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Research Article
Control of Gray Mold Disease of Tomato by Postharvest Application of Organic Acids and Salts

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Abstract

Background and Objective: Tomato is the major fruit crop produced and exported in Morocco. This commodity is faced to many threats. The most important tomato diseases caused commercially significant losses, in Morocco and worldwide, is gray mold caused by *Botrytis cinerea*. This study was aimed to find out an alternative to synthetic fungicides used in the control of the polyphagous devastating fungus '*Botrytis cinerea*' using common food additives. **Materials and Methods:** Thirty seven organic acids and salts considered as common food additives were tested *in vitro* against this pathogen using the agar dilution method. Compounds with the best antifungal activity, selected after one-way analysis of variance, were tested *in vivo* on artificially inoculated tomato fruit. **Results:** At 0.02 M, EDTA, copper sulfate and sodium metabisulfite completely inhibited the mycelial growth and sporulation of *B. cinerea*. The lowest Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) were recorded in sodium metabisulfite treatment. The conidia germination was inhibited by ammonium molybdate and sodium metabisulfite treatments at only 10 mM. The nine most active chemicals in the *in vitro* trials were tested *in vivo* on tomato fruit. The incidence and the severity of gray mold were significantly reduced by EDTA, potassium carbonate, sodium bicarbonate, sodium carbonate, sodium metabisulfite and sodium salicylate compared to 100% (incidence and the severity) in the control. **Conclusion:** The results of the current study suggest that these salts are potentially useful as postharvest GRAS compounds to control *B. cinerea* on tomato fruit.

Key words: Antifungal activity, *Botrytis cinerea*, food additives, GRAS compounds

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is a major fruit crop that is grown around the world for both fresh produce markets and processed food industries[1]. About 1.23 million tons of tomato is annually produced in Morocco[2] and constitutes the major exported fruit crop, playing therefore a major role in the national economic development. However, tomato fruit is subjected to multitude of pathogenic microorganisms attacks and its intensive culture has generated and amplified many phytosanitary problems. In addition to phytopathogenic bacteria, viruses and deleterious microorganisms[3,4], fungal attacks are important factors that reduce the fruit quality and performance of the crop.

Gray mold is known as one of the most destructive fungal diseases of tomato, caused by *Botrytis cinerea*[5]. It is responsible for significant losses of tomato for both those grown in the fields and in green houses[6]. *Botrytis cinerea* is very harmful because it can attack different organs of the host crop including leaves, stems and fruits[7]. The decay is more prevalent under wet season and after harvest. Measures employed to manage this fungal disease are mainly based on the repetitive use of synthetic chemical fungicides (e.g., benzimidazoles and dicarboximides). The frequency of treatments during a season ranges from one or two to more than twenty applications. However, the frequent use of fungicides is becoming increasingly restricted due to stringent regulation, pathogen resistance development and growing public concern about chemical residues in fruit[8].

Various decay control methods that are alternatives to conventional synthetic fungicides to manage gray mold disease have been therefore assayed. These alternative methods include cold storage in conventional controlled atmospheres, application of heat treatments[9], use of ionizing radiations[10], use of biological control agents[3,11], or dips in solutions of food additives or other natural or synthetic compounds, with known and low toxicity, Generally Recognized as Safe (GRAS) compounds by most of food authorities' worldwide[12]. Such these compounds have minimal adverse effects on the environment, cost effective and accepted by consumers[8].

Several studies have shown the effectiveness of some salts to control various pathogens of many crops. Carbonates and bicarbonates have been demonstrated to have the ability to reduce the incidence of a wide range of pathogenic fungi. For example, sodium bicarbonate have been used to control *Phytophthora infestans*, *Phytophthora erythroseptica*[13], *Penicillium digitatum*[14], *Penicillium expansum*[15] and *B. cinerea* in table grape[8], in *Geranium*[16], in apple[17] and in cherry fruit[18]. Potassium carbonate was also used to control *B. cinerea* in table grape[8] and in *Geranium*[16]. Sodium metabisulfite was known for its ability to control gray mold in pear fruit[19] and potato silver scurf caused by *Helminthosporium solani*[20]. In addition, ethylenediaminetetraacetic acid (EDTA) was also shown to reduce the incidence of *B. cinerea*[17,21]. Derckel *et al.*[22] observed that treatment of grapevine berries by salicylic acid reduced the incidence of gray mold by inducing chitinase activity in fruits. Previous studies have shown that chitosan reduces decay incidence caused by *B. cinerea* in tomato fruit stored at 20°C[23].

The present work was performed to evaluate the efficacy of some selected known and commonly used organic acids and salts for *in vitro* and *in vivo* control of *B. cinerea,* the causal agent of tomato gray mold.

MATERIALS AND METHODS

Fungal pathogen culture: *Botrytis cinerea* was isolated from naturally infected tomato plants and was the most aggressive isolate in our collection. This isolate was deposited at the Laboratory of Biotechnology and Valorization of the Natural Resources under the number SR4. The pathogen was maintained on Potato Dextrose Agar (PDA) and stored at 4°C with periodic transfer through tomato fruit to maintain its aggressiveness.

Tomato fruit: Tomato fruit (*Lycopersicon esculentum*) cv 'Pitenza' was harvested from the greenhouse of the AZURA company in Souss Massa region (western centre of Morocco), only healthy and commercially mature fruits were used in the *in vivo* test.

