International Journal of Agriculture and Crop Sciences.
Available online at www.ijagcs.com
IJACS/2014/7-14/1352-1358
ISSN 2227-670X ©2014 IJACS Journal

Antifungal Evaluation of Some Inorganic Salts Against Three Phytopathogenic Fungi

Masoud Zaker*

Department of plant protection, Shahrood Agricultural Research Center,P. O. Box No: 36155 – 313, Shahrood, Iran.

Corresponding author email: Masoudzaker35@gmail.com

ABSTRACT: Four inorganic salts namely, ammonium bicarbonate (AB), potassium bicarbonate (PB), sodium bicarbonate (SB) and Calcium chloride (CC) were screened for their antifungal activity against spore germination as well as mycelial growth of three plant pathogenic fungi namely Fusarium oxysporum, Alternaria alternata and Botrytis cinerea causal agents of potato dry rot, tomato leaf spot and grape gray mold respectively under labaratory condition. Poisoned food technique and spore germination assay were used for conducting these experiments. Among tested salts, potassium bicarbonate performed highest antifungal activity against all three phytopathogenic fungi followed by sodium bicarbonate (p=0.01). Significant differences were also observed between ammonium bicarbonate (AB), calcium chloride (CC), potassium bicarbonate (PB) and sodium bicarbonate (SB) in inhibiting spore germination as well as mycelial growth of three tested fungi when their concentrations (100 & 200 mM) were considered (p=0.01). The best inhibitory effect in both experiments were achieved by PB (200 mM) followed by SB (200 mM), PB (100 mM) and SB (100 mM) while the least inhibitory effect on all tested fungi was observed by CC (100 mM).
***Key words**:* Antifungal activity, Bicarbonates, Fusarium oxysporum, Alternaria alternata, Botrytis cinerea

INTRODUCTION

Some organic and inorganic salts used in the food industry as preservatives and antimicrobial agents have been found suitable alternatives for synthetic fungicide in plant disease control (Russell and Gould, 1991). These compounds have shown broad spectrum antimicrobial activity with low mammalian toxicity (Olivier et al., 1999), possess biocompatibility (Horst et al., 1992), and are generally recognized as safe (GRAS). Moreover, they are less sensitive to environmental conditions than other alternatives such as biological control agents, which may make them useful for the efficient control of plant pathogens. Several documents are available proving this fact (Reuveni et al., 1994; Smilanick et al., 1999; Ehret et al., 2002; Hervieux et al., 2002; Bombelli and Wright, 2006; Jamar et al., 2007; Mecteau et al., 2008; El-Mougy and Abd-el-Kader, 2009; Mitre et al., 2010; Wenneker and Kanne, 2010). Kuepper et al. (2001) gathered several research works on benefits of sodium bicarbonate as a safe fungicide for management of several plant diseases. Palmer et al. (1997) found that ammonium, potassium and sodium bicarbonates could inhibit the colony growth of Botrytis cinerea at concentrations as low as 20 mM.. Abd-el-Karim (2007) tested potassium or sodium bicarbonate alone or in combination with Nerol against early blight of potato caused by Alternaria solani. Under laboratory condition, complete mycelial inhibition was obtained by potassium or sodium bicarbonate at 2% and Nerol at 0.50%. Under greenhouse condition, potassium bicarbonate, sodium bicarbonate and Nerol at 1.0, 2.0 and 0.50% respectively reduced the early blight incidence by more than 70.6% but under field condition, potassium bicarbonate at 2.0% plus Nerol at 0.5% was most effective treatment and reduced the disease incidence by more than 86.8 % during two successive seasons. Karabulut et al. (2003) found that baking soda (sodium bicarbonate) significantly reduced the total number of decayed grape berries caused by Botrytis cinerea, Alternaria spp., or Aspergillus niger after storage for 30 days at 1°C followed by 2 days at 20°C. In their study, potassium bicarbonate also substantially inhibited the growth of the three fungal strains. Ziv and Zitter (1992) showed that spraying of sodium, potassium and ammonium bicarbonates all provided good control of Alternaria leaf blight (Alternaria cucumerina) in greenhouse grown muskmelon. Trukkan (2013) evaluated the efficacy of 26 different salts for the control of Fusarium oxysporum f.sp. cepae, the causal agent of onion basal rot and reported that 15 salts including ammonium bicarbonate, completely inhibited its mycelial growth at 2% concentration. Baking soda ($NaHCO_3$) or $KHCO_3$ were found capable of significantly reducing the mycelial growth of *Fusarium* species in Czapek Dox broth as low as 0.2 g/100 mL. In this connection, the mycelial growth of *Fusarium oxysporum* was inhibited by greater than 95% and the bicarbonate component of baking soda was responsible for the inhibitory effect (Hang and Woodams, 2003). Arslan et al. (2009) tested the efficacy of eight food additives as possible alternatives to synthetic fungicides for the control of soilborne pathogens (Fusarium oxysporum f. sp. melonis, Macrophomina phaseolina, Rhizoctonia solani, and Sclerotinia sclerotiorum) and found that the ED_{50}, minimum inhibition concentration (MIC), and minimum fungicidal concentration (MFC) values of ammonium bicarbonate and potassium sorbate were more toxic to soilborne pathogens compared to other food additives. Ammonium bicarbonate controlled all fungi at 2% and both ammonium bicarbonate and potassium sorbate increased the pH of soil. The rate of pH increase was higher in ammonium bicarbonate.

