

EFFECT OF CALCIUM SALTS ON POSTHARVEST FUNGAL PATHOGENS *IN VITRO*

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SUMMARY

In the recent years, several studies have shown that calcium salts may have potential as environmentally compatible, nontoxic fungicides for controlling postharvest fungal pathogens. Therefore, the objective of this study was to evaluate and compare the effects of calcium chloride and calcium hydroxide on *in vitro* mycelial growth, spore germination and germ tube growth of *Colletotrichum acutatum*, *C. gloeosporioides*, *Alternaria alternata*, and *Penicillium expansum*. The obtained results showed that the fungal isolates grew similarly or stimulated in the presence of 1 and 1.5% calcium salts compared to the control. After seven days of incubation, reduction of mycelial growth was observed only on PDA supplemented with 2% calcium salts. Calcium chloride and calcium hydroxide at 1.5% and 2.0% concentrations significantly decreased spore germination and germ tube growth of all fungal isolates. The results of this study show that the tested calcium salts can be used as an alternative treatment against postharvest fungal pathogens *C. acutatum*, *C. gloeosporioides*, *A. alternata* and *P. expansum*.

Key words: postharvest fungal pathogens, calcium salts, *in vitro* effect

INTRODUCTION

Synthetic fungicides have been routinely used to control postharvest diseases. However, the development of fungicide resistance and an increasing environmental concern over fungicide residues in food have stimulated to find alternative means for controlling postharvest decay (Holmes and Eckert, 1999). Among alternatives to synthetic chemicals, several inorganic salts and organic, lipophilic acids and their salts have shown increasing evidence of efficacy in controlling plant pathogens. Many of these salts are widely used in industry as preservatives and antimicrobials (Russell and Gould, 1991) and have shown some advantages for utilization as postharvest chemicals, such as a broad-spectrum antimicrobial activity with low mammalian toxicity (Olivier et al., 1998), and biocompatibility (Horst et al., 1992).

Several studies have been undertaken in re-

cent years identifying the fungicidal properties of many different antimicrobial salts. Sodium carbonate, sodium bicarbonate, potassium carbonate, potassium bicarbonate, ammonium bicarbonate, and potassium silicate have been tested for inhibition of fungal pathogens on fruits, vegetables, field crops and ornamentals. These salts demonstrated *in vitro* and/or *in vivo* inhibition of *Fusarium tricinctum*, *F. graminearum*, *F. sporotrichioides*, *Aspergillus ochraceus*, *A. flavus*, *A. niger*, *P. griseofulvum*, *P. notatum*, *P. digitatum*, *P. expansum*, *Botrytis cinerea*, *Helminthosporium solani*, *C. acutatum*, and *C. gloeosporioides* (DePasquale et al., 1990; Olivier et al., 1998; Smilanick et al., 1999; Karabulut et al., 2001; Hervieux et al., 2002; Conway et al., 2007; Hasan et al., 2012). Sorbic and propionic acids and their salts are used as preserving additives in milled corn and have been tested for the suppression of mycotoxins and storage molds (Rusul et al., 1987; BuaZZi and Marth, 1991).

Calcium is a key plant nutrient that has a significant role in cell functions, including reducing softening and senescence of fruits (Conway et al., 1991). Many disorders of crops, such as bitter pit in apple, cork spot in pear, and blossom end rot in tomato that are caused by calcium deficiency could be reduced by calcium spraying (Kader, 2002). The most common form of calcium supplement is calcium chloride, however, many proprietary products containing calcium in other forms, or combined with other nutrients are available.

Few studies have examined the potential role of calcium supplementation in the postharvest period for reducing decay (Conway et al., 1994). Saftner et al. (1997) reported that postharvest calcium treatment of apples provided broad-spectrum protection against *P. expansum* and *B. cinerea*, and Alternaria rot of the apple cultivar Nittany was effectively managed with pre- and postharvest calcium applications (Biggs et al., 1993; Biggs et al., 2000; Maouni et al., 2007). Biggs (1999) examined the effects of calcium salt solutions on two *Colletotrichum* species originated from apple fruits. The results verified the effect of calcium salts on reduction of disease severity, expressed as lesion diameter. Also, there are few reports about effects of calcium salts on infection of tropical fruits by *Colletotrichum* spp. (Chillet et al., 2000; Mahmud et al., 2008; Madani et al., 2014). The ability of calcium to reduce the development of postharvest diseases of fruit has been attributed mainly to the formation of calcium cross-linkages in the cell wall, resulting in decreased effectiveness of cell wall-macerating enzymes secreted by the pathogen (Conway et al., 1988).

