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Ethanol on the postharvest control of anthracnose in 'Kumagai' guava

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ABSTRACT: The aim of this study was to evaluate the effects of ethanol on the control of *Colletotrichum* spp. in 'Kumagai' guava and on the fruit physical-chemical attributes. For this purpose, three assays were carried out. For the first assay, fruit were exposed to ethanol vapor (0, 1, 2, 4, and 8 $mL \cdot kg^{-1}$), applied in hermetic containers. On the second, ethanol was applied by immersion for 2 minutes in different concentrations (20, 30, 40, and 50%). On the last assay, fruit were immersed in 30, 40, and 50% ethanol for 2, 5, and 10 minutes. Fruit that were not exposed to volatile (first assay) or water treated (second and third assay) were used as control. In all assays, guavas were first inoculated with a conidial suspension of *Colletotrichum* spp. and after 2 hours, fruit were subject to ethanol. Fruit were stored at 25 °C / 80% RH for 8 days and assessed for incidence, rot severity and physical-chemical properties. Conidial germination and mycelial growth of *Colletotrichum* spp. were evaluated on *in vitro* assays. It was observed that 40 and 50% of ethanol, applied by fruit immersion for 2 min, reduced incidence and anthracnose severity with no changes in the guava physical-chemical attributes, when fruit were stored at 25 °C. Applied as a vapor, ethanol reduced only the incidence, but did not reduce anthracnose severity in guavas stored at 25 °C. Under cold storage, fruit treated with 40% ethanol exhibited a reduction of 19% in anthracnose severity. *In vitro*, ethanol vapor at 70 and 140 $\mu L \cdot L^{-1}$ inhibited *Colletotrichum* spp. mycelial growth rate and when incorporated in potato-dextrose-agar, ethanol (30, 40 and 50%) inhibited *Colletotrichum* spp. mycelial growth rate and conidial germination.

Key words: *Psidium guajava*, *Colletotrichum* spp., alternative control, ethylic alcohol.

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INTRODUCTION

One of the factors that affect the quality of guavas is the occurrence of rots, especially anthracnose, caused by *Colletotrichum* spp. (Pandey et al. 1997, Fischer et al. 2011). *Colletotrichum* spp. penetration may occur in green fruit, during pre-harvest. In this case, the pathogen is able to survive in quiescent form and the symptoms are manifested once the fruit ripening process has initiated (Moraes et al. 2013). The disease symptoms are characterized by rounded, necrotic and soaked light brown lesions on the surface of the fruit. Under appropriate conditions it becomes depressed, growing irregularly and, subsequently, it coalesces.

Fungicide application is still the main control method to avoid the development of rots during postharvest. However, there is a lack of registered products, especially for this period, focused on guava culture. Also, a global concern about environmental pollution and health risks promoted by agricultural pesticides, in addition to the possibility of pathogen resistance and the removal of some products from the market, has led to increased research involving the use of alternative agents to disease postharvest control.

In this regard, several studies have shown the positive effects of ethanol on the control of postharvest rots in diverse cultures (Pesis 2005), usually applied by evaporation or fruit immersion. Under this aspect, Lichter et al. (2002) reported that immersion of grape bunches in ethanol at 33, 40, or 50% resulted in a reduction of disease, being as or more effective than sulfur dioxide (SO_2) without altering fruit appearance. Karabulut et al. (2004) verified complete inhibition of *Botrytis cinerea* spores germination after 10 seconds of exposure to 30%, or more, of ethanol at 24 °C. Furthermore, the immersion of naturally infected grapes for 30 s in ethanol (30%) at 24 °C, reduced 50% the disease incidence after 35 days of storage at 1 °C. Chervin et al. (2005) concluded that ethanol vapor (2 $mL \cdot kg^{-1}$) was as effective as SO_2 in controlling *B. cinerea* in 'Chasselas' grapes, with no differences between treated and control fruit, reported as a result of the sensory evaluation.

