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Araştırma
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Antifungal effect of carbonate and bicarbonate salts against *Botrytis cinerea*, the casual agent of grey mould of kiwifruit

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Abstract

Control of grey mould of kiwifruit caused by *Botrytis cinerea* has been accomplished by postharvest application of synthetic fungicides. However, the development of resistant fungal strains and increasing public concern over food safety and the environment are driving a search for alternative disease control strategies. In the present study, the inhibitory effect of carbonate and bicarbonate salts of ammonium, potassium and sodium against *B. cinerea* were investigated in both *in vitro* and *in vivo* experiments. Ammonium carbonate, ammonium bicarbonate, sodium carbonate, sodium bicarbonate, potassium carbonate and potassium bicarbonate completely inhibited the mycelial growth of *B. cinerea* at 10, 25, 25, 50, 50 and 75 mM, respectively. With the exceptions of few, carbonate and bicarbonate salts totaly halted spore germination at lower concentrations than that of the mycelial growth of fungus. Complete inhibitory activity of ammonium carbonate exhibited spore germination at 10 mM, whereas same concentration of sodium carbonate reduced spore germination of fungus by 98.75%; however, the difference between this and the effects of first salt was not statistically significant ($P<0.05$). The lowest minimum inhibition concentration (MIC) and EC_{50} values were also recorded in ammonium carbonate treatment. *In vivo*, however, with the exception of 100 mM ammonium carbonate, five other carbonate and bicarbonate salts significantly reduced the incidence of grey mould on kiwifruits (cv. Hayward). Moreover, potassium bicarbonate was detected to be the most effective salt for *in vivo* control of disease, and the difference between the effects of the lowest and highest concentrations of the salt was not statistically significant ($P<0.05$). Results from this study may provide an important basis for further study on the uses of carbonate and bicarbonate salts in the control of grey mould in kiwifruit at wider semi-commercial conditions.

Anahtar kelimeler: Kiwifruit, *Botrytis cinerea*, carbonate and bicarbonate salts, alternative control

Kivilerde kurşuni küfe neden olan *Botrytis cinerea*'ya karşı karbonat ve bikarbonat tuzlarının antifungal etkisi

Öz

Botrytis cinerea tarafından neden olunan kurşuni küfün kontrolü sentetik fungisitlerin hasat sonrası uygulaması ile başarılmaktadır. Ancak, dirençli fungal ırkların gelişimi, gıda güvenliği ve çevre konusundaki toplumun artan endişesi alternatif hastalık kontrol stratejileri için bir arayışa yol açmıştır. Mevcut çalışmada, amonyum, potasyum ve sodyumun karbonat ve bikarbonat tuzlarının *B. cinerea*'ya karşı engelleyici etkileri hem *in vitro* hem de *in vivo* denemelerle araştırılmıştır. Amonyum karbonat, amonyum bikarbonat, sodyum karbonat, sodyum bikarbonat, potasyum karbonat ve potasyum bikarbonatın sırası ile 10, 25, 25, 50, 50 and 75 mM konsantrasyonları *in vitro*'da *B. cinerea*'nın miselyal gelişimini tamamen engellemiştir. Birkaç istisna dışında, karbonat ve bikarbonat tuzları fungusun miselyal gelişmesinden daha düşük konsantrasyonlarda spor çimlenmesini tamamen engellemiştir. Amonyum karbonatın spor

çimlenmesini tam engelleme aktivitesi 10 mM'da ortaya çıkmış, halbuki sodyum karbonatın aynı konsantrasyonu spor çimlenmesini % 98.75'e kadar azaltmıştır. Ancak ilk ve ikinci tuz etkileri arasındaki farklılık istatistiksel olarak önemsiz bulunmuştur ($P<0.05$). En düşük minimum engelleme konsantrasyonu (MIC) ve EC_{50} değerleri de amonyum denemelerinde kaydedilmiştir. Ancak in vivo'da amonyum karbonatın 100 mM konsantrasyonu hariç, diğer beş karbonat ve bikarbonat tuzu kivilerde kurşuni küfü önemli oranda azaltmıştır. Dahası potasyum bikarbonatın *in vivo* hastalık kontrolünde en etkili tuz olduğu belirlenmiştir ve bu tuzun en düşük ve en yüksek konsantrasyonlarının etkileri arasındaki farklılık istatiksel olarak önemli bulunmamıştır ($P<0.05$). Bu çalışmadan elde edilen sonuçlar, daha geniş yarı ticari koşullarda kivilerde kurşuni küf kontrolünde karbonat ve bikarbonat tuzlarının kullanımıyla ilgili daha ileri çalışmalar için önemli bir temel sağlayabilir.