Postharvest fungal decay may cause significant losses to the apple production and storage in Serbia. Therefore, the present study was conducted in order to examine and compare the effects of two calcium salts on *in vitro* growth, spore germination and germ tube elongation of the postharvest pathogens *C. acutatum*, *C. gloeosporioides*, *A. alternata*, and *P. expansum*.

MATERIAL AND METHODS

Pathogens

C. acutatum, *C. gloeosporioides*, *A. alternata*, and *P. expansum* were isolated from decayed apple fruits. The fungi were maintained on potato dextrose agar (PDA) at 4°C.

Effect of calcium salts on mycelial growth *in vitro*

Pure calcium salts used in this study were calcium chloride and calcium hydroxide. Salts were prepared in sterile deionized distilled water, added to autoclaved warm (~50°C) PDA to provide a final concentration of 1, 1.5, and 2% (w/v). An agar disc (Ø5 mm) taken from an active colony of tested fungal pathogens was placed in the center of each of three replicate Petri plates. PDA not supplemented with calcium salts served as a control. The all Petri plates were incubated at 25°C, and growth was assessed after 7 days.

Effect of calcium salts on spore germination and germ tube elongation *in vitro*

Spore suspensions of all tested fungal pathogens were obtained from 2- week-old cultures incubated at 25°C by flooding the cultures with sterile-distilled water containing 0.05% (v/v) Tween 80, and filtered through four layers of sterilized cheesecloth. Spore concentration was determined with a hemacytometer, and adjusted to 1×10^6 conidia/ml. Aliquots of 100 μ l of the pathogen suspension were transferred to glass tubes containing 5 ml potato dextrose broth (PDB), and then the tested chemicals were added to each tube to achieve the proposed concentration. All tubes were put on a rotary shaker at 110 rpm at 25°C. After 18 h incubation, 100 spores of pathogens were measured for germination rate. Spores were considered germinated when germ tube length was equal to or greater than spore length.

The efficacy of each treatment was calculated according to the following formula: $R(\%) = K-T/K \times 100$, where R is reduction, K is spore germination or germ tube growth in control medium, and T is spore germination or germ tube growth in a medium with tested calcium salts.

Statistical Analysis

All treatments consisted of three replicates, and experiments were repeated twice. The data were analyzed by analysis of variance (ANOVA). Mean values were compared using Duncan's multiple range test, and significance was evaluated at $P < 0.05$.

RESULTS AND DISCUSSION

Calcium chloride and calcium hydroxide at 1% stimulated mycelial growth of *C. acutatum*

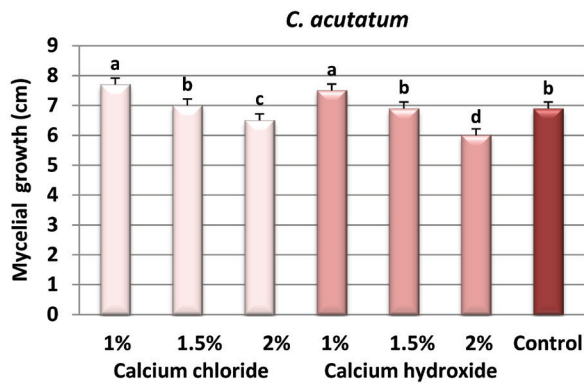


Fig. 1. Effect of calcium salts on mycelial growth of *C. acutatum*. Means with the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$).

Sl. 1. Efekat kalcijumovih soli na porast micelije *C. acutatum*. Vrednosti sa istim slovom nisu statistički značajno različite na osnovu Duncan's multiple range testa ($P < 0.05$).

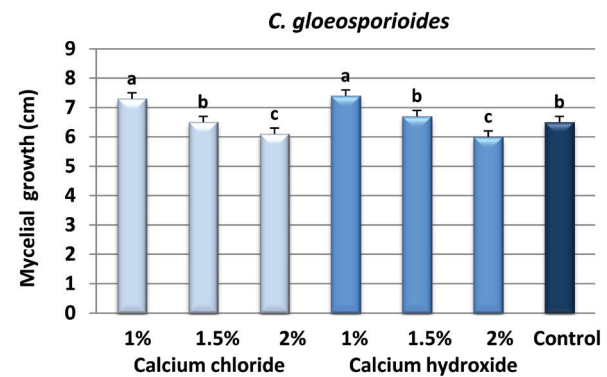


Fig. 2. Effect of calcium salts on mycelial growth of *C. gloeosporioides*. Means with the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$).

Sl. 2. Efekat kalcijumovih soli na porast micelije *C. gloeosporioides*. Vrednosti sa istim slovom nisu statistički značajno različite na osnovu Duncan's multiple range testa ($P < 0.05$).

