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Efficacy of Calcium Salts on Controlling *Phytophthora pistaciae*, the Cause of Pistachio (*Pistacia vera* L.) Gummosis

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ABSTRACT

Research in plant disease management focuses on developing safe methods for humans and the environment in order to prevent the entry of harmful chemicals in food. Simple inorganic salts have low cost and are safe enough to be used as pesticides. *Phytophthora* genus causes crown and root rot (gummosis) in crops and leads to great losses in some pistachio (*Pistacia vera* L.) production areas annually. In this study, the potential efficacy of different calcium salts with various concentrations on *Phytophthora pistaciae* control was examined. This was done to find out a safe way of material usage instead of synthetic pesticides. Samples from the crown of a pistachio tree, with gummosis, were cultured on lima bean agar to isolate and purify the pathogen. *P. pistaciae* identification was done using the PCR method with ITS4 and ITS6 primers. Pathogenicity of isolate was confirmed through tests on apples, pistachio branches, and pistachio seedlings. Different concentrations of seven calcium salts (chloride, nitrate, sulfate, oxide, hydroxide, phosphate, and carbonate) were applied which reduced mycelial growth and 3000ppm of calcium oxide inhibited it completely. Most of the salts reduced the number of sporangia, zoospores, and cyst germination percentage. Calcium oxide and calcium hydroxide also caused hyphae branching and deformation of some sporangia. The results suggested that some calcium salts, especially calcium oxide, could control the growth of *P. pistaciae in vitro*. Future *in vivo* studies are needed to examine whether calcium salts can reduce the disease severity and spread of the pathogen in the environment if those are sprinkled over the soil around the infested crown.

Introduction

Nowadays, the research in plant disease management emphasizes on the creation of safe alternatives for disease control (Olle *et al*. 2014). Safety for humans, animals, and the environment is of great importance and entering of harmful chemicals in food must be stopped (Chandler *et al*. 2008). Safe pesticides are classified into three main groups: 1) natural minerals, 2) micro-organisms (microbial pesticides), and 3) organic matter, produced by plants. Simple mineral salts, which are in the first group, are formed of a negatively charged anion (single or multi atomic) and a positively charged cation. These varieties of salt have some properties which make them suitable for use in disease management programs, including their low cost and being adequately safe for humans, animals, and the environment (Deliopoulos *et al*. 2010). Calcium is an essential element for living beings, including plants. Calcium ions maintain the structural and functional integrity of cell membranes and cell walls, increase host resistance to certain pathogenic microorganisms,

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and increase the tolerance to abiotic stresses (Campanella *et al*. 2002).

Phytophthora species are fungus-like plant pathogens which cause huge economic losses in crops around the world and damage to natural ecosystems (Moradi *et al*. 2017). In the genus *Phytophthora*, both sexual and asexual reproductions are reported. In asexual reproduction, the sporangia produce mobile zoospores, which lose their mobility after release and get converted to cysts. These cysts can germinate and be the source of new hyphae and mycelium (Khodaparast 2013). Pistachio (*Pistacia vera* L.) is an important horticultural crop of Iran and some other countries (Aghasi Kermani *et al*. 2017). Root and crown rot (gummosis) is among the damaging factors to pistachio that causes great losses in some pistachio–producing regions (Davoodi *et al*. 2013). If the disease is not controlled properly, it can reduce the number of trees by up to 80% over 5–10 years (Moradi 2015). Most of the *Phytophthora* species can be the cause of pistachio gummosis (Mostowfizadeh-ghalamfarsa and Mirsoleimani 2013). Among them, two species, namely *P. pistaciae* and *P. drechsleri*, are the most frequently occurring ones (Banihashemi and Moradi 2004).

Different approaches are recommended to reduce disease and its severity, including cultural, chemical, and biological approaches (Moradi 2015). Of these, chemical pesticides are applied most frequently. There are also reports of the use of lime (calcium carbonate) to disinfect the crown and root of pistachio trees (Moradi and Masoomi 2011). Calcium ion activity against *Phytophthora* root rot of some crops, including citrus and avocado, is well known (Zentmyer and Lewis 1975). In *P. nicotiana*, it is reported that some calcium salts reduce mycelial growth, zoospore production and viability, inoculum density in soil, and sour orange root infection (Campanella *et al*. 2002). In soybean, the stem rot that is caused by *P. sajae* was recovered by some types of calcium salts. However, there was no significant relationship between reduced mycelial growth *in vitro* and reduced disease in greenhouse plants. The researchers mentioned that the inhibition of disease may be due to the plants response to calcium and not the pathogen response. Calcium may exert its effect by increasing plant resistance. Thereby, plant disease reduction may be due to multiple effects of calcium on the inhibition of zoospore production and pathogen growth, along with the plant response to calcium (Sugimoto *et al*. 2008).