Key words: Kivi, *Botrytis cinerea*, karbonat ve bikarbonat tuzları, alternatif control

Introduction

Kiwifruit [*Actinidia deliciosa* (A. Chev.) C. F. Liang & A. R. Ferg.] has been recently introduced as a potential commercial crop in Turkey. According to FAO records in 2016, Turkey was the world's seventh largest kiwifruit producer with a total cultivation area of 24.108 da from which 41.640 t of fruits are currently harvested (FAO 2016, TUİK 2016). Kiwifruit production is negatively affected by pre and postharvest pathogens. After harvest, postharvest fungal diseases including *Botrytis cinerea*, *Diaporthe actinidiae*, *Sclerotinia sclerotiorum*, *Botryosphaeria dothidea*, *Cadophora luteo-olivacea* and *Mucor piriformis* can cause losses of kiwifruit (Brook 1986). Moreover, the losses can even reach 32 % in certain countries, such as Korea (Koh et al. 2005). *B. cinerea*, which causing stem-end rot, is regarded as the most important because environmental conditions prevailing during storage facilities are favourable to its development and infection (Pennycook, 1985; Sommer, 1985; Koh et al. 2003). Disease agent may cause serious economic losses on a wide range of matured fruit through secretion of a number of endo-polygalacturonases (EPGa enzymes) involved in pathogenesis (ten Have et al. 1998). The main control measure against the disease by applying fungicides such as iprodione, fenhexamide, imazalil, folpet, or cyprodinil+fludioxonil (Delen, 2016). Traditional chemical fungicides have had measurable success, but *B. cinerea* has developed resistance to common fungicides used to control grey mold. For example, populations of *B. cinerea* resistance to benzimidazole (benomyl) and dicarboximide (iprodione) fungicides has been documented in the United States and Canada (Northover and Matteoni, 1986; Moorman and Lease, 1992). However, the negative effects of these chemicals on both the environmental and public health have led to their banning in many countries (Fan et al. 2008). The use of natural compounds, such as organic and inorganic salts either alone or in combination with other control methods, appears to represent one of the best alternatives to synthetic fungicides for grey mold disease (Soylu et al. 2010; Türkkan and Erper 2014). The main advantages of using salt compounds include their relatively low mammalian toxicity, a broad spectrum of modes of action and relatively low cost (Olivier et al. 1998). Bicarbonate and carbonate salts of ammonium, sodium and potassium have been shown to inhibit fungal pathogens of fruits, field crops, vegetables and ornamentals (DePasquale et al. 1990 a, b; Ziv and Zitter 1992; Punja and Gaye 1993; Aharoni et al. 1997; Palmer et al. 1997; Campanella et al. 2002; Palou et al. 2002; Arslan et al. 2009; Erper et al. 2011; Latifa et al. 2011). With the exception of potassium carbonate, the remaining other bicarbonate and carbonate salts are generally recognised as safe (GRAS) by the United States Food and Drug Administration (FDA) (Anonymous 2016).

The objective of the present study was to evaluate the efficacy of carbonate and bicarbonate salts of ammonium, potassium and sodium for the control of *B. cinerea*. Inhibitory effects of the salts on the mycelial growth and conidial germination of the fungus were evaluated by *in vitro* tests, then the effect of salts on kiwifruit against *B. cinerea* were determined in *in vivo* test.

Material and Methods

Fungal isolate

The isolate of *B. cinerea* used in the study was isolated from decayed postharvest fruit of kiwifruit in Samsun province in Black Sea Region of Turkey. The isolate was maintained on potato dextrose agar (PDA; BD Difco, Sparks, USA). PDA slants were stored at 4 °C.