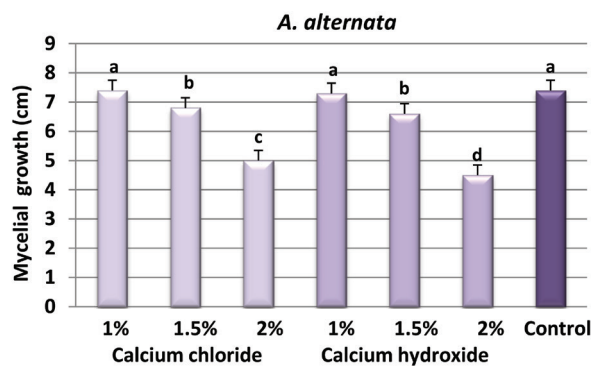


Fig. 3. Effect of calcium salts on mycelial growth of *A. alternata*. Means with the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$).

Sl. 3. Efekat kalcijumovih soli na porast micelije *A. alternata*. Vrednosti sa istim slovom nisu statistički značajno različite na osnovu Duncan's multiple range testa ($P < 0.05$).

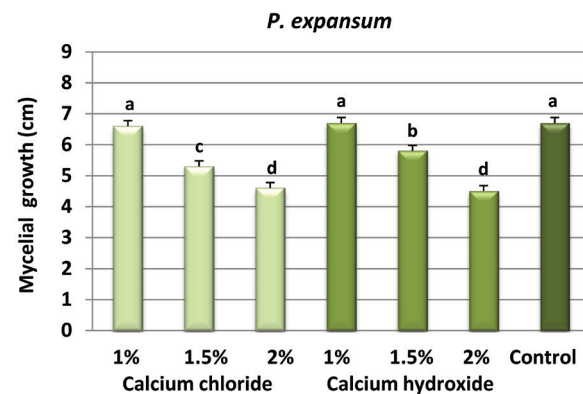


Fig. 4. Effect of calcium salts on mycelial growth of *P. expansum*. Means with the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$).

Sl. 4. Efekat kalcijumovih soli na porast micelije *P. expansum*. Vrednosti sa istim slovom nisu statistički značajno različite na osnovu Duncan's multiple range testa ($P < 0.05$).

and *C. gloeosporioides* relative to the control. The mycelial growth at 1.5% calcium salts was similar compared to the control (Fig. 1-2). Also, no reduction in growth was observed when *A. alternata* and *P. expansum* were cultured on PDA supplemented with 1% calcium chloride and calcium hydroxide (Fig. 3-4). In this experiment, only the calcium salts at 2% concentration reduced mycelial growth of all tested pathogens, relative to the control. Our results are in agreement with those of other researchers who demonstrated that the fungal isolates grew similarly or stimulated in the presence of calcium

salts compared to the control (Biggs, 2004; Madani et al., 2014). Tian et al. (2002) recorded that calcium chloride at 2% inhibited the growth of *R. stolonifer*, although calcium chloride was tolerated by *A. alternata* and *P. expansum* *in vitro* where their growth was highly affected only at 6% concentration (Maouni et al., 2007). Calcium salts also have been shown to reduce mycelial growth *in vitro* and reduce incidence and severity of infection of peach fruits and shoots by *Monilinia fructicola* and *Leucostoma persoonii*, respectively (Biggs and Peterson, 1990; Biggs et al., 1997).

Table 1. Effect of calcium salts on *in vitro* conidial germination and germ tube growth of postharvest fungal pathogens.**Tabela 1.** *In vitro* efekat kalcijumovih soli na klijavost konidija i porast klicine cevi skladišnih gljivičnih patogenata.

Treatment Tretman	Conc. %	Pathogen Patogen							
		<i>C. acutatum</i>		<i>C. gloeosporioides</i>		<i>A. alternata</i>		<i>P. expansum</i>	
		RSG* (%)	RGTG** (%)	RSG (%)	RGTG (%)	RSG (%)	RGTG (%)	RSG (%)	RGTG (%)
Calcium chloride	1	16.8d***	35.3d	16.3c	29.4d	21.1d	38.9d	31.5d	46.6c
	1.5	24.3c	47.4c	23.4b	41.5c	38.8c	49.6c	44.3c	59.3b
	2	38.5a	57.1a	38.2a	53.1a	52.7a	63.8a	61.1a	70.5a
Calcium hydroxide	1	16.2d	34.1d	13.3d	30.4d	22.5d	37.5d	32.8d	47.6c
	1.5	23.6c	46.1c	24.7b	40.9c	38.1c	47.5c	44.6c	59.8b
	2	32.8b	53.3b	37.9a	48.7b	47.4b	59.8b	57.8b	70.2a

RSG* - Reduction of spore germination; RGTG** - Reduction of germ tube growth; *** - Means in column followed by the same letter are not significantly different according to the Duncan's multiple range test ($P < 0.05$).