***In vitro* screening of chemicals:** The inhibitory effects of 37 organic acids and salts (Table 1) on mycelial growth of *B. cinerea* were determined by the agar dilution technique. An aqueous solution of each compound was prepared in sterile distilled water and was added aseptically to molten (45°C) sterile Potato Dextrose Agar (PDA) to obtain tow final concentrations of 0.02 and 0.2 M[8], then the medium was poured into Petri dishes (15-20 mL PDA per plate). Plates without chemicals were used as control. *Botrytis cinerea* hyphal discs (5 mm in diameter) were cut from the periphery of actively growing colonies (7-10 days cultures) and transferred aseptically to three replicate Petri dishes containing PDA medium with chemicals. Inoculated plates were arranged in a complete randomized block design and incubated in dark at 25°C. Radial growth was daily measured

Table 1: Tested chemicals

Compounds	Chemical formulas	Molecular weight	Companies
Ammonium acetate	$C_2H_7NO_2$	77.08	Riedel-de Haën
Ammonium carbonate	$(NH_4)_2CO_3$	96.09	Riedel-de Haën
Ammonium chloride	NH_4Cl	53.491	ASDS
Ammonium dihydrogen phosphate	$NH_4H_2PO_4$	115.03	Fluka chemika
Ammonium molybdate	$(NH_4)_6Mo_7O_{24}$, $4H_2O$	1235.86	Labosi
Ammonium sulfate	$(NH_4)_2 SO_4$	132.14	Flukachemika
Aspartic acid	$C_4H_7O_4N$	133.11	BDH chemicals
Boric acid	H_3BO_3	61.83	Labosi
Calcium carbonate	$CaCO_3$	100.09	Flukachemika
Calcium chloride	$CaCl_2$	110.98	Lobachemie
Calcium hypochlorite	$CaCl_2O_2$	142.99	Flukachemika
Calcium nitrate	$Ca(NO_3)_2$ 4 H_2O	236.14	Panreac
Calcium sulfate	$CaSO_4$	136.14	Scharlau
Citric acid	$C_2H_8O_7$, H_2O	210.14	BDH chemicals
Copper sulfate	$CuSO_4$, 5 H_2O	249.68	Labosi
EDTA	$C_{10}H_{14}N_2Na_2O_8$, $2H_2O$	372.24	Panreac
Glutaric acid	$C_5H_8O_4$	132.12	Flukachemika
Magnesium chloride	$MgCl_2$	203.31	Lobachemie
Nicotinic acid	C_5H_4NCOOH	123.11	Flukachemika
Potassium acetate	$C_2H_3KO_2$	98.14	Riedel- de Haën
Potassium carbonate	K_2CO_3	138.21	Panreac
Potassium chloride	KCl	74.55	BDH chemicals
Potassium phosphate, dibasic	K_2HPO_4	174.18	Riedel- de Haën
Sodium acetate	$C_2H_3O_2Na$	82.03	BDH chemicals
Sodium bicarbonate	$NaHCO_3$	84.01	Riedel- de Haën
Sodium carbonate	Na_2CO_3	105.99	Flukachemika
Sodium chloride	NaCl	58.44	Labosi
Sodium metabisulfite	$Na_2S_2O_5$	190.1	Sigma- Aldrich
Sodium molybdate	Na_2MoO_4	241.95	Flukachemika
Sodium nitrite	$NaNO_2$	68.9953	Sigma- Aldrich
Sodium phosphate, dibasic	Na_2HPO_4	177.99	Riedel- de Haën
Sodium salicylate	$C_7H_5NaO_3$	160.11	Sigma- Aldrich
Sodium sulfate	Na_2SO_4	142.04	Flukachemika
Sodium sulfite	Na_2SO_3	126.04	Flukachemika
Sodium thiosulfate	$Na_2S_2O_3$, $5H_2O$	248.18	Scharlau
Trisodium citrate	$Na_3C_6H_5O_7$, $2H_2O$	294.1	Flukachemika
Zinc sulfate	$ZnSO_4$	161.47	SDFCL

at tow perpendicular colony diameter, for 7 days, until the growth in the control plate reached the edge of the plate. The antifungal activity was expressed in terms of percentage of Mycelial Growth Inhibition (MGI) and calculated with the following formula[24]:

$$MGI\ (\%) = \frac{\text{Average colony in control} - \text{Average colony diameter in treated plate}}{\text{Average colony diameter in control}} \times 100 \quad (1)$$

The Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC) were determined in parallel for the compounds that allowed more than 50% of mycelial growth inhibition when tested at 0.2 M[25].

Fungicidal or fungistatic effect of tested chemicals on *B. cinerea* was determined by transferring the plugs from treatment with no growth to PDA medium without chemicals. Treatments in which mycelial growth did not occur after additional one week of incubation were considered as compounds that suppressed or inhibited *B. cinerea* growth and therefore having fungicide effect.

Effect of chemicals on fungal sporulation: Fungal sporulation was assessed using PDA medium supplemented with chemicals as described above. Once fungal colony in control reached the edge of the plate, amended as well as control plates were flooded with 10 mL of distilled water containing 0.05% (w/v) Tween 80 and spores were gently harvested from the medium using bacteriological loop. The resulted suspension was filtered through a Buchner funnel and spores counts were determined using a Neubauer Heamacytometer[26]. The result was expressed as percent sporulation inhibition using the following formula[24]:

$$\text{Sporulation inhibition (\%)} = \frac{\text{Mean number of spores in control} - \text{Mean number of spores in treatment}}{\text{Mean number of spore in control}} \times 100 \quad (2)$$