Ethanol inhibitory action is complex, but probably the primary target site is the plasma membrane. It promotes protein denaturation and removes the lipids from the cell membrane of microorganisms, inhibitis the uptake of various nutrients, affecting its viability (Ingram and Buttke 1985; Dao and Dantigny 2011).

Therefore, this work focused in evaluate the hypothesis that ethanol acts directly on the control of *Colletotrichum* spp. in 'Kumagai' guavas, stored at 25 °C or under refrigeration (10 °C), not interfering in the fruit physical-chemical attributes.

MATERIAL AND METHODS

In vitro effect of ethanol on the development of *Colletotrichum* spp.

In order to verify that the ethanol acts directly on the development of the fungus, the germination of conidia and mycelial growth of *Colletotrichum* spp. was evaluated under different concentrations of alcohol. The fungus isolate was obtained from naturally infected guavas, and grown in potato-dextrose-agar (PDA) for 8-days in growth chambers at 25 °C, with photoperiod of 12 hours. To evaluate the conidia germination, a spore suspension of *Colletotrichum* spp. was prepared by adding distilled water in a petri dish containing the fungus. The suspension was filtered with gauze and conidia quantification was determined in a hemacytometer (10^4 $conidia \cdot ml^{-1}$).

Polystyrene plates were divided into four quadrants. In each quadrant was deposited 40 mL of spore suspension added to 40 µL of the alcohol (LabSynth®) in different concentrations (30, 40, and 50%). For the control treatment, 40 µL of the spore suspension was added to 40 µL of sterile distilled water. Germination of conidia was evaluated after 24 h, at 25 °C. The experimental design was completely randomized with five plates per treatment and one plate as experimental unit. The evaluations were performed by counting 50 conidia per quadrant. It was considered germinated the conidia which presented a germ tube of equal or greater size than the spore.

The effect of ethanol on the mycelial growth was evaluated by transferring mycelia discs with 3 mm of diameter, taken from the edge of the colonies with 8 days of cultivation, to the center of plates containing PDA media, incorporated with alcohol (30, 40, and 50% v/v). For the control treatment, mycelia discs were transferred to the center of plates containing only PDA media. In a second assay, discs with 3 mm of diameter, taken from the edge of colonies of *Colletotrichum* spp., cultivated for →

8 days in PDA media, were transferred to Petri dishes, with the same culture media, which remained open for an hour inside hermetic containers (200 L), at 25 °C, with air circulation, where it was exposed to ethanol vapor (0, 17.5, 35, 70, and 140 $\mu L \cdot L^{-1}$). After the treatment, plates were kept at 25 °C in growth chambers and were evaluated every 2 days, by measuring the colony diameter (cm) in two opposite directions. Eight repetitions were used per treatment with one plate as experimental unit.

The collected data was used to calculate the MGR (mycelial growth rate), as demonstrated by Peres et al. (2003), according to the mathematical equation:

$$MGR = M_1.N_1^{-1} + M_2.N_2^{-1} + ... + M_n.N_n^{-1}$$

where, M_1 = mycelial growth in the first day and N_1 = number of days.

All the data were subject to ANOVA and means compared by Tukey's test at 5%.

In vivo effect of ethanol on the control of anthracnose in guavas stored at 25 °C