Post-harvest application of several bicarbonates has also been reported to control different diseases of stored fruits and vegetables. For instance, Mlikota and Smilanick (2001) found that when single grape berries were inoculated with Botrytis cinerea and sprayed with water, or 500 mM potassium bicarbonate, sodium bicarbonate and ammonium bicarbonate and incubated for 7 days at 14^{O}C., the incidence of gray mold were 24.2, 8.4, 6.4, and 4.2% respectively and the addition of 200 µg/ml chlorine to the bicarbonate salts significantly decreased gray mold incidence. Nigro et al. (2006) also screened 19 inorganic and organic salts for controlling table grape storage rots under *in vitro* and *in vivo* tests and reported that several salts reduced the growth of *Botrytis cinerea* on amended (0.1–2%, w/v) glucose agar, however, only calcium chloride, potassium carbonate, sodium bicarbonate and sodium carbonate significantly reduced the incidence of grey mould on small table grape bunches (cv. Italia). PC, SB and SC showed a similar effect in the *in vitro* (inhibition of mycelial growth and conidia germination of *B. cinerea*) and *in vivo* tests (reduction of incidence of grey mould on table grape), whereas CC was effective only *in vivo*. Fagundes et al. (2013) found that some food additives or 'generally recognized as safe' (GRAS) compounds reduced mycelial growth of Botrytis cinerea and Alternaria alternata in PDA Petri dishes amended with food preservatives at 0.2, 1.0, or 2.0% (v/v) after 3, 5, and 7 days of incubation at 25 °C. In overall, the best results for reduction of gray mold on cherry tomato fruit were obtained with coatings containing 2.0% of potassium carbonate, ammonium phosphate, potassium bicarbonate, or ammonium carbonate, while 2.0% sodium methylparaben, sodium ethylparaben, and sodium propylparaben were the best ingredients for coatings against tomato black rot (A. alternata). The purpose of this study was to find out the best in vitro antifungal activity of tested salts against three plant pathogenic fungi for their further evaluations under green house and field conditions.

MATERIALS AND METHODS

Chemicals

The inorganic salts used in this study were purchased from Merck Chemicals (Darmstadt, Germany) (Table 1).

Table 1 . Salts used

Compound	Chemical formule	Molecular weight (g mol $^{-1}$)	Company
Ammonium bicarbonate (AB)	$(NH_4)HCO_3$	79.07	Merck (Germany)
Potassium bicarbonate (PB)	$KHCO_3$	100.12	Merck (Germany)
Sodium bicarbonate (SB)	$NaHCO_3$	84.01	Merck (Germany)
Calcium chloride (CC)	$CaCl_2 \cdot {}_2H_2O$	110.98	Merck (Germany)

Fungal isolates

Phytopathogenic cultures of *Fusarium oxysporum* from potato tubers, *Alternaria alternata* from tomato leaves and *Botrytis cinerea* from grape were isolated and purified following single spore method. The agar slants of purified fungi were stored at 4°C and served as stock cultures.