Effect of chemicals on spore germination: The effect of tested chemicals on spore germination or conidia viability was determined only for compounds which showed a MGI value equal or lower than 50%. The conidia suspension was prepared from 10 days old culture of *B. cinerea* grown on PDA at 25°C, by flooding the culture with 10 mL of sterile distilled water containing 0.05% (w/v) Tween 80 as described above. Conidia were recovered by filtering the suspension through two layers of cheesecloth to remove hyphal fragments. The conidia concentration of the suspension was adjusted to 1×10^5 conidia mL^{-1} using a Neubauer Hemocytometer through dilution[26]. The germination of conidia of *B. cinerea* was determined in five different concentrations of 0.002, 0.005, 0.01, 0.02 and 0.04 M of the tested compounds. The aqueous solution of chemicals was prepared in Malt extract broth. Aliquots (40 µL) of conidia suspensions (1×10^5conidia mL^{-1}) were aseptically transferred in triplicate to sterile depression slides containing 40 µL of Malt extract broth supplemented with the different concentrations of retained chemicals. Inoculated depression slides were placed in Petri plates with a moist filter paper and incubated at 25°C for 16 h. Each slide was then fixed with fuchsin acid solution to stop further germination[14]. Conidia germination was determined in three microscopic fields using 10×40 ocular micrometers. At least 100 conidia within each replicate were observed. A conidium was scored as germinated when the length of the germ tube extended to at least twice the diameter of the conidium itself[27]. Germination was assessed microscopically at various concentrations of tested chemicals compared with control treatment. The results were expressed as percent conidia germination using the formula[28] below. Each treatment included three replicates and the test was conducted twice.

$$\text{Germination (\%)} = \frac{\text{Mean number of germinated conidia in treated slides}}{\text{Mean number of germinated conidia in control}} \times 100 \quad (3)$$

Effect of medium pH on mycelial growth of *Botrytis cinerea*: Since the medium initial pH could be affected by the incorporated chemicals, the effect of pH on the colony growth of *B. cinerea* was studied. The PDA medium was adjusted at 2, 4, 6 and 12 with 1 N HCl or NaOH. Hyphal plugs (5 mm diameter) cut form actively growing colony of *B. cinerea* were transferred aseptically to three replicate Petri dish plates containing PDA medium at different pH. Radial growth was determined daily by measuring colony size along two perpendicular axes. Percentage of colony growth which is the ratio of colony growth at the various pH medium was compared with that of the control.

***In vivo* effect of selected chemicals on gray mold development in artificially wounded and inoculated tomato fruits:** Based on the *in vitro* antifungal activity, only chemicals with a MIC value equal or lower than 200 mM were retained. Healthy and commercially mature fruits were washed with tap water, disinfected by immersion for 2 min in sodium hypochlorite solution (0.1%), rinsed in sterile distilled water then air dried prior to wounding. One wound at the equatorial side (5 mm wide, 3 mm deep) was performed per fruit.

The wounds were treated with 20 µL of aqueous solutions of retained chemicals at concentrations of 100, 200 and 300 mM. Controls were treated with the same volume of sterile distilled water under the same conditions. After 4 h incubation, in the laboratory at room temperature, each wound was inoculated with 10 µL of an aqueous conidia suspension of *B. cinerea* at the concentration of 1×10^6 conidia mL^{-1} [29]. Treated fruits were placed on plastic tray in cardboard boxes and incubated at 25°C and at high relative humidity of about 95% for 5 days. Gray mold incidence and lesion diameters of the overall treated tomato fruits were determined daily. All treatments were arranged in a complete randomized block design. Fifteen tomato fruits constituted a single replicate and each treatment was replicated three times and the experiment was conducted twice. The incidence and severity of gray mold disease were calculated as follows[25,30]:

$$\text{Disease incidence (\%)} = \frac{\text{Mean number of rotten fruits}}{\text{Mean number of total fruits}} \times 100 \quad (4)$$

$$\text{Disease severity (\%)} = \frac{\text{Average lesion diameter of treatment}}{\text{Average lesion diameter of control}} \times 100 \quad (5)$$

In all the experiments, the possible phytotoxic effect of tested compounds manifested as drying or browning around the treated wounds on tomato fruit was visually examined.

Statistical analysis: All data were subjected to statistical Analysis of Variance (ANOVA). One-way analysis of variance was performed for the *in vitro* studies. In *in vivo* experiments, two-way analysis of variance was performed using STATISTICA software, version 6, Stat-Soft, 2001, France. Percentage values of both *in vitro* and *in vivo* experiments

were subjected to arcsine-square root transformation before ANOVA, non-transformed means are shown. Tukey's HSD test was used as post-hoc test to separate treatments which were significantly different at $p<0.05$[13].

RESULTS

Effects of chemicals on mycelium growth and sporulation: The results showed that most of the selected food additives inhibited significantly ($p<0.05$) the mycelium growth and sporulation of *B. cinerea* (Table 2). However, copper sulfate, EDTA and sodium metabisulfite inhibited completely the growth and sporulation of the pathogen at the concentrations of 0.02 and 0.2 M. In addition, complete inhibition of mycelial growth was achieved by ammonium carbonate, potassium carbonate, sodium bicarbonate, sodium carbonate, sodium nitrite, sodium phosphate dibasic, sodium salicylate and zinc sulfate when tested at 0.2 M. At the same concentration, nicotinic acid, potassium phosphate dibasic and potassium chloride reduced the mycelial growth by more than 80%. Nicotinic acid and potassium phosphate dibasic were found to be more effective than potassium chloride since they completely inhibited the spore production. However, even if potassium chloride reduced the colony growth it has also a stimulating effect of spore production (-42.71%). Calcium carbonate and calcium hypochlorite did not show any