Physiologically developed and green 'Kumagai' guavas were harvested at Campinas/SP, and selected by its uniformity of color, absence of injuries and diseases. After that, the fruit were inoculated with a chromatography syringe (Hamilton®) perforating two mm deep the fruit epidermis, in the equatorial region, and inserting 10 µL of the spore suspension of *Colletotrichum* spp. (10^5 conidia$\cdot mL^{-1}$) (Gupta and Pathak 1990, with modifications). Two hours after the inoculation, fruit were exposed to different ethanol concentrations. Three assays were carried out to evaluate ethanol effects on the control of anthracnose. In the first assay, fruit were placed inside hermetic containers (200 L), with air circulation at 25 °C. The specific concentrations corresponding to 0, 1, 2, 4 and 8 $mL \cdot kg^{-1}$ of ethanol were deposited in filter papers, inside of Petri dishes, which were subsequently placed in the bottom of the containers. The fruit were exposed to ethanol for one hour. For the second assay, fruit were immersed for two minutes in 20, 30, 40, and 50% v/v of ethanol. As a control, fruit were immersed in water at ambient temperature. In this assay, the evaluation consisted in anthracnose incidence, severity and the physical-chemical attributes of not inoculated fruit. For determination of peel and pulp color, a colorimeter Hunter Lab - MiniScan XE Plus, L C °H system [L represents luminance, C represents chroma, and °H (Hue), the color angle (0° to 360° - 0°: Red; 90°: yellow; 180°: green; and 270°: blue)], with readings in two opposite points in the equatorial region of the fruit; Soluble solids content was determined with the fruit juice, obtained by the centrifugation of two fruit per repetition, directly read in a digital refractometer Pal - 1 (Atago®), and the results were expressed in °Brix; Titratable acidity was determined by titration, with sodium hydroxide solution (NaOH) 0.1 N until pH 8.10 (pH meter Digimed®), using 10 g of juice, diluted in 90 mL of distilled water. The results were expressed as percentage citric acid. Quality analyses were evaluated in two fruits as experimental unit and five repetitions were performed.

On the third assay, fruit were immersed in concentrations of 30, 40, and 50% of ethanol, for 2, 5, and 10 minutes. As a control, fruit were immersed in water at ambient temperature. Fruit were stored at 25 ± 2 °C / 80 ± 5 % RH during eight days and evaluated, every 2 days, for incidence (percentage of occurrence) and rot severity (lesion diameter).

The obtained data were used to calculate the Area Under Disease Progress Curve (AUDPC), as the equation described by Shaner and Finney (1977).

All the assays were conducted in a completely randomized design and means compared by Tukey's test at 5%. Anthracnose incidence and severity were evaluated with five repetitions composed of three (first assay) or four fruit (second and third assays) as experimental unit. For the third assay, the statistical analyses were performed in factorial arrangement (3 × 3).

In vivo effect of ethanol on the control of anthracnose in guavas stored under refrigeration

Physiologically developed and green 'Kumagai' guavas were harvested at Campinas/SP, selected and inoculated as described above. Based on the obtained results in previous trials, after 2 h from the inoculation, fruit were treated with 40% ethanol by immersion for 2 min. Fruit immersed in water for 2 min were used as control. Once dry, the fruit were kept under refrigeration (10 °C ± 1 °C / 90 ± 5% UR) for 15 days, followed by a transference to 25 ± 2 °C / 80 ± 5 %UR, where it were kept for more six days. The evaluation consisted in incidence and →

severity of anthracnose in the fruit, every two days, after the transference to 25 °C. The obtained data were used to calculate the Area Under Disease Progress Curve (AUDPC), as the equation described by Shaner and Finney (1977).

The experimental design was completely randomized, with five repetitions composed of four fruit as experimental unit, and the means were compared by Tukey's test at 5%.

RESULTS AND DISCUSSION

In vitro effect of ethanol on the development of *Colletotrichum* spp.

Ethanol, at all evaluated concentrations, completely inhibited mycelial growth rate of *Colletotrichum* spp., when incorporated into the culture media, and also impaired the fungus conidial germination (88% of germination in the control treatment and 4.85 of mycelial growth rate; data not showed). When ethanol was applied by evaporation, it was observed that the concentrations of 70 and 140 $\mu L \cdot L^{-1}$ completely inhibited the development of *Colletotrichum* spp. (Table 1), whereas lower concentrations did not alter, significantly, mycelial growth rate.