Effect of salts on spore germination

The spore germination assay (Plascencia-Jatomea et al., 2003) was used for evaluating the effect of salt solutions on spore germination of selected fungi. Conidia of selected fungi (6×10^5) were suspended using an agitator (150 rpm for 10 minutes at 24°C) in 50 mL conical flasks containing 20 mL of each salt solution (100& 200 mM) or double distilled water (control). Samples (600 µL) were withdrawn at intervals (6, 9, 12 and 24 h) from the flasks and placed into microtubes. Conidia were recovered by centrifugation (Mecteau et al., 2002) and incubated in 500 µL of malt extract broth (MEB) at 24°C with agitation to favour germination. After 24 h of incubation, a sample of the conidial suspensions were examined under light microscope using a hemacytometer in order to rate the germination of conidia. The level of mortality was based on 100 conidia with four replicates and expressed as a percentage: {[germinated conidia (control) – germinated conidia (salt solution)] / germinated conidia (control)}×100.

Effect of salts on mycelial growth

For evaluating antifungal property of selected salts in controlling mycelial growth of selected fungi the food poisoned technique described by Schmitz (1930) was used. Pure isolate of selected fungi were grown in Petri dishes on PDA unamended (control) or amended with test salts (100 & 200 mM) at 24°C. PDA agar disks (6 mm diam of actively growing mycelia of selected phytopathogenic fungi were used to inoculate the plates. For each plate, diam of the colony was determined after 10 days of incubation period. Colony diam was measured as the average of the longest diam and the shortest diam with four replicates. Inhibition of mycelial growth was calculated as follows: [(control radial growth – salt amended radial growth) / control radial growth] x 100.

Statistical analysis

The factorial design, based on completely randomized design was used for analysing the data of this study. Results were analyzed in MSTAT-C and means were compared by Duncan's multi range test.

RESULTS AND DISCUSSION

Antifungal activity of different salts against spore germination and mycelial growth of Fusarium oxysporum, Alternaria alternata and Botrytis cinerea

Among four inorganic salts, potassium bicarbonate performed highest antifungal activity against F. oxysporum, A. alternata and B. cinerea followed by sodium bicarbonate. Ammonium bicarbonate also provided acceptable antifungal property but calcium chloride showed lowest effect against these pathogens in both the experiments. There was a significant difference (p=0.01) between salts irrespect of their concentrations in inhibiting the mean spore germination and mycelial growth of all three selected fungi (Fig 1, 3 & table 2). Results revealed significant difference at 1% probability level in respect of spore germination inhibition by salts {For F. oxysporum (46.87%) and For B. cinerea (44.85%)} compared to control while no significant difference was observed in respect of their mycelial growth inhibition (MGI). On the other hand, as it is shown in Figures 2 and 4, significant differences were also observed between AB, CC, PB and SB against mean spore germination as well as mycelial growth of three tested fungi when teir concentrations (100 & 200 mm) were considered (p=0.01). The best inhibitory effect in both experiments were achieved by PB (200 mM) followed by SB (200 mM), PB (100 mM) and SB (100 mM) while the least inhibitory effect on all tested fungi was observed by CC (100 mM) (Fig 2 & 4 and Table 2). According to Duncan's multiple range test (Tables 2, 3 and 4) , potassium bicarbonate at 200 mM followed by sodium bicarbonate at 200 mM demonstrated best activity for inhibiting the spore germination as well as mycelial growth of tested pathogens (p=0.05)