Table 2: *In vitro* effects of 37 test chemicals on mycelial growth and sporulation of *Botrytis cinerea*

	Inhibition (%)			
	Mycelial growth		Sporulation	
Chemicals	0.02 M	0.2 M	0.02 M	0.2 M
Ammonium acetate	$13.52\pm2.94^{i-l}$	$76.86\pm2.37^{a-c}$	$56.80\pm3.25^{g-k}$	84.47 ± 3.20^{ab}
Ammonium carbonate	$82.35\pm0^{a-c}$	100 ± 0 a	100 ± 0^{a}	ND
Ammonium chloride	0.00 ± 0^{l}	$1.37\pm1.35^{j-l}$	$76.58\pm6.16^{b-e}$	25.65 ± 3.72^{op}
Ammonium dihydrogen phosphate	0.00 ± 0^{l}	0.00 ± 0^{l}	$58.93\pm1.96^{f-j}$	$65.47\pm2.11^{c-h}$
Ammonium molybdate	$15.68\pm2.78^{h-l}$	$67.84\pm1.48^{b-d}$	85.54 ± 4.05^{ab}	100 ± 0^{a}
Ammonium sulfate	$2.15\pm3.24^{j-l}$	0.00 ± 0^{l}	31.98 ± 5.53^{no}	$40.56\pm0.81^{k-o}$
Aspartic acid	$3.33\pm2.22^{j-l}$	$4.31\pm6.00^{j-l}$	$63.13\pm2.54^{d-i}$	84.68 ± 4.51^{ab}
Boric acid	0.00 ± 0^{l}	$73.52\pm1.55^{b-d}$	$79.20\pm5.60^{b-d}$	100 ± 0^{a}
Calcium carbonate	0.00 ± 0^{l}	0.00 ± 0^{l}	100 ± 0^{a}	100 ± 0^{a}
Calcium chloride	0.58 ± 0.58^{kl}	$31.17\pm0.58^{f-i}$	$54.98\pm5.29^{h-l}$	87.91 ± 5.13^{ab}
Calcium hypochlorite	0.00 ± 0^{l}	$71.76\pm11.89^{b-d}$	100 ± 0^{a}	100 ± 0^{a}
Calcium nitrate	0.00 ± 0^{l}	$9.80\pm5.89^{i-l}$	$33.48\pm6.87^{m-o}$	$62.09\pm9.21^{e-i}$
Calcium sulfate	0.00 ± 0^{l}	$2.74\pm0.89^{j-l}$	$38.56\pm2.45^{l-o}$	$49.86\pm8.10^{h-m}$
Citric acid	$4.9\pm6.55^{j-l}$	$74.9\pm4.00^{a-c}$	$77.19\pm4.55^{b-e}$	100 ± 0^{a}
Copper sulfate	100 ± 0^{a}	100 ± 0^{a}	ND	ND
EDTA	100 ± 0^{a}	100 ± 0^{a}	ND	ND
Glutaric acid	0.00 ± 0^{l}	$41.37\pm3.9^{e-g}$	$53.18\pm4.93^{h-l}$	$75.03\pm2.96^{b-f}$
Magnesium chloride	0.00 ± 0^{l}	0.00 ± 0^{l}	$41.89\pm3.69^{k-o}$	$65.08\pm2.88^{c-i}$
Nicotinic acid	0.00 ± 0^{l}	$81.76\pm3.85^{a-c}$	$79.21\pm5.60^{b-d}$	100 ± 0^{a}
Potassium acetate	$7.84\pm1.22^{i-l}$	$71.76\pm2.56^{b-d}$	81.38 ± 1.82^{bc}	100 ± 0^{a}
Potassium carbonate	86.47 ± 5.12^{ab}	100 ± 0^{a}	$72.58\pm10.59^{b-g}$	ND
Potassium chloride	0.00 ± 0^{l}	$81.96\pm3.73^{j-l}$	-103.21 ± 7.96^{s}	-42.710 ± 6.54^{q}
Potassium phosphate dibasic	0.00 ± 0^{l}	$81.96\pm4.33^{a-c}$	$44.09\pm7.04^{j-n}$	100 ± 0^{a}
Sodium acetate	$26.47\pm13.5^{f-j}$	$71.76\pm6.14^{b-d}$	-34.94 ± 4.62^{q}	$48.54\pm3.83^{j-n}$
Sodium bicarbonate	$84.11\pm1.76^{a-c}$	100 ± 0^{a}	$53.05\pm1.93^{h-l}$	ND
Sodium carbonate	$81.49\pm0.88^{a-c}$	100 ± 0^{a}	$63.39\pm4.24^{d-i}$	ND
Sodium chloride	$16.66\pm1.69^{g-l}$	$13.13\pm1.48^{i-l}$	9.69 ± 4.04^{p}	83.05 ± 4.89^{b}
Sodium metabisulfite	100 ± 0^{a}	100 ± 0^{a}	ND	ND
Sodium molybdate	0.00 ± 0^{l}	$40.88\pm17.41^{e-h}$	$78.09\pm5.79^{b-e}$	100 ± 0^{a}
Sodium nitrite	$49.41\pm7.45^{d-f}$	100 ± 0^{a}	83.83 ± 4.52^{ab}	ND
Sodium phosphate dibasic	$25.88\pm8.62^{f-k}$	100 ± 0^{a}	-107.16 ± 18.12^{s}	ND
Sodium salicylate	86.47 ± 1.55^{ab}	100 ± 0^{a}	100 ± 0^{a}	ND
Sodium sulfate	$4.90\pm6.12^{j-l}$	0.98 ± 1.69^{kl}	-60.67 ± 3.38^{r}	76.98 ± 5.54^{be}
Sodium sulfite	0.00 ± 0^{l}	$76.86\pm3.54^{c-e}$	$76.35\pm4.81^{b-e}$	84.26 ± 2.86^{ab}
Sodium thiosulfate	0.00 ± 0^{l}	$10.39\pm1.48^{i-l}$	$34.77\pm2.56^{m-o}$	82.67 ± 1.43^{b}
Trisodium citrate	0.00 ± 0^{l}	$76.86\pm3.54^{a-c}$	84.09 ± 4.64^{ab}	84.58 ± 3.27^{ab}
Zinc sulfate	87.45 ± 1.48^{ab}	100 ± 0^{a}	$63.01\pm3.45^{d-i}$	ND