Phytophthora-induced damage to high–value pistachio crop, moreover health and environmental concerns regarding the use of synthetic fungicides as well as possible resistance creation in target pathogens show the need for researchers to find safer, more stable, and more effective ways to control this pathogen. Therefore, the aim of this study was to isolate *P. pistaciae* from pistachio trees with crown rot and evaluate the effect of some mineral calcium salts on mycelial growth, sporangium and zoospore production, cyst germination, and sporangium morphology of the isolate.

Materials and Methods

Isolation, purification and identification of P. pistaciae

Samples were taken from infected trees and transferred to the laboratory in ice boxes. The infected tissue of the crown and root were cut into small pieces and cultured on Lima Bean Agar (LBA) medium and kept in 26 °C in darkness for seven days. The plates were examined for suspected fungal growth every day. To purify the isolates, a hyphal tip method was used. For molecular identification, total DNA was extracted from mycelium using fungi DNA isolation kit (DENAzist Asia, Iran). PCR reaction was performed with ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') primers (Grünwald *et al*. 2011) and followed programs of 3 minutes at 94°C (1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C) for 35 cycles, and 10 minutes at 72°C. The PCR product was then sequenced. The obtained sequences were aligned in NCBI database with BLAST software.

Pathogenicity tests

Apple

The immature fruits were inoculated with the edge of three–day–old mycelia of the isolate. The inoculation area was covered by paraffin and fruits were kept at 26°C for seven days. The fruits were examined for observing the presence of fungal growth to determine the aggressiveness of the isolate.

Pistachio twig

After disinfection of pistachio twigs, a hole was made in the xylem and a three–day–old plug of mycelia was used for inoculation. The inoculation area was covered by parafilm and the twigs were kept at 26°C. The inoculated twigs were examined after two weeks of colonization.

Pistachio seedlings

The crowns of one–month–old pistachio seedlings were inoculated by a plug of actively growing mycelia in order to assess the pathogenicity under greenhouse conditions. The mortality rates were assessed one month after inoculations.

The impacts of Calcium Salts on P. pistaciae

Mycelial growth

Stock solutions with 3000ppm concentration in distilled water were prepared by solid salts of calcium oxide, calcium hydroxide, calcium carbonate, calcium sulfate, calcium phosphate, calcium nitrate, and calcium chloride. Since the solubility of the salts, except calcium nitrate and calcium chloride, was very low in the water, pure HCl was dropped and stirred until the salts were completely dissolved. All solutions were then adjusted to pH 6.5. LBA media were prepared with 250, 500, 1000, 1500, 2000 or 3000ppm concentrations of the salts. With higher concentrations of calcium salt, a higher amount of agar was needed to solidify the medium. The concentration of agar was then found to be 10, 10, 12, 16, 20, 24, and 32g/l in control and 250, 500, 1000, 1500, 2000, and 3000ppm modified media with salts, respectively. LBA media without calcium salt were also used as control. Plugs from the edge of a growing mycelium were cultured on the media and kept at 26°C. Colony diameter was measured by a ruler after two, four, and six days. The experiment was repeated four times.

Sporangium and zoospore production

To prepare LBA, 23g of ground–bean per liter was autoclaved, filtered, and poured in sterile Petri dishes. Plugs from the edge of a growing mycelium of the *P. pistaciae* were cultured overnight. From each salt, 1500 and 3000ppm aquatic solutions were prepared. As much as 8ml of each solution was poured into a Petri dish and 2ml of a sporangium inductor solution was added to it. The inducer receipt is expressed below. Four plugs with young hyphae were put into each Petri dish. The dishes were placed at 26°C under fluorescent light at a distance of 20cm. After 24 hours, dishes were investigated under light microscopy and the number of sporangia around each plug was counted. The dishes were then placed in the refrigerator for an hour and were taken out for zoospores to be released from sporangia. Moreover, 1ml of zoospore suspension from each Petri dish was poured into a microtube and vortexed gently for 20 seconds in order to slow the zoospores' movement. The density of zoospores in suspension was assessed by a hemocytometer. The experiment was repeated four times.

Sporangium inducer: 3.08 g of calcium nitrate, 1.49g magnesium sulfate, and 0.51g of potassium nitrate were dissolved in 1l of distilled water. To that, 1ml of iron chelate solution (0.65g of EDTA, 0.37g potassium hydroxide and 1.24g of iron sulfate in 50ml of distilled water) was added. The solution was then autoclaved (Broembsen and Deacon, 1996).