Similarly, Karabulut et al. (2004) observed complete inhibition of *B. cinerea* spore germination after 10 s of exposure to 30% or more of ethanol at 24 °C and a reduction of 56% in *B. cinerea* spores treated for 30 s with 20% ethanol (Karabulut et al. 2005). Gabler et al. (2004) had the same result for *Alternaria alternate* spores after 30 s of exposure to 30% ethanol. Lichter et al. (2002) reported that 40% of ethanol completely inhibited the germination of *B. cinerea* conidia, but no positive results of the product were verified on the mycelial growth of the pathogen. According to the authors, the main ethanol target, as a stressor, is the lipid membrane of microorganisms, but it can also exert other effects on fungal cells.

Table 1. Mycelial growth rate (MGR) of *Colletotrichum* spp., obtained from naturally infected guavas, exposed to ethanol vapor for 1 h and kept for eight days at 25 °C, after treatment.

Ethanol ($\mu L \cdot L^{-1}$) *	MGR
0	2.19 a
17.5	2.18 a
35	2.03 a
70	0 b
140	0 b
CV(%)	34,87

*Applied by evaporation, in hermetic containers, with air circulation. Average of eight repetitions. Means followed by the same letter, in the column, do not differ significantly from each other by Tukey's test ($p \leq 0.05$).

In vivo effect of ethanol on the control of anthracnose in guavas stored at 25 °C

In the first assay, when ethanol was applied as a vapor, it was verified that the alcohol was not effective in reducing anthracnose severity (Table 2), although resulted in a reduction of 25.5% in the disease incidence of fruit treated with 4 $mL \cdot kg^{-1}$ of ethanol.

Similar results were obtained when ethanol was applied by immersion (second assay). Ethanol, in the highest evaluated concentration (50%), decreased by 31.3% the number of fruit with anthracnose symptoms, not reducing, significantly, the lesions diameter (Table 3).

It was observed that after seven days of storage at 25 °C, ethanol did not affect the brightness (L), hue angle (H °) and chroma (Chroma) in the peel of the fruit. For the pulp color, results indicated an increase in H °, which was less pronounced

Table 2. Area under the disease progress curve (AUDPC) for incidence and severity of anthracnose in 'Kumagai' guavas inoculated with *Colletotrichum* spp., treated with ethanol by evaporation (1 h), and stored at 25 °C / 80% RH for eight days.

Ethanol ($mL \cdot kg^{-1}$)	AUDPC (incidence)*	AUDPC (severity)*
0	180 a	1.43 a
1	146 ab	1.26 a
2	166 ab	1.17 a
4	134 b	1.06 a
8	150 ab	1.28 a
CV%	13.48	27.75

*Average of five repetitions, with three fruits as experimental unit. Means followed by the same letter, in the column, do not differ significantly from each other by Tukey's test ($p \leq 0.05$).

Table 3. Area under the disease progress curve (AUDPC) for incidence and severity of anthracnose in 'Kumagai' guavas inoculated with *Colletotrichum* spp., treated with ethanol by immersion (2 min), and stored at 25 °C / 80% RH for eight days.

Ethanol (%)	AUDPC (incidence)*	AUDPC (severity) *
0	335 a	4.51 a
20	335 a	4.57 a
30	350 a	4.77 a
40	290 ab	3.97 a
50	230 b	3.70 a
CV%	11.82	13.78

*Average of five repetitions, with four fruits as experimental unit. Means followed by the same letter, in the column, do not differ significantly from each other by Tukey's test ($p \leq 0.05$).

in fruit treated with 50% of ethanol. The obtained values for L and chroma remained nearly constant along the storage, indicating no influence by the treatments. The other physical-chemical attributes evaluated (soluble solids and titratable acidity) were not influenced by the different concentrations of ethanol (Tables 4,5). These findings corroborates with Zhang et al. (2007) and Wang et al. (2011).

Under this aspect, Siddiqui et al. (2005) reported that 'Banarsi Surkha' guavas, submitted to ethanol vapor for 2 h, showed lower mass loss and greater pulp firmness during storage. The alcohol reduced the activity of enzymes like polygalacturonase, β – galactosidase, cellulase and the ethylene production. Authors observed as well that the treatment reduced soluble solids content in guavas. Loquat fruit treated with ethanol vapor (300 $\mu L \cdot L^{-1}$) exhibited lower firmness and higher extractable juice as compared to the control. The treatment also maintained higher contents of soluble solids and vitamin C even after 8 days at 20 °C (Wang et al. 2015).