Fruit

Kiwifruits (cv. Hayward) were obtained from commercial orchards in Samsun, Turkey. The fruit had not received any pre-harvest fungicide treatment at least in the month before harvest, and were used immediately following harvest or after short storage at +1 °C.

Chemicals

Bicarbonate and carbonate salts of ammonium, potassium and sodium were purchased from Merck Chemicals (Merck, Darmstadt, Germany).

Effect of salts on mycelial growth and spore germination

The effect of bicarbonate and carbonate salts of ammonium, potassium and sodium on mycelial growth of *B. cinerea* were assayed according to Türkkan (2015) with a slight modification. 10, 25, 50, 75, or 100 mM concentrations of the salts added to autoclaved and cooled PDA medium at 50°C. For each salt, a 20 mL aliquot of ameliorated PDA medium was aseptically dispensed into 9-cm-dia. Petri plate, with an unamended PDA plate used as a control. A 5-mm-dia. mycelial disk cut from a 7-day-old fungal culture was placed in the center of each plate, which was then sealed with Parafilm and incubated at at 25°C. Mycelial growth was measured daily at two perpendicular colony diameters until growth reached the edge of the Petri plates in the control. Mycelial growth values were converted into the inhibition percentage of mycelial growth inhibition (MGI) in relation to the controls using the formula, MGI (%) = [(dc – dt) / dc] × 100, where dc and dt represent mycelial growth diameter in the control and amended Petri plates, respectively. Each treatment was replicated 4 times and was repeated twice.

To assess the effects of the six salts on *B. cinerea* spore germination, 20 µL aliquots of conidial suspensions (1×10^4 conidia/mL) were plated on 1 % agar flake (9 mm in diameter) mounted with different concentrations (0, 10, 25, 50, 75, or 100 mM) of bicarbonate and carbonate salts of ammonium, potassium and sodium, and then incubated in petri plates at 25°C, after 24 h of incubation, conidial germination rates were evaluated by counting of 100 conidia on per treatment replicate under an Olympus CX-31 compound microscope at 100 to 400x magnification. The level of mortality was based on 100 spores and expressed as a percentage: {[germinated spores (control) - germinated spores (salt solution)]/ germinated spores (control) }× 100 (Mecteau et. al. 2002).

EC_{50} and MIC values of salts

The effective doses of salts that cause 50% inhibition (EC_{50}) of mycelial growth and spore germination were determined by probit analysis (IBM SPSS Statistics). The minimum inhibition concentration (MIC) that completely inhibited the mycelial growth/spore germination was determined was also determined in parallel experiments.

Fruit inoculation

Kiwifruits were punctured with a probe with a 3 x 3 mm tip that had been inoculated with *B. cinerea* spore suspension just before apply. For the treatment of each salt (25 µL) at 100 mM and 250 mM concentrations was applied to the wounds, followed by inoculation with of *B. cinerea* conidial suspension (20 µL; 1×10^4 conidia/mL). A control treatment of distilled water (25 µL) followed by inoculation with conidial suspension (20 µL) of *B. cinerea* was performed. The kiwifruits were placed in plastic boxes (20x15 cm). After incubation at 20 ± 1°C and 85 ±5% R.H. for 7 days, image of the lesion area on kiwifruit was copied to a transparent paper. Images were then scanned into a digital format using a Mustek 1200 UB Plus desktop scanner (Mustek Systems, Inc., China), and the final versions of scanned images were saved as bmp 24-bit file. Lesion area was measured by using public domain software (Digimizer, Version 4.0.0.0 for MS Windows 2005–2011). The ratio of lesion area in the salts amended kiwifruit to that of control was determined as percentage inhibition. Treatment was replicated 8 times for each salt (Nunes et al. 2001).

Statistical analysis

The statistical analysis was performed using the statistical software SPSS (version 19, Property of SPSS, Inc.; IBM Company, Chicago, USA). Results were separately subjected to one-way analysis of variance (ANOVA), and significant differences between means were determined by using the Tukey–HSD test ($P<0.05$).