Cyst germination

As much as, 1ml of zoospore suspension from each Petri dish was poured into a microtube and vortexed gently for 70 seconds in order to inhibit the zoospores movement completely. The cysts were left to germinate for 1 hour at 26°C for germination. The density of the cysts and the germinated cysts was counted by a hemocytometer. The experiment was repeated four times.

Morphology of isolate

Deformation in morphology of mycelium and sporangia (1500 and 3000ppm of salts) were studied via light microscopy assays.

Statistical analysis

The average values of mycelial growth, sporangium and zoospore production, and cyst germination were determined for each replication. The data were analysed using Proc GLM procedures (SAS Release Version 9.0). Comparisons of means were made using Duncan's new multiple range test at 5 % probability. The charts were plotted with Excel software.

Results

Isolation, purification and identification of P. pistaciae

From infected crown tissues, *Phytophthora pistaciae* was grown on LBA medium and was identified based on the morphological and molecular features (Fig. 1). In PCR assays, a band with size of 940bp was produced, which was then sequenced. The obtained sequences showed 99% similarity with *P. pistaciae* DNA in the NCBI database.

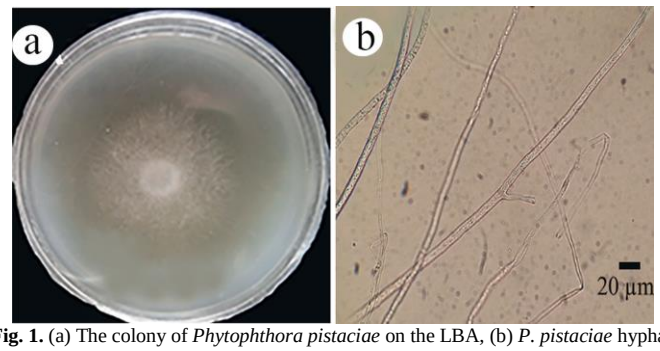

Fig. 1. (a) The colony of *Phytophthora pistaciae* on the LBA, (b) *P. pistaciae* hyphae

Pathogenicity of isolate

Overall, the inoculations of apple fruits and pistachio twigs showed the ability of the isolate to produce disease. All the seedlings were inoculated with *P. pistaciae*, which showed 100% mortality after two months. In all the inoculations, *P. pistaciae* was isolated from the infected tissue.

Mycelial growth

Two days after culture

After two days, in media containing calcium salts with 250 and 500ppm concentrations, no significant differences was observed with control plates in growth inhibition. But these concentrations in media containing calcium oxide and calcium carbonate showed a significant reduction in the growth of *P. pistaciae*. In media with all salts, at concentrations of 1000, 1500, 2000, and 3000ppm, growth was reduced significantly. The highest differences were observed in 1500, 2000, and 3000ppm of calcium oxide, 2000 and 3000ppm of calcium phosphate, and 3000ppm of calcium carbonate, in which no mycelial growth was observed (Fig. 2).