When ethanol was applied in different concentrations for different immersion time intervals, no significant interaction between these two factors was verified. It is valid to emphasize that the shortest time interval of immersion (2 min) promoted a pronounced reduction in rot incidence. Besides reducing the number of fruit with symptoms, the treatments also reduced significantly the lesions diameter (Table 6). The major control of anthracnose was evident under concentrations of 40 and 50% of ethanol, with a decrease of 73 and 77% in the severity, and 52 and 50% in rot incidence, respectively.

Ethanol effectiveness to control anthracnose in guavas can be assigned to its fungistatic or fungicide property. Therefore, ethanol application, in the concentrations presented in this work, may have interfered directly on the functions of

Table 4. Peel and pulp color of 'Kumagai' guavas treated with ethanol by immersion (2 min), and stored at 25 °C / 80% RH for seven days.

Treatments	Peel color*		
	L	H°	Chroma
First day	62.31	117.14	33.95
Control	65.51 a	109.27 a	37.82 a
20%	67.76 a	107.61 a	39.28 a
30%	66.96 a	108.82 a	39.27 a
40%	67.54 a	109.23 a	38.57 a
50%	66.61 a	107.94 a	38.54 a
CV%	6.6	4.19	5.5
Treatments	**Pulp color***		
	L	H°	Chroma
First day	80.06	92.20	20.90
Control	80.45 a	97.55 a	20.10 a
20%	80.83 a	97.40 a	21.08 a
30%	80.32 a	97.50 a	20.19 a
40%	80.04 a	96.82 ab	20.68 a
50%	78.91 a	95.98 b	20.88 a
CV%	1.4	5.3	4.1

*Average of ten repetitions. Means followed by the same letter, in the column, do not differ significantly from each other by Tukey's test ($p \leq 0.05$).

Table 5. Soluble solids and titrable acidity of 'Kumagai' guavas treated with ethanol by immersion (2 min), and stored at 25 °C / 80% RH for seven days.

Treatment	Soluble solids* (°Brix)	Titrable acidity* (% ác. cítrico)
First day	8.60	0.59
Control	7.56 a	0.62 a
20%	7.18 a	0.66 a
30%	7.26 a	0.64 a
40%	7.72 a	0.66 a
50%	7.52 a	0.58 a
CV%	7.1	11.2

*Average of five repetitions. Means followed by the same letter, in the column, do not differ significantly from each other by Tukey's test ($p \leq 0.05$).

Table 6. Area under disease progress curve (AUDPC) to severity (cm) and incidence (%) of anthracnose in 'Kumagai' guavas inoculated with *Colletotrichum* spp., treated by immersion in ethanol and stored at 25 °C / 80 % RH for eight days.

AUDPC (Severity, cm)*				
	Immersion interval			
Control: 5.12 Control × Factorial**	2 min	5 min	10 min	Mean^{ns}
Ethanol 30%	2.02	2.72	2.68	2.47 a
Ethanol 40%	1.36	4.30	3.26	2.97 a
Ethanol 50%	1.16	3.88	5.63	3.56 a
Mean*	1.51 B	3.63 A	3.85 A	Time × Conc.^{ns}
CV% 23.76				
AUDPC (Incidence, %)*				
Control: 240 Control × Factorial**	2 min	5 min	10 min	Mean^{ns}
Ethanol 30%	140	210	165	171.7 a
Ethanol 40%	115	185	175	158.3 a
Ethanol 50%	120	170	175	155.0 a
Mean**	125.0 B	188.3 A	171.7 A	Time × Conc.^{ns}
CV% 13.99				

*Means followed by the same capital letter, in line, or lower case letter, in the column, do not differ significantly by Tukey's test ($p \leq 0.05$). ^{ns} = not significant; **.*** = significant at 5% or 1%, respectively.