ND: Not determined due to complete inhibition of mycelial growth. Mean values followed by different letter(s) within column are significantly different at $p<0.05$ according to Tukey's test. Values are given as Mean±SD

inhibitory effect on mycelial growth of *B. cinerea*. However they completely inhibited the spore production at only 0.02 M (Table 2).

Results of MIC and MFC study are shown in Table 3. Among the 11 chemicals that completely inhibited the mycelial growth of *B. cinerea,* ammonium carbonate, EDTA, potassium carbonate, sodium carbonate, sodium metabisulfite, sodium nitrite and zinc sulfate were fungicidal rather than fungistatic. Indeed, mycelial plugs transferred from PDA supplemented with chemicals to free PDA failed to grow after additional one week of incubation at 25°C (Table 3).The lowest MIC values were recorded for sodium metabisulfite, EDTA and copper sulfate and were, respectively 10, 15 and 20 mM. However, among them, sodium metabisulfite and EDTA appear to be more effective since they showed a MFC value at only 15 mM, while the MFC value of copper sulfate is greater than 200 mM (Table 3).

Sodium carbonate, potassium carbonate, sodium nitrite and zinc sulfate were also effective against *B. cinerea*, with MIC values that ranged between 35 and 75 mM and MFC values equal or less than 200 mM (Table 3).

Effect of chemicals on conidia germination: Among the 37 tested compounds on *B. cinerea* mycelial growth, only 22 compounds that inhibited the growth with more than 50% were tested on germination of conidia (Table 4). There were significant differences ($p<0.05$) between the control and the treatments at the various studied concentrations. Ammonium molybdate and sodium metabisulfite showed the strongest inhibition effect on spore germination where they completely inhibited the germination at the concentrations of 10, 20 and 40 mM. In addition, five other compounds such as calcium hypochlorite, copper sulfate, potassium carbonate, sodium carbonate and sodium sulfite also inhibited completely spore germination but only at the concentration of 40 mM (Table 4). The results also showed that at 40 mM, the percentage of *B. cinerea* spore germination inhibition in media amended with citric acid, sodium bicarbonate, sodium nitrite and zinc sulfate was higher than 89%, with only 2.3% germination obtained in zinc sulfate. Moreover, citric acid and sodium bicarbonate exhibited an important potential to reduce the germination even at the lower concentration (2 mM), with inhibition values of 74.08 and 69.67%, respectively (Table 4).

The EDTA and sodium salicylate have shown high inhibitory effect on mycelial growth of *B. cinerea* but they did not show any inhibitory effect on spore germination. Remaining compounds reduced slightly the spore germination of *B. cinerea* when tested at 40 mM (Table 4).

Effect of medium initial pH on mycelial growth of *Botrytis cinerea*: The effect of medium initial pH on mycelial growth of *B. cinerea* was studied at various pH. Obtained results showed that the optimum growth of *B. cinerea* is observed at medium pH ranged between 6 and 10. Under acidic pH (bellow 6) and very basic

Table 3: Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) values of selected chemicals and their toxicity at 0.2 M

Chemicals	MIC (mM)	MFC (mM)	Toxicity at 0.2 M
Ammonium acetate	>200	>200	ND
Ammonium carbonate	100	200	Fungicidal
Ammonium molybdate	>200	>200	ND
Boric acid	>200	>200	ND
Calcium hypochlorite	>200	>200	ND
Citric acid	>200	>200	ND
Copper sulfate	20	>200	Fungistatic
EDTA	15	15	Fungicidal
Nicotinic acid	>200	>200	ND
Potassium acetate	>200	>200	ND
Potassium carbonate	50	100	Fungicidal
Potassium phosphate dibasic	>200	>200	ND
Sodium carbonate	35	100	Fungicidal
Sodium acetate	>200	>200	ND
Sodium bicarbonate	200	>200	Fungistatic
Sodium metabisulfite	10	15	Fungicidal
Sodium nitrite	75	150	Fungicidal
Sodium phosphate dibasic	150	>200	Fungistatic
Sodium salicylate	75	>200	Fungistatic
Sodium sulfite	>200	>200	ND
Trisodium citrate	>200	>200	ND
Zinc sulfate	75	200	Fungicidal

ND: Not determined, the complete inhibition of mycelial growth is reached above 200 mM

Table 4: *In vitro* effect of 22 compounds on conidia germination of *Botrytis cinerea*