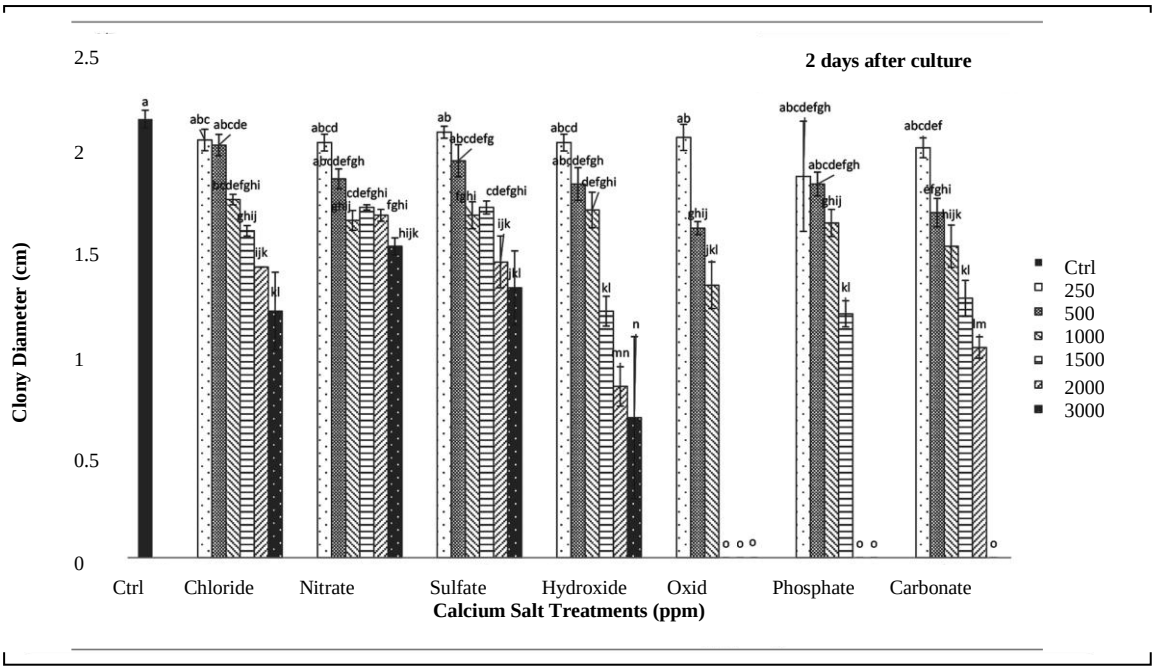

Fig. 2. Effect of calcium salts on colony diameter of *P. pistaciae* isolate after two days

Four days after culture

After four days, in media with all salts and all concentrations, growth reduction was significant compared to control. This reduction was obviously higher with increasing concentrations of salts. The complete inhibition of growth was only observed in medium containing 3000ppm of calcium oxide (Fig. 3).

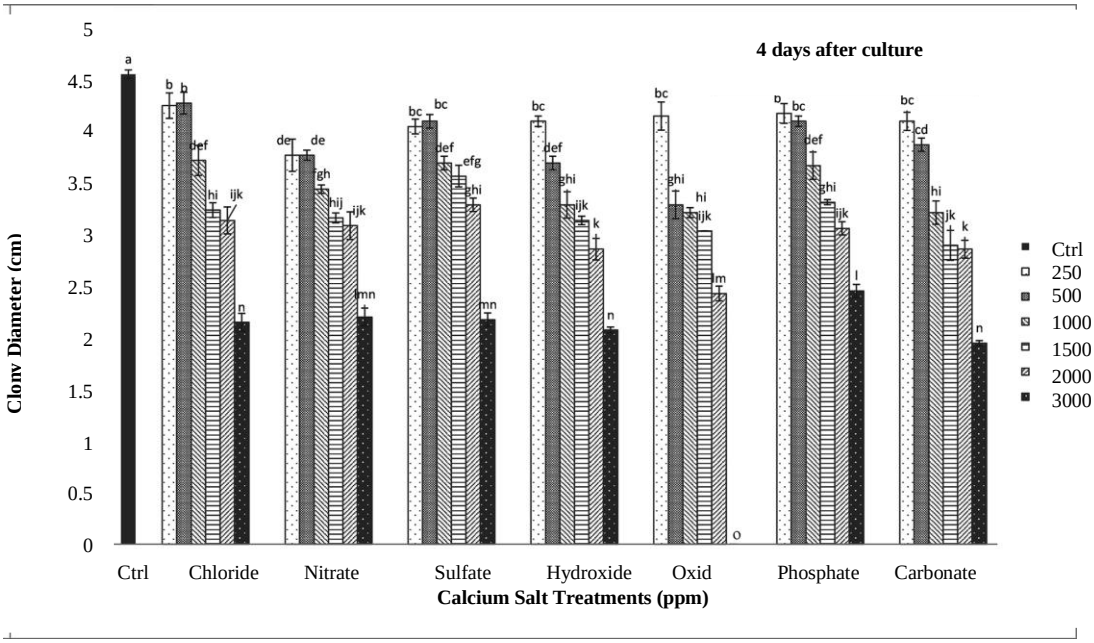

Fig. 3. Effect of calcium salts on colony diameter of *P. pistaciae* isolate after four days

Six days after culture

After six days, no significant difference with control was observed in media containing calcium nitrate, calcium oxide and calcium carbonate with 250ppm concentration. However, in 500ppm and higher concentrations of all these varieties of salt, growth reduction was significant, as compared to the control. In media containing calcium chloride, calcium sulfate, calcium hydroxide, and calcium phosphate at all concentrations, growth reduction was significant. In media with all kinds of salt, growth reduction was higher with increased salt concentration. The complete inhibition of growth (colony diameter zero) was only seen in media containing 3000ppm of calcium oxide (Fig. 4).