Result and Discussion

Ammonium carbonate completely inhibited the mycelial growth of *B. cinerea* at 10 mM, whereas five other carbonate and bicarbonate salts inhibited mycelial growth at rates from between 43.59 and 67.71% at same concentration ($P<0.05$) (Table 1). Moreover, ammonium bicarbonate, potassium

bicarbonate, potassium carbonate, sodium bicarbonate and sodium carbonate concentrations of 25, 75, 50, 50 and 25 mM, respectively, were required to completely inhibit mycelial growth of the fungus. However, ammonium bicarbonate and ammonium carbonate totaly inhibited spore germination of the fungus even at the lowest concentration tested. Sodium carbonate also reduced spore germination by 98.75%, and the difference between this and the effects of the first group of salts was not statistically significant ($P<0.05$). In contrast, potassium and sodium bicarbonate affected slightly the spore germination of the fungus at 10 mM and was no significant different from that of the control ($P<0.05$). Potassium carbonate, sodium bicarbonate and potassium bicarbonate concentrations of 25, 50 and 50, respectively, were required to completely inhibit spore germination of the fungus. In line with the present study, previous studies have also shown carbonate and bicarbonate salts including ammonium, sodium and potassium to have inhibitory effects on the mycelial growth of different fungi (Palmer et al. 1997; Palou et al. 2001; Droby et al. 2003; Latifa et al. 2011 Youssef et al. 2012). Palmer et al. (1997) observed that ammonium carbonate and bicarbonate salts had a greater inhibitory effect on *B. cinerea* than other carbonate and bicarbonate salts, and ammonium bicarbonate also halted colony formation of the fungus at 20 mM (fungitoxic), whereas potassium bicarbonate was unable to completely inhibit mycelial growth, even at the highest concentrations tested (150 mM = 1.5 %). Similarly, Bombelli and Wright (2006) found that potassium bicarbonate reduced the mycelial growth of *B. cinerea* to some extent (65%) at 0.5%; and were required to complete inhibitory activity at 1% and higher concentrations. Droby et al. (2003) determined that sodium bicarbonate completely inhibited mycelial growth of *B. cinerea* and *Penicillium expansum* at 0.7 and 0.8%, respectively. Moreover, some researhers showed that sodium carbonate and bicarbonate salts exhibited fungistatic rather fungicidal activity against many fungi, unlike ammonium carbonate and bicarbonate (Punja and Grogan, 1982; Depasquale and Montville, 1990b; Palou et al. 2001; Latifa et al. 2011). The inhibitory effect of bicarbonate salts on fungi was probably due to the reduction in fungal cell turgor pressure which resulted in collapse and shrinkage of hyphae and spores, and consequent inability of fungi to sporulate (Fallik et al. 1997). Palmer et al. (1997) also reported that the ammonium cation also contributed greatly to fungal growth inhibition, although the bicarbonate anion primarily affected fungal growth. Of bicarbonate and carbonate salts examined in the present study, ammonium carbonate was found to have the greatest toxicity against both mycelial growth and spore germination of *B. cinerea*, followed by ammonium bicarbonate and sodium carbonate (Table 2). According to their MIC and EC_{50} values, carbonate salts of ammonium, potassium and sodium were more toxic than bicarbonate salts of those. MIC values were consistent with EC_{50} values in the salts tested.

Table 1. Effect of carbonate and bicarbonate salts of ammonium, potassium and sodium on the mycelial growth and spore germination of *Botrytis cinera*

Salts	Concentration (mM)	% Inhibition	
		Mycelial growth	Spore germination
Ammonium bicarbonate	10	67.71 d[a]	100.00 a
	25	100.00 a	100.00 a
Ammonium carbonate	10	100.00 a	100.00 a
Potassium bicarbonate	10	43.59 g	1.25 d
	25	73.94 cd	76.50 b
	50	81.82 b	100.00 a
	75	100.00 a	100.00 a
Potassium carbonate	10	49.41 fg	30.75 c
	25	88.32 b	100.00 a
	50	100.00 a	100.00 a
Sodium bicarbonate	10	51.07 ef	2.00 d
	25	74.37 c	78.00 b
	50	100.00 a	100.00 a
Sodium carbonate	10	57.09 e	98.75 a
	25	100.00 a	100.00 a
Control	0	0.00 h	0.00 d

[a]Means followed by the same letter are not significant different according to the Tukey's HSD ($P<0.05$).