Colletotrichum spp. plasma membrane, delaying or inhibiting its development and, consequently, the development of anthracnose on the fruit.

Romanazzi et al. (2007) evaluated the effects of ethanol (10 and 20%), applied by immersion, to control gray mold in 'Thompson Seedless' and 'Autumn Seedless' grapes, and observed that the alcohol reduced the percentage of berries infected by *B. cinerea*. Likewise, Fischer et al. (2016) reported a significant reduction in the incidence of anthracnose (*Colletotrichum* spp.) in 'Pedro Sato' guavas immersed in 50% of ethanol for 5 minutes followed by sodium hypochlorite (0.2 $g \cdot L^{-1}$ active chlorine), on the sixth day of storage at 22 °C. Wang et al. (2015) stated that ethanol treatment (300 $\mu L \cdot L^{-1}$) in loquat fruit, inoculated with *C. acutatum* and stored at 20 °C for 8 days, presented a reduction of 30% in both pathogen incidence.

Candir et al. (2012) verified that the packaging of grapes with perforated polyethylene and Antimold® 80 (ethanol vapor-generating sachets) was as effective as the treatment with SO_2 in reducing the incidence of postharvest rots in naturally infected or artificially inoculated grape clusters. The promising results for anthracnose control in this work were verified in artificially inoculated guavas; future experiments should be done to verify the ethanol efficacy in naturally infected fruit.

In vivo effect of ethanol on the control of anthracnose in guavas stored under refrigeration

The obtained results in previous assays revealed that ethanol (40 % / 2 min) significantly reduced the incidence and severity of anthracnose on guavas kept at 25 °C, with no significative alterations in the peel and pulp color, as well as in the soluble solids content and titratable acidity. Thus, fruit treated with ethanol by immersion were submitted to a new evaluation, after storage under refrigeration followed by transference to 25 °C. The results showed that ethanol (40 % / 2 min) significantly reduced the severity of anthracnose in guavas (19% of reduction), although was not effective in reducing the number of fruit with rot symptoms (Table 7). Cold storage is frequently used to extend shelf life of perishable products. Allied with ethanol it can contributes to control postharvest pathogens, as studied by Karabulut et al (2005), that verified a reduction of 31% in the incidence of *B. cinerea* in 'Flame seedless' grapes stored for 10 days at 15 °C.

Therefore, the ethanol does not appear to have much effectiveness in reducing anthracnose in guavas when the fruit are stored for a long period. Zhang et al. (2007) observed that in low temperature storage, ethanol had a brief accumulation with a substantial decline during shelf-life. Chinese bayberries, submitted to ethanol vapor (1000 $\mu L \cdot L^{-1}$) for five days at 0 °C and two days at 20 °C, had lower decay incidence. The maintenance of ethanol effect during storage is still a challenge. Ethanol efficacy declines during prolonged storage, because its residues are low and short-lived (Karabulut et al. 2005) and apparently it is not efficient in protecting the fruit for long periods.

Table 7. Area under the disease progress curve (AUDPC) for incidence (%) and severity (cm) of anthracnose in 'Kumagai' guavas inoculated with *Colletotrichum* spp., immersed in ethanol, and stored at 10 °C / 90% RH for 15 days followed by six days at 25 °C / 80% RH.

	AUDPC (Incidence %)*	AUDPC (Severity cm)*
Control	395 a	11.94 a
Ethanol (40% / 2 min)	380 a	9.73 b
CV%	6,29	11,60

*Average of five repetitions with four fruit as experimental unit. Means followed by the same letter, in the column, do not differ significantly from each other by Tukey's test ($p \leq 0.05$).

CONCLUSION

The ethanol at 40 or 50% applied by immersion of the fruit for 2 min reduces the development of anthracnose in 'Kumagai' guavas stored at 25 °C, acts directly on the pathogen and does not alter the physical-chemical attributes of the fruit. Under refrigeration, ethanol reduces anthracnose severity. Thus, it may be a potential control agent in integrated management programs.

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