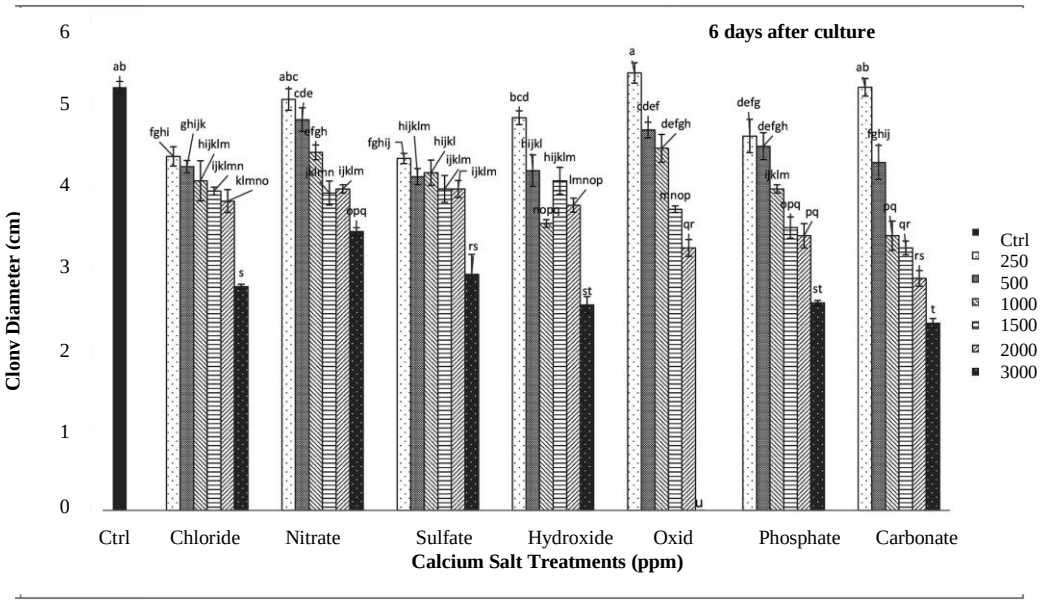

Fig. 4. Effect of calcium salts on colony diameter of *P. pistaciae* isolate after six days.

Sporangium and zoospore production

Calcium salt treatments had different effects on sporangium production (Fig. 5). Calcium chloride (3000ppm) and calcium nitrate (1500 and 3000ppm) did not affect the sporangium production rate, compared to the control. Calcium chloride (1500ppm) increased sporangium production. However, calcium sulfate, calcium hydroxide, calcium oxide, calcium phosphate, and calcium carbonate in both concentrations decreased sporangium production (Fig. 6). The effect of calcium salts on zoospore production was consistent with its effects on the production of sporangium (Figs. 7 and 8).

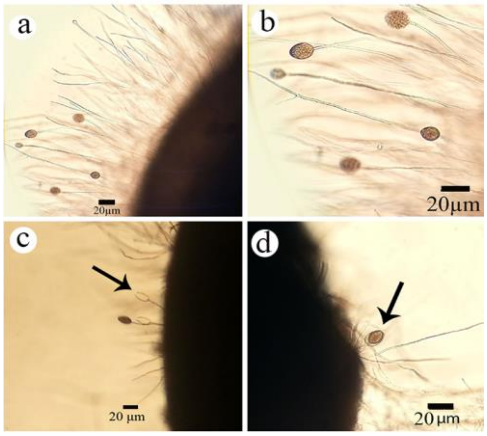

Fig. 5. (a, b) sporangia of *P. pistaciae* isolate, (c) sporangia devoid of zoospores, (d) a sporangium growing directly from a previous one

