoplastic transport. Symplastic transport from treated leaves contributes to a lesser extent. However, when Ridomil Gold was applied as a soil drench in experimental pots, its efficacy was reduced by 8-17% compared to immersing branches in the fungicide solution. This observation may indicate the apoplastic transport of Ridomil Gold within the plant. Fungicides with apoplastic transport have the ability to move through phloem tissue at high concentrations. Furthermore, crown and stem uptake, as well as acropetal transport in seedlings, may occur, leading to the inhibition of fungal colonization.

In contrast, other fungicides based on phosphite applied as soil drenches were more effective in reducing fungal colonization compared to immersing branches in the fungicides. This suggests that phosphite-based fungicides may have both direct and indirect modes of action in pistachio seedlings. When using the immersion method, the fungicides directly prevent the development of



Phytophthora species on the branches.

Brown *et al.* (2011) proposed that a late-summer foliar application of potassium phosphonate on the aboveground parts of Persian walnut trees, at a rate of 7 liters/ha, can potentially decrease the mortality rate and damage caused by *P. citricola*.

Türkölmez & Derviş (2017) conducted greenhouse experiments and found that foliar sprays of fosetyl-Al and phosphorous acid, as well as soil drenches of metalaxyl-M, significantly reduced the severity of crown and root rot in apricot and cherry trees caused by *P. palmivora*. They also noted that symptoms development was suppressed after two or three foliar applications of phosphorous acid at a concentration of 2 g a.i./l, as observed in field experiments.

Numerous studies have reported that the application of phosphite-based fungicides on various crops can exhibit a complex mode of action against *Phytophthora* species (Smillie *et al.* 1989; Grant *et al.* 1990). For instance, potassium

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phosphonate, fosetyl-Al, and phosphorous acid have been shown to have a direct mode of action against *Phytophthora* species (Fenn & Coffy 1985; Grant *et al.* 1990). These fungicides can induce changes in fungal metabolism at low concentrations without affecting fungal growth (Grant *et al.* 1990). Additionally, they can indirectly stimulate natural defense mechanisms within the plant (McGrath 2004; Smillie *et al.* 1989). Studies have indicated that fosetyl-Al can be converted to potassium phosphonate by certain plant species (Cohen & Coffey 1986; McGrath 2004).

In general, concentrations of 1120 μ g/ml, 320 μ g/ml, 80 μ g/ml, and 40 μ g/ml of Elite, Potassium phosphonate, phosphorous acid, and Ridomil Gold, respectively, demonstrated significant inhibitory effects on the growth of *Phytophthora* species under laboratory and greenhouse conditions. These findings provide valuable insights for further research and investigation into the management of crown and root rot in pistachio trees.

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چکیدہ

پسته که اهمیت اقتصادی زیادی در ایران دارد، به شدت حساس به بیماری پوسیدگی طوقه و ریشه (معروف به گموز یا انگومک) است. برای ارزیابی اثربخشی قارچکشهای سیستمیک در کنترل این بیماری مخرب، غلظتهای مختلفی از فوزتیل آلومینیوم ([®]Ellt)، فسفونات پتاسیم، متالاکسیل (Ridomil[®] Gold) و اسید فسفریک در برابر بیمارگرهای اصلی عامل گموز، از جمله Ridomino citrophthora citrophthora (و rcyptogea و Pistaciae در شرایط درون شیشهای (in vitro) و در محل (in situ) آزمایش شدند. یافتهها نشان دادند که با افزایش غلظت قارچکشها در محیط کشت، میزان رشد بیمارگر به میزان قابل توجهی کاهش می اید. در غلظتهای ۱۲۰، ۲۰، ۸۰ و ۴۰ میکروگرم در میلی لیتر، به تریب، الیت، فسفونات پتاسیم، اسید فسفریک و ریدومیل، رشد بیمارگر را به طور کامل مهار کردند. تمامی قارچکشهای مورد استفاده، باعث کاهش میزان کلونیزاسیون سرشاخهها شدند که از ۷۳ و ۲۰۰ درصد به ترتیب برای فسفونات با غلظت ۳۰ میکروگرم در میلی لیتر، غلظت ۲۰ میکروگرم در میلی لیتر متغیر بود. در مایهزنی طوقه نهالهای پسته، توانایی قارچکشها در کاهش کلونیزاسیون قارچ در میلی لیتر، ایت، اسید فسفورس، فسفانات و ریدومیل کاهش یافت. این نکته قابل ذکر است که بین قارچکشها با بنیان فسفیتی تفاوتی از نظر آماری در بازدارندگی از در درختان پسته ارائه می دود.

كلمات كليدى: اسيد فسفونيك، فوزتيل آلومينيوم، فيتوفتورا، متالاكسيل، مهار شيميايي