Table 2. EC_{50} and MIC values of bicarbonate and carbonate salts of ammonium, potassium and sodium tested against *Botrytis cinerea*

Salts	Mycelial growth		Spore germination	
	EC_{50}[a]	MIC[b]	EC_{50}	MIC
Ammonium bicarbonate	<10	25	<10	10
Ammonium carbonate	<10	10	<10	10
Potassium bicarbonate	12.77	75	19.99	50
Potassium carbonate	10.35	50	11.49	25
Sodium bicarbonate	10.98	50	19.44	50
Sodium carbonate	<10	25	<10	25

[a]The concentration that caused 50% reduction.

[b]Minimum inhibition concentration.

In vivo trials, unlike 100 mM ammonium carbonate, five other carbonate and bicarbonate salts significantly reduced the incidence of grey mould symtomps on kiwifruits (P<0.05) (Table 3 and Figure 1). Among them, the most effective salt was determined to be potassium bicarbonate for *in vivo* control of the disease, followed by sodium carbonate, sodium bicarbonate, potassium carbonate, ammonium bicarbonate and ammonium carbonate, respectively. Generally, the differences between inhibitory effects of increased concentrations of the salts against the disease were statistically significant, whereas 100 mM (1%) and 250 mM (2.5%) concentrations of potassium bicarbonate did not (P<0.05). These results are consistent with those of previous studies. Aharoni et al. (1997) showed that 2% sodium bicarbonate has potential for controlling *Rhizopus*, *Alternaria* and *Fusarium* decay on different melons during prolonged storage and shelf-life, while maintaining fruit quality. Bombelli and Wright (2006) reported that potassium bicarbonate at concentrations below 2%, demonstrated no protective effect against *B. cinerea* on tomato fruits, and moreover, applicationf of 2 % potassium bicarbonate to be efficient in preventing an attack by the pathogen for two weeks. However, phytotoxic effects that caused depreciation of fruit quality have been reported by several reserchers in melon, pepper and tomatoes when applying potassium and sodium bicarbonate concentrations higher than 2% (Aharoni et al., 1997; Fallik et al., 1997; Bombelli and Wright 2006).

Table 3. Effect of carbonate and bicarbonate salts of ammonium, potassium and sodium on the development of grey mould caused by *Botrytis cinerea* on kiwifruit

Salts	Concentration (mM)	Lesion area (cm^2)
Ammonium bicarbonate	100	3.92 bcd[a]
	250	3.97 bc
Ammonium carbonate	100	4.49 a
	250	4.22 bc
Potassium bicarbonate	100	2.61 g
	250	2.39 g
Potassium carbonate	100	4.35 bc
	250	3.02 fg
Sodium bicarbonate	100	4.01 bc
	250	3.02 fg
Sodium carbonate	100	3.56 def
	250	3.15 efg
Control (positive)		5.21 a

[a]Means followed by the same letter are not significant different according to the Tukey's HSD (P<0.05).

Figure 1. Activity of potassium bicarbonate (A) and ammonium carbonate (B) against grey mould of kiwifruit caused by *Botrytis cinerea* on double wounded kiwifruit as compared to inoculated control (C).

In conclusion, in the present study with carbonate and bicarbonates of ammonium, potassium and sodium, notable differences were determined between results of *in vitro* and *in vivo* trials. Wisniewski et al. (1998) found no correlation between the inhibitory effects *in vitro* and *in vivo* of different compounds used against *P. digitatum* and *B. cinerea*. For these reasons, our primary screenings were performed *in vivo* and we did not initially asses the toxicity of substances *in vitro*. Bicarbonate and carbonate salts have broad spectrum antimicrobial properties and are generally recognised as safe (GRAS) compounds which do not require expensive testing and validation by regulatory agencies. They are therefore very promising candidates for postharvest grey mould, especially in fresh commodities to which the application of synthetic fungicides is banned.

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