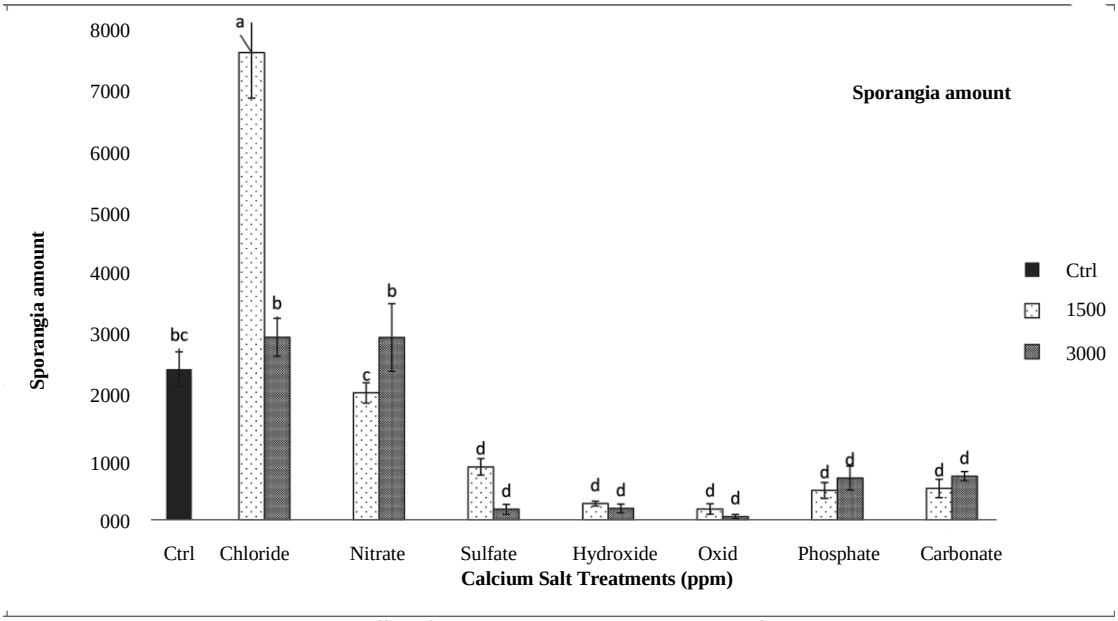

Fig. 6. The effect of calcium salt treatments on the amount of sporangia

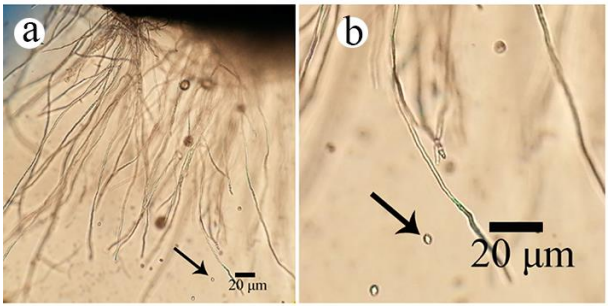

Fig. 7. Zoospores of *P. pistaciae* isolate

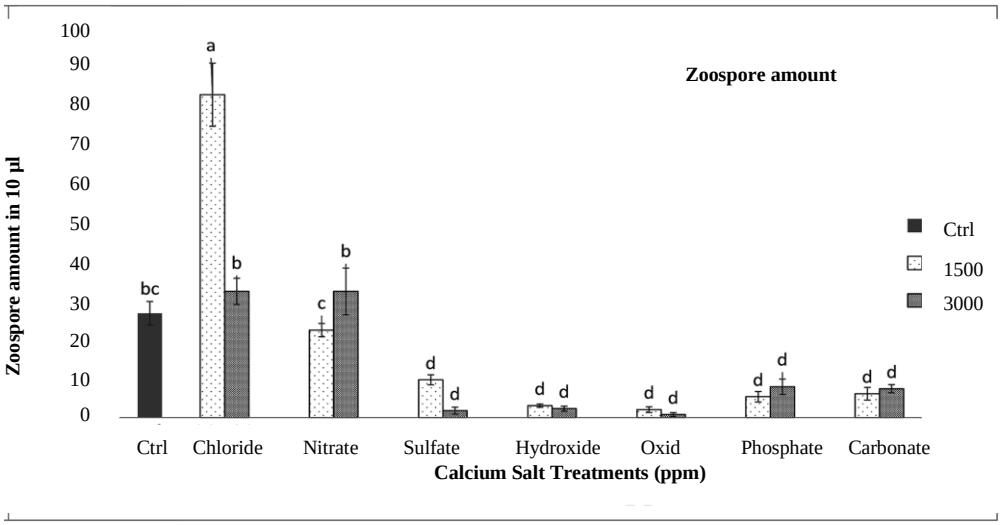

Fig. 8. The effect of calcium salt treatments on the realizing of zoospores

Cyst germination

Zoospores convert to immobile cysts, which have the ability to germinate and produce new hyphae (Fig. 9). Calcium salts had different effects on cyst germination. Calcium chloride and calcium nitrate significantly increased germination percentage, which was significantly higher in 3000 than in 1500ppm

concentration. Calcium sulfate at 1500 and 3000ppm concentrations increased and decreased cyst germination, respectively. Calcium hydroxide, calcium oxide, calcium phosphate, and calcium carbonate in both concentrations inhibited cyst germination completely (Fig. 10).

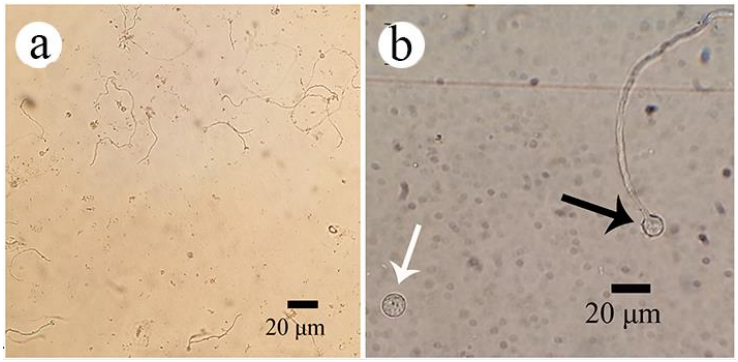

Fig. 9. (a) Cysts of *P. pistaciae* isolates (b) a germinating cyst (black arrow) and an intact cyst (white arrow)