Antifungal activity of many organic and inorganic salts specially those of bicarbonates have been reported by several researchers worldwide in controlling plant pathogenic fungi. The fungicidal effect of bicarbonates might be due to the bicarbonate compound of salts (Hang and Woodams, 2003). Results of present study appproved the in vitro effectiveness of PB and SB in reducing growth and spore germination of F. oxysporum, A. alternata and B. Cinerea, the causal agents of potato dry rot, tomato leaf spot and grape gray mold respectively. Several reports are in favour of present findings, for instance Abd-El-Kareem (2007) reported that under lab. Condition, complete inhibition of Alternaria solani was obtained with potassium or sodium bicarbonate at 2% and under greenhouse condition, potassium and sodium bicarbonate at 1.0 and 2.0 % reduced the early blight incidence by more than 70.6 %. Bombelli and Wright (2006) also found that an in vitro treatment of tomato fruits with 1% $KHCO_3$ controlled B. cinerea by a fungistatic action. In another study Karabulut et al. (2003) examined the yeast Metschnikowia fructicola, ethanol, and sodium bicarbonate, alone or in combinations to table grapes on vines 24 h before harvest to control the incidence of postharvest diseases and in four experiments, all significantly reduced the total number of decayed berries caused by Botrytis cinerea, Alternaria spp., or Aspergillus niger after storage for 30 days at 1°C followed by 2 days at 20°C. Palmer et al. (1997) found that ammonium, potassium, and sodium bicarbonates could inhibit the colony growth of B. cinerea at concentrations as low as 20 mM. According to another study, baking soda ($NaHCO_3$) or $KHCO_3$ were found capable of significantly reducing the mycelial growth of *Fusarium* oxysporum by greater than 95% in Czapek Dox broth at as low as 0.2 g/100 mL. (Hang and Woodams, 2003). Nigro et al (2006) reported that several salts could reduce the growth of *Botrytis cinerea* on amended (0.1–2%, w/v) glucose agar, but under green house condition only calcium chloride, sodium bicarbonate, potassium carbonate and sodium carbonate significantly reduced the incidence of gray mold on small table grape bunches (cv. Italia). Their result related to effectiveness of sodium bicarbonate agaist this pathogen is in agreement with our results while their findings in regard of calcium chloride effectiveness against this fungus is not in favour of our results. In contrary to our findings, Mecteau et al. (2008) reported that calcium chloride and sodium bicarbonate at 0.2 M inhibited the mycelial growth of F. solani var. coeruleum only by 42.4 and 12.5% while other salts such as potassium sorbate, aluminium acetate, aluminium chloride and sodium benzoate controlled its growth by 100% at the same concentrations. Fagundes et al. (2013) also reported that among several salts the best results for reduction of gray mold on cherry tomato fruit (B. cinerea) were obtained with coatings containing 2.0% of potassium carbonate, potassium bicarbonate, or ammonium carbonate, while these compounds were not effective against black rot of cherry tomato caused by Alternaria alternata.

However there are some reports in contrary to our results but more documents are available in favour of proving the antifungal efficacy of potassium and sodium bicarbonates.