RSG* - Redukcija klijavosti konidija; RGTG** - Redukcija porasta klicine cevi; *** - Vrednosti u koloni označene istim slovom nisu statistički značajno različite na osnovu Duncan's multiple range testa ($P < 0.05$).

Conidial germination assay were conducted to test the potential for calcium chloride and calcium hydroxide at different concentrations to inhibit germination of postharvest fungal pathogens. The both of calcium salts were effective in reducing the spore germination and germ tube growth of *C. acutatum*, *C. gloeosporioides*, *A. alternata* and *P. expansum*. In most cases, in treatments with 1 and 1.5% calcium salts, inhibition percentage did not exceed 50%. The spore germination of all tested pathogens was decreased significantly on PDA supplemented with 2% calcium salts (Table 1.). Also, inhibition of germ tube growth was greater, and increases with salts concentration (Table 1.). Our findings are in agreement with those of Eryani-Raqeeb et al. (2009) who demonstrated that high concentration of calcium reduced spore germination of the papaya anthracnose pathogen. Droby et al. (1997) observed inhibition of spore germination and germ tube elongation of *P. digitatum* in culture, as well as inhibition of polygalacturonase activity. Wisniewski et al. (1995) have indicated that calcium chloride reduced germination and germ tube elongation of *B. cinerea* and *P. expansum in vitro*. Increasing concentrations of calcium chloride (25–175mM) resulted in decreased spore germination and germ tube growth of both pathogens, but the greatest effect was observed in the case of *B. cinerea*.

Reduced spore germination shows that the pathogen may be more sensitive to calcium at the conidial stage relative to the mycelial growth stage.

The mechanisms by which calcium salts inhibited fungal spore germination and germ tube elongation are not known. One hypothesis is that high concentration of extracellular calcium may increase calcium in the cytosol to toxic levels. With a minimum level of calcium ion concentration being necessary for normal cell growth, any limitation in regulation of intracellular calcium level may result in reduced organism development (Droby et al., 1997). Calcium ions may reduce the incidence of fungal infection by directly inhibiting fungal growth and by inhibiting cell wall-degrading enzymes produced by the pathogens. These effects were probably due the toxicity of higher concentration of calcium on pathogens by affecting the osmotic balance in the fungal cells and inhibition of pectinolytic enzymes (Miceli et al., 1999).

Several studies have shown that calcium salts may have potential as environmentally compatible, nontoxic fungicides for controlling postharvest pathogens. Our results support these findings by showing that calcium chloride and calcium hydroxide restrict *in vitro* conidial germination and germ tube growth of *C. acutatum*, *C. gloeosporioides*, *A. alternata*, and *P. expansum*.

ACKNOWLEDGMENT

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project TR 31018.

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(Received: 31.01.2014.)
(Accepted: 28.02.2014.)

IN VITRO EFEKAT KALCIJUMOVIH SOLI NA GLJIVIČNE SKLADIŠNE PATOGENE

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REZIME

Tokom poslednjih nekoliko godina, više studija je pokazalo da u kontroli gljivičnih skladišnih patogena soli kalcijuma mogu imati potencijal kao ekološki kompatibilni, netoksični fungicidi. Stoga je cilj ovog istraživanja bio da se u *in vitro* uslovima procene i uporede efekti kalcijum hlorida i kalcijum hidroksida na porast micelije, klijavost konidija i rast klicine cevi gljiva *C. acutatum*, *C. gloeosporioides*, *A. alternata*, i *P. expansum*. Dobijeni rezultati su pokazali da je u prisustvu 1 i 1,5% kalcijumovih soli porast izolata gljiva u tretmanima sličan ili povećan u odnosu na kontrolu. Nakon 7 dana inkubacije, redukcija porasta micelije je utvđena samo na PDA podlozi sa 2% soli kalcijuma. Kalcijum hlorid i kalcijum hidrok- sid u koncentracijama od 1,5 i 2,0% značajno smanjuju klijavost konidija i rast klicinih cevi svih ispitivanih izolata gljiva. Rezultati ove studije pokazuju da se testirane soli kalcijuma mogu primeniti kao alternativni tretman u kontroli gljivičnih skladišnih patogena, *C. acutatum*, *C. gloeosporioides*, *A. alternata* i *P. expansum*.

Ključne reči: gljivični skladišni patogeni, soli kalcijuma, *in vitro* efekat

(Primljeno: 31.01.2014.)

(Prihvaćeno: 28.02.2014.)