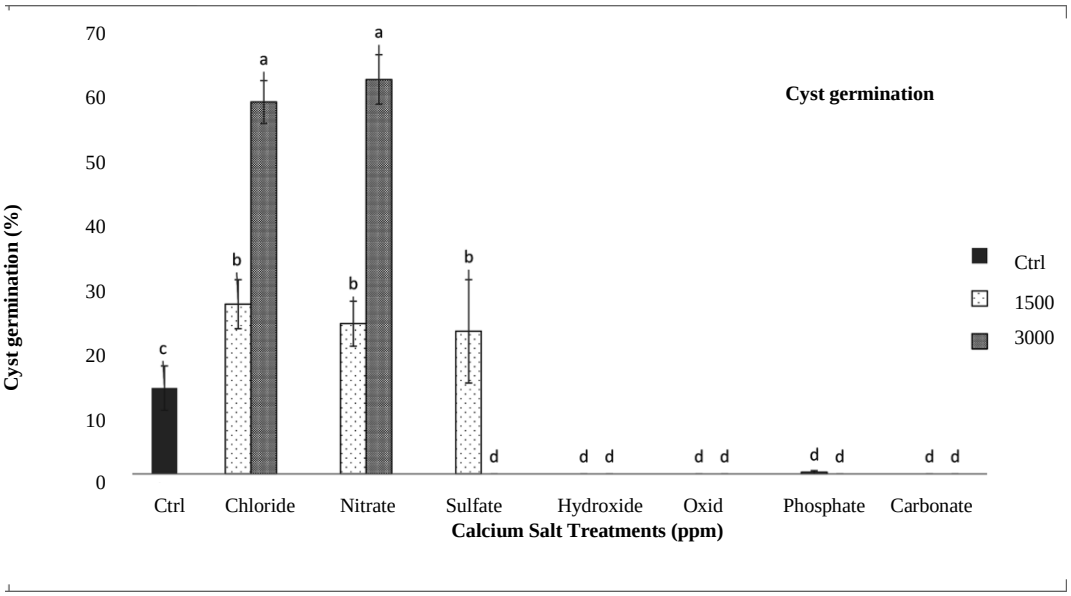

Fig. 10. The effect of calcium salt treatments on cyst germination percentage

Morphology of isolate

Most varieties of calcium salt did not have any obvious effect on the morphology of isolates. Only in calcium oxide and calcium hydroxide treatments, hyphae were highly branched and some sporangia showed deformation (Fig. 11).

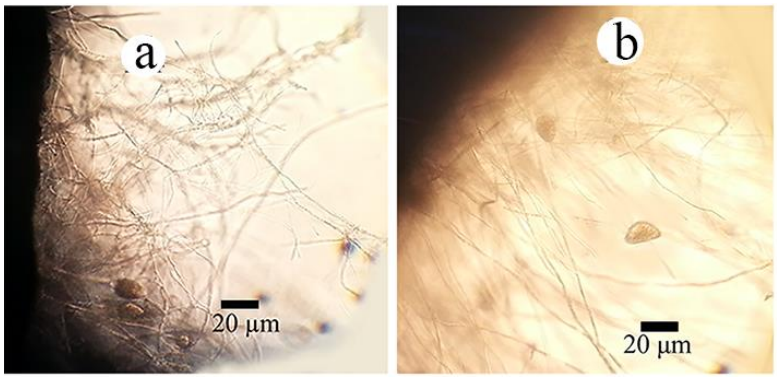

Fig. 11. (a) Branched hyphae of *P. pistaciae* isolate under the influence of calcium oxide, (b) deformation of *P. pistaciae* sporangia under the influence of calcium oxide

Discussion

The ITS4 and ITS6 primers that are located inside the 28s and 18s rRNA genes, respectively, are usually used for amplification of ITS1 and ITS2 spacer regions that are diverse and effective in detecting most of *Phytophthora* species (Cooke *et al*. 2000). Analysis of spacer regions in rDNA of a wide range of different isolates of *P. pistaciae* showed high degree of sequence similarity (Mostowfizadeh-ghalamfarsa and Mirsoleimani 2013). In this study, the isolate was confirmed as *P. pistaciae* because of 99% similarity with *P. pistaciae* DNA in NCBI database.