	Spore germination (%)				
	Concentration (mM)				
Chemicals	2	5	10	20	40
Ammonium acetate	93.64±0.45^{cd}	88.67±0.28^{d}	80.33±0.57^{e}	78.33±0.39^{f}	60.15±0.87^{c}
Ammonium carbonate	94.78± 0.84^{c-e}	88.05±1.02^{d}	82.36±1.3^{e}	79.67±0.92^{f}	71.71±0.72^{d}
Ammonium molybdate	98.06± 0.97^{e-g}	97.45±0.9^{gh}	0.00±0.0^{a}	0.00±0.0^{a}	0.00±0.0^{a}
Boric acid	100.00±0.0^{g}	98.71±0.84^{h}	97.33±0.52^{gh}	96.90±0.95^{h}	80.45±0.65^{ef}
Calcium hypochlorite	97.62±0.84^{d-g}	96.87±1.57^{gh}	93.45±1.24^{f-h}	0.0±0.0^{a}	0.00±0.0^{a}
Citric acid	25.92± 0.36^{a}	23.12±0.85^{b}	20.87±0.96^{d}	11.49±0.89^{b}	8.71±1.14^{b}
Copper sulfate	98.33±0.67^{e-g}	95.71±0.46^{gh}	14.53±0.54^{c}	0.00±0.0^{a}	0.00±0.0^{a}
EDTA	97.87± 0.18^{e-g}	96.05±0.73^{gh}	96.83±0.99^{gh}	95.34±0.89^{h}	94.79±0.82^{hi}
Nicotinic acid	97.50±0.51^{d-g}	95.33±0.35^{gh}	81.67±1.08^{e}	71.04±0.98^{e}	60.45±0.62^{c}
Potassium acetate	97.79±1.01^{fg}	93.62±0.34^{fg}	90.67±1.57^{fg}	87.33±0.54^{g}	80.15±0.5^{e}
Potassium carbonate	90.71±0.84^{c}	15.67±1.32^{a}	5.05±0.54^{b}	3.67±1.12^{ab}	0.00±0.0^{a}
Potassium phosphate, dibasic	98.05±0.59^{e-g}	97.87±0.95^{gh}	96.94±0.68^{h}	96.05±0.23^{h}	94.38±0.97^{i}
Sodium acetate	95.87±0.90^{c-f}	92.34±1.17^{e-g}	89.87±1.74f	88.67±0.83^{g}	88.96±2.13^{fg}
Sodium bicarbonate	30.33±1.08^{b}	26.67±1.79^{b}	20.67±0.79^{d}	17.16±0.70^{c}	10.83±1.26^{b}
Sodium carbonate	92.71±2.01^{c-e}	90.11±1.4^{de}	88.45±2.51^{f}	1.34±0.9^{ab}	0.00±0.0^{a}
Sodium metabisulfite	92.76±2.08^{c}	75.67±1.71^{c}	0.35±0.61^{a}	0.00±0.0^{a}	0.00±0.0^{a}
Sodium nitrite	98.87±1.42^{d-g}	95.34±1.08^{gh}	93.28±1.12^{f-h}	91.71±0.93^{h}	10.85±0.92^{b}
Sodium phosphate, dibasic	96.04±1.49^{c-g}	91.67 ±0.83^{d-f}	82.36±.57^{e}	78.85±1.57^{f}	70.16±2.55^{d}
Sodium salicylate	98.79±0.36^{f-g}	97.45±1.78^{gh}	95.04±1.52^{gh}	93.67±0.86^{h}	90.15±1.78^{gh}
Sodium sulfite	97.96±0.88^{fg}	96.04±1.08^{gh}	11.67±1.41^{c}	10.85±.62^{ab}	0.00±0.0^{a}
Trisodium citrate	97.05±1.24^{d-g}	95.67±1.34^{g}	89.71±2.13^{fg}	70.34±1.77^{e}	69.94±2.36^{d}
Zinc sulfate	98.67±0.44^{f-g}	96.16±1.12^{gh}	95.80±0.95^{gh}	26.03±1.16^{d}	2.30±0.85^{ab}
Control	100.00±0.0^{g}	100.00±0.0^{h}	100.00±0.0^{h}	100.00±0.0^{h}	100.00±0.0^{h}

Means followed by different letter(s) within each column are significantly different at p<0.05 according to Tukey's test. Values are given as Mean±SD

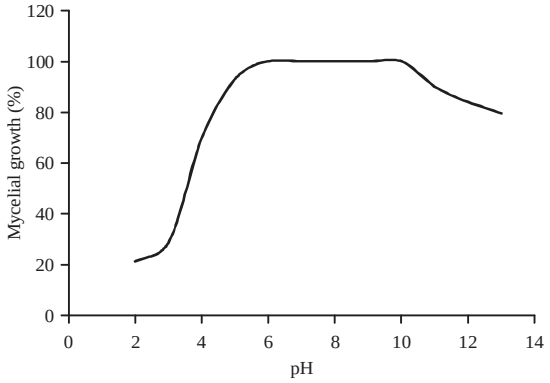

Fig. 1: Effect of medium pH on *in vitro Botrytis cinerea* mycelial growth

pH (above 10), mycelial growth of *B. cinerea* was significantly reduced (Fig. 1).

***In vivo* effect of chemicals on gray mold development in artificially inoculated and wounded tomato fruit:** The data presented in Fig. 2 show that the selected chemicals reduced significantly the incidence of gray mold on tomato fruit in a dose dependent manner (p<0.05) under laboratory conditions. After 5 days of incubation, the selected compounds reduced the incidence of the disease to less than 30% when tested at 300 mM (Fig. 2), with the exception of copper sulfate and zinc sulfate that showed 91.67 and 69.45% of disease incidence, respectively. The lowest values of 5.56, 5.56 and 8.34% were recorded with sodium bicarbonate, sodium salicylate and EDTA, respectively (Fig. 2).