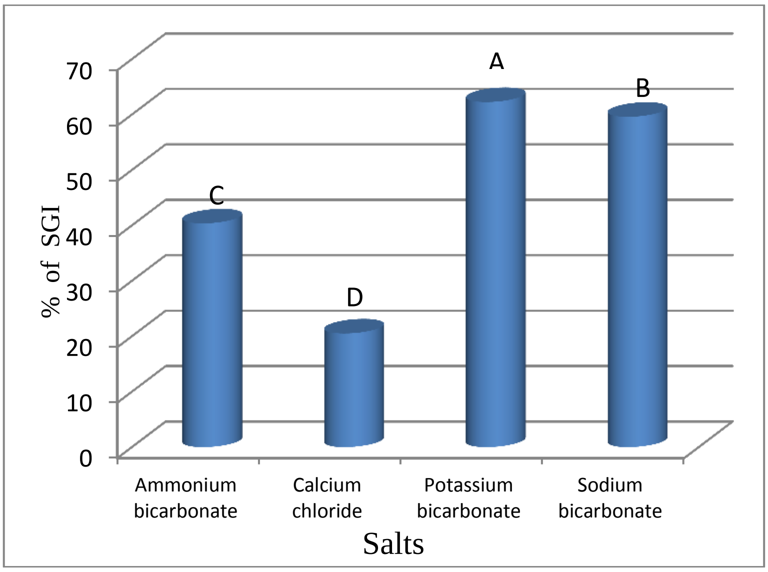

Figure 1. Effect of different salts on spore germination of selected fungi

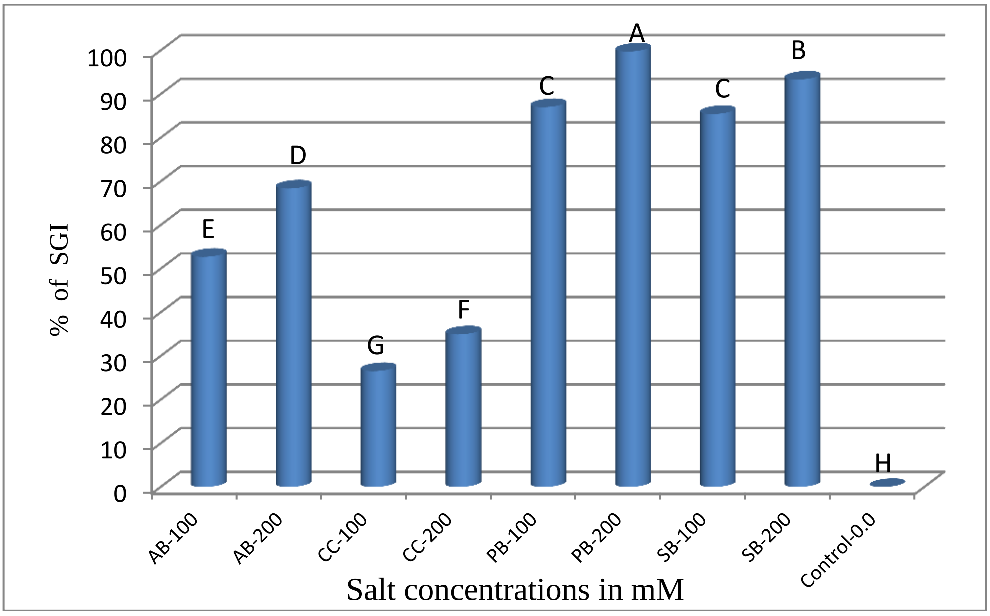

Figure 2. Effect of different treatments on mean spore germination of selected fungi