Mycelial growth reduction was observed in media containing calcium salt of all types, and the most effective one was calcium oxide at 3000ppm concentration which completely inhibited the growth. Campanella *et al*. (2002) examined the effect of 10 types of calcium salt for controlling the *P. nicotianae* both *in vitro* and on sour orange seedlings. Their results showed that calcium oxide, calcium propionate, calcium carbonate, and calcium nitrate decreased the growth of colony. The most effective salt was calcium oxide that reduced the growth by as much as 46–57%. They concluded that the effects of calcium oxide could be attributed to the value of pH in the medium that was 10.7, which is far from the optimal value (6–6.5) for the growth of *Phytophthora*. Another study showed that the effect of calcium salts against *Botrytis cinerea* was not just for pH but also due to the fact that the salts were harmful on the enzyme activity of pathogen (Biggs *et al*. 1997). Boumaaza *et al*. (2015) also showed that calcium chloride with concentrations up to 150ppm, increased the mycelial growth of *B. cinerea in vitro* and decreased it at higher concentrations.

The effects of calcium salts were varied on sporangium and zoospore production such as decreased, increased or no effects compared to the control. Different effects of calcium salts may support the role of anionic compounds as well as evidence on the role of calcium ions. For example, at a concentration of 2 to 10mM, calcium increased the formation of sporangia (Allen and Nandra 1975). Higher concentrations of this ion decreased zoospore release from sporangia (Von Broembsen and Deacon 1997) and zoospore mobility (Byrt *et al*. 1982). Based on the results, inhibition of zoospore production in most of the salt treatments could be due to high concentration of calcium ions. Campanella *et al*. (2002) showed that in *P. nicotianae*, calcium gluconate, calcium lactate, and calcium acetate, which are organic salts, increased zoospore production. But increased zoospore production in this study occurred due to the application of calcium chloride, which is an inorganic salt. Therefore, the use of calcium chloride in 1500ppm concentration can be useful for the production of *P. pistaciae* zoospores *in vitro* in lab experiments. Furthermore, it was shown in *B. cinerea* that calcium chloride slightly increased the spore production (Boumaaza *et al*. 2015).

Organic salts such as calcium gluconate, calcium lactate, and calcium acetate, increased cyst germination percentage in *P. nicotianae* (Campanella *et al*. 2002). The same results were observed with calcium chloride, calcium nitrate, and calcium sulfate in this study. Boumaaza *et al*. (2015) also showed that calcium chloride in 50ppm concentration increased the spore germination in *B. cinerea*, while other varieties of calcium salt reduced it. In *P. nicotianae* only calcium oxide reduced the germination of cysts (Campanella *et al*. 2002). While in this study, salts of calcium hydroxide, calcium oxide, calcium phosphate, and calcium carbonate in both examined concentrations reduced cyst germination down to zero.

Calcium sulfate, calcium hydroxide, calcium oxide, calcium phosphate, and calcium carbonate in both the investigated concentrations significantly decreased sporangium and zoospore production and cyst germination. Therefore, these may effectively prevent spore production and germination in the soil around the tree and may reduce the severity of the disease and spread of pathogen in the environment if sprinkled on the soil around the crowns of the infected trees.

Calcium oxide and calcium hydroxide caused branching of hyphae. It is well known that calcium plays a major role in changing the polarity of cells and creating branches in those. Calcium entering from some points of the cell creates a directional transmission system in cell. Therefore, vesicles containing the cell wall material accumulate at those points. This causes the formation of branches at those points (Lyndon 1996).

Naturally sporangia of *Phytophthora* have morphological diversity, even within species. For example, in *P. citrophthora*, sporangia can be spherical, ovoid, inverted pear-shaped, elliptical or papillate, and some have asymmetrical shape. Sporangia of *P. nicotianae* may be spherical, ovoid, pear-shaped, elliptical or papillate. *P. syringae* may have ovoid, inverted pear–shaped or relatively papillate sporangia that usually originate in succession from a primary sporangium (Mounde *et al*. 2012). The isolate of this study had ovoid to elliptical sporangia and some of them became asymmetric in shape under the influence of calcium oxide and calcium hydroxide. Since calcium plays a key role as a second messenger in maintaining the cell shape and changing the cell polarity, deformation in the structure of hyphae and sporangia is not unexpected. Campanella *et al*. (2002) also reported that calcium oxide created a drastic change in sporangia morphology of *P. nicotianae*.

Conclusions

Overall, the results of this study support the potential of inorganic calcium salts in controlling *P. pistaciae*, which is the cause of pistachio gummosis. Therefore, these varieties of salt may somehow replace the synthetic pesticides that are harmful for human and the environment.

Conflict of Interest

The authors declare that they have no conflict of interest

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