Regarding the severity of the disease which is the ratio of lesion diameter at various chemical concentrations compared with that of the control, results indicated that EDTA, potassium carbonate, sodium bicarbonate, sodium carbonate, sodium metabisulfite and sodium salicylate reduced significantly (p<0.05) the severity to less than 30% at 300 mM. Even at the lowest concentration of 100 mM (p<0.05), EDTA, potassium carbonate and sodium carbonate reduced the severity by >50% (Fig. 3).

Despite its effect in reducing disease incidence, sodium salicylate treatment leads to a yellowish halo around the wound. However in the copper sulfate treatment, a drying of the rind around the wound was observed.

DISCUSSION

The results obtained from this study demonstrated that several compounds generally recognized as safe inhibit,

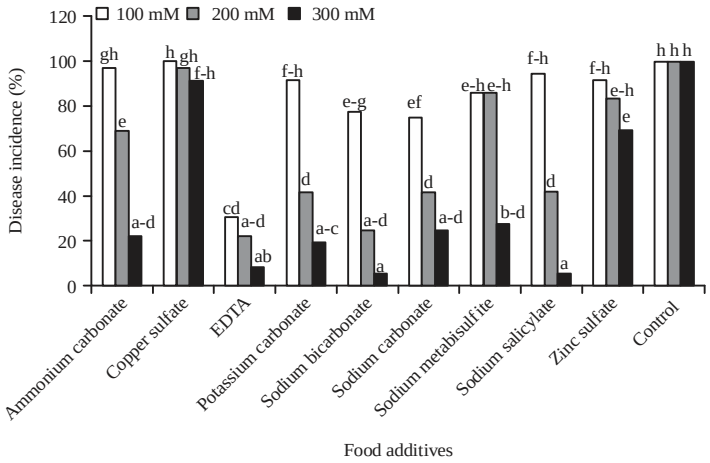

Fig. 2: Gray mold incidence on tomato fruit treated with various food additives at different concentrations and stored at 25°C and 95% relative humidity for 5 days

Significant differences (p<0.05) between means are indicated by different letters above histogram bars

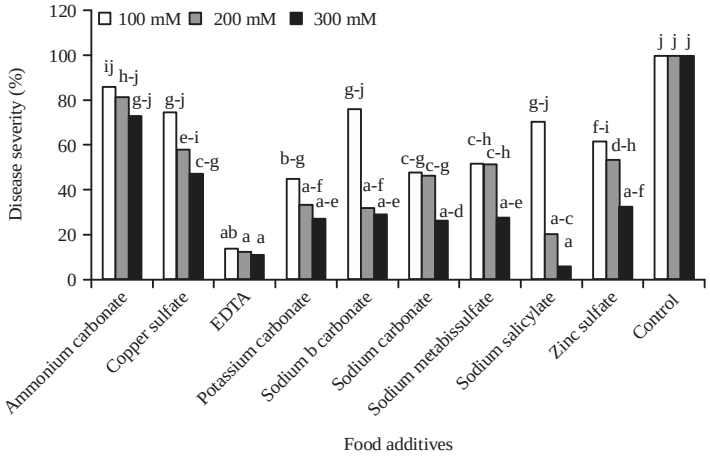

Fig. 3: Gray mold severity on tomato fruit treated with the various food additives at different concentrations and stored at 25°C and 95% relative humidity for 5 days

Significant differences (p<0.05) between means are indicated by different letters above histogram bars

affected significantly the growth of *B. cinerea* under both *in vitro* and *in vivo* conditions. Sodium metabisulfite, EDTA, copper sulfate, zinc sulfate, sodium salicylate, potassium carbonate, sodium bicarbonate, ammonium carbonate and sodium carbonate have completely inhibited the mycelial growth of *B. cinerea*. These results are consistent with those of Palmer *et al.*[16], who reported that, ammonium carbonate, sodium metabisulfite, potassium carbonate, sodium bicarbonate and sodium carbonate inhibited or reduced the mycelial growth of *B. cinerea*. Other studies reported that sodium metabisulfite, copper sulfate, sodium bicarbonate, potassium carbonate, sodium carbonate and EDTA were the most effective food additives that control the mycelial growth of *B.cinerea*[8,13,17,18].

The inhibitory effect of sodium metabisulfite, EDTA, ammonium carbonate, sodium carbonate and potassium carbonate have also been reported on the growth of several fungal species such as *Geotrichum candidum*[30] and *Penicillium italicum*[24] the major postharvest fungal pathogens on citrus fruit. In addition, potassium carbonate, ammonium carbonate and sodium bicarbonate have also been reported to have an inhibitory effect on *Penicillium digitatum* causal agent of citrus green mold[31]. In a previous study, Hervieux *et al.*[20] showed that potassium carbonate, sodium metabisulfite, sodium bicarbonate and sodium carbonate have completely inhibited the mycelial growth of *Helminthosporium solani* on potato. Current results showed that most of the tested compounds that inhibited the mycelial

growth of *B. cinerea* also inhibited the spore production, which is in agreement with the results of Hervieux *et al.*[20] and Mills *et al.*[13], who reported that the inhibition of mycelial growth is related to the inhibition of the fungal sporulation.

However, some compounds such as calcium carbonate, calcium hypochlorite and sodium chloride did not show any remarkable effect on mycelial growth but have resulted in a complete inhibition of *B. cinerea* sporulation.