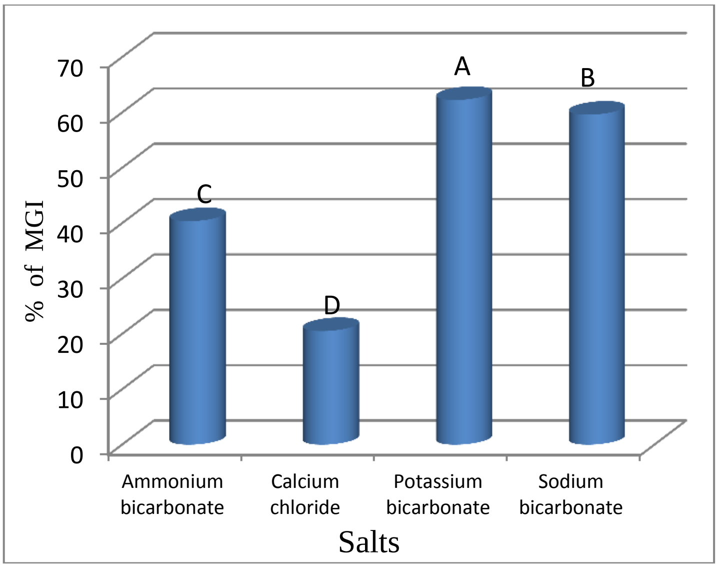

Figure 3. Effect of different salts on mycelial growth of selected fungi

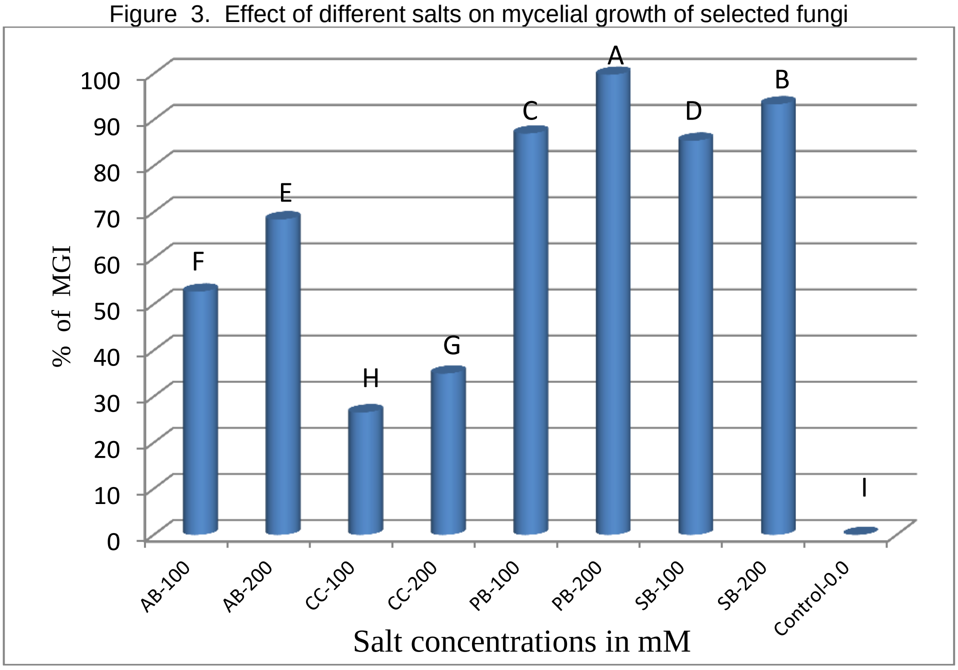

Figure 4. Effect of different treatments on mean mycelial growth of selected fungi

Table 2 . Analysis of variance table for effect of four inorganic salts against three plant pathogenic fungi

K value	Source	DF	Mean Square % of SGI	Mean Square % of MGI
1	Replication	3	20.511	12.617
2	Factor A	2	51.357**	10.272
4	Factor B	3	13566.923**	13252.366**
6	AB	6	5.908	8.040
8	Factor C	2	76640.750**	63383.043**
10	AC	4	18.905	2.993
12	BC	6	3432.648**	3368.594**
14	ABC	12	2.713*	2.322*
-15	Error	105	9.743	6.268
	Total	143		
Coefficient of Variation			6.83%	6.02%

** = Significant at 1% level of significance
* = Significant at 5% level of significance
SGI = spore germination inhibition
MGI = mycelial growth inhibition

Table 3 . Inhibitory effect of different treatments on spore germination of selected fungi

Treatment	% of spore germination inhibition		
	F. oxysporum	A. alternata	B. cinerea
AB-100 mM	55.85 G	52.55 GH	49.78 H
AB-200 mM	69.90 F	66.90 F	68.38 F
CC-100 mM	29.63 KL	24.60 M	25.42 LM
CC-200 mM	37.85 I	32.88 JK	34.20 IJ
PB-100 mM	88.90 CD	87.15 DE	85.03 DE
PB-200 mM	100.00 A	100.00 A	99.30 A
SB-100 mM	86.68 DE	85.22 DE	84.45 E
SB-200 mM	93.63 B	93.57 B	92.85 BC
Control-0.00 mM	0.00 N	0.00 N	0.00 N

Values in columens followed by same letters are not significantly different (p=0.05) according to Duncan's multiple range test.

Table 4. Inhibitory effect of different treatments on mycelial growth of selected fungi

Treatment	% of mycelial growth inhibition		
	F. oxysporum	A. alternata	B. cinerea
AB-100 mM	52.80 G	52.75 G	50.65 G
AB-200 mM	62.95 F	62.95 F	60.50 F
CC-100 mM	23.95 IJ	19.80 K	20.88 JK
CC-200 mM	28.25 H	25.55 HI	25.05 HI
PB-100 mM	80.57 C	80.03 CD	79.25 CD
PB-200 mM	95.35 A	95.35 A	94.78 A
SB-100 mM	75.20 E	77.25 CDE	76.75 DE
SB-200 mM	84.78 B	86.43 B	85.07 B
Control-0.00 mM	0.00 L	0.00 L	0.00 L

Values in columens followed by same letters are not significantly different (p=0.05) according to Duncan's multiple range test.

CONCLUSION

Results of present study approved the antifungal efficacy of potassium and sodium bicarbonate against three important fungal pathogens. These compounds are effective against powdery mildew of several crop plants and ornamentals specially potassium bicarbonate has been formulated commercially under trade names of Armicarb-100, Kaligreen and Mildstop and is used for controlling such diseases worldwide. These compounds should be tested against diseases caused by F. oxysporum, A. alternata and B. cinerea under green house and field conditions.

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