In the MIC and MFC studies, the results showed that *B. cinerea* has differential sensitivity to various tested chemicals, as demonstrated by the variations in its MIC and MFC values. The lowest MIC value was recorded for sodium metabisulfite (10 mM) followed by EDTA (15 mM), sodium carbonate (35 mM) and potassium carbonate (50 mM), which is in agreement with the results of Latifa *et al.*[24] and Talibi *et al.*[30], who also reported that sodium metabisulfite and EDTA registered the lowest MIC against *P. italicum* and *G. candidum.* In another study, Olivier *et al.*[32] reported that sodium carbonate and potassium carbonate were more effective against *H. solani* on potato. Olivier *et al.*[32] also indicated that sodium carbonate was more toxic than the other carbonic salts. Sodium carbonate and sodium bicarbonate are common food additives permitted with no restriction for many applications in North American regulations including organic agriculture[14]. When comparing their MIC and MFC values, presented data showed that *B. cinerea* was more sensitive to sodium carbonate treatment than sodium bicarbonate. Moreover, sodium bicarbonate exhibited a fungistatic activity rather than fungicidal.

Among the 22 chemicals tested on spore germination, ammonium molybdate, calcium hypochlorite, copper sulfate and sodium metabisulfite were found to completely inhibit the spore germination at 20 and 40 mM. Similar results were also reported by Talibi *et al.*[30], who indicated that sodium metabisulfite and ammonium molybdate completely inhibited spore germination of *G. candidum*. Moreover, in accordance with the results of Mills *et al.*[13], copper sulfate and sodium metabisulfite inhibited the spore germination of a wide range of postharvest plant pathogens. In contrast, our results showed that, although sodium salicylate and EDTA have an effect on mycelial growth, they did not affect the spore germination of *B. cinerea.*

Considering the fact that the use of these chemicals could modify the pH of the growing medium, the effect of pH modifications on fungal growth was assessed. The results showed that *B. cinerea* grew at both acidic and alkaline pH, which is similar to the behavior of *G. candidium*[30] and *P. italicum*[33] under the same conditions. In another study, Byrde and Willetts[34] reported that *Monilinia* sp. grew at varied pH (from 1.5 to 9.0) with optimum growth occurred under acidic pH. Similarly, for *B. cinerea*, the optimum growth was obtained in the range of 6.0 to 10.0, indicating that colony growth was not significantly affected by pH modifications. This ability of pathogens to grow over a wide range of pH shows that differences in mycelial growth in chemical amended media cannot be only due to pH modifications, which is in agreement with the results of Hervieux *et al.*[20], who demonstrated that the pH have no direct effect on the inhibition of fungal growth.

The adaptation of fungal species to a wide range of pH could be explained by several mechanisms known as "pH homeostasis"[35] that are present in the cell membranes of pathogens. These mechanisms maintain the stability of macromolecules such as enzymes and therefore the growth and metabolism of these microorganisms[36] through the regulation of ions transport across membranes even when the extra cellular pH varies significantly; and this by means of the selectivity and energy coupling with the translocation of solutes[37].

Although *in vitro* tests are important steps in selecting chemicals with anti-botrytis activity, *in vivo* tests are also needed to check whether the positive results obtained in the *in vitro* tests could be confirmed and validated. Current results showed that EDTA, sodium bicarbonate and sodium salicylate were the most effective compounds in reducing gray mold incidence on tomato fruit, which are in lines with the results obtained by Droby *et al.*[17], who reported that EDTA and sodium bicarbonate reduced significantly the incidence of gray mold on apple. The EDTA and sodium bicarbonate reduced the gray mold incidence on tomato fruit to 8.34 and 5.56%, respectively at a concentration of 300 mM. Sodium bicarbonate has been reported to be very effective in reducing gray mold incidence caused by *B. cinerea* on grapes and cherries[8,18,38], green mold incidence on citrus fruit[14] and silver scurf incidence on potato tuber[20]. Additionally, EDTA has also been reported to reduce the incidence and the severity of sour rot caused by *G. candidum*[30] and green mold caused by *P. digitatum* on citrus fruit[39].

Furthermore, potassium carbonate and sodium carbonate reduced significantly the incidence of gray mold compared with the control at a concentration of 300 mM. Both compounds were also found to reduce the incidence of the decay caused by *Monilinia fructicola*, *B. cinerea*, *G. candidum* and *P. expansum* in many stone fruit[40].

Ammonium carbonate reduced the incidence of tomato gray mold by more than 77% which is consistent with the results of Talibi *et al.*[30], who reported that ammonium carbonate reduced the incidence of sour rot by more than

51%. Obtained results may be explained by the high pH value of host- tissues due to ammonium carbonate solution. Indeed, high pH value is detrimental to fungi[41]; Palmer *et al.*[16] reported that ammonium salts are effective under alkaline rather than acidic conditions, where the production of ammonia gas NH_3 is favored over the form NH_4^+ which is ineffective.

CONCLUSION

In conclusion, it was observed that among the 37 common food additives tested in this study, EDTA, sodium bicarbonate, sodium salicylate and sodium metabisulfite were found to have significant anti botrytis activity in both *in vitro* and *in vivo* conditions. These chemicals could therefore be considered as potential alternative candidates for the control of gray mold disease of tomato fruit, which could significantly reduce the abusive use of synthetic fungicides. However, further studies with selected antifungal compounds are warranted so as to characterize their mode of action and also to define the impact of their application on tomato fruit quality and storability before proceeding to a commercial formulation.

SIGNIFICANCE STATEMENT

Fungicide resistance is becoming a serious problem. This study identified candidates of salts compounds for developing alternative methods to control postharvest gray mold of tomato fruit.

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