Preformed Antifungal Compounds of Citrus Fruit: Effect of Postharvest Treatments with Heat and Growth Regulators

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Heat treatment (36 °C, 3 days) of lemon fruit *Citrus limon* (L.) Burm. inhibited both the decline of antifungal activity in the flavedo tissue and the loss of the preformed antifungal material citral and drastically reduced the decay percentage. Dips in gibberellic acid (50 and 100 ppm) or in 2,4-dichlorophenoxyacetic acid (200 ppm) inhibited the decay development of stored fruit and slowed the decline of citral content and of the antifungal activity in the flavedo. On the contrary, ethylene treatment (25 or 50 ppm for 3 days at 25 °C) reduced the citral content and antifungal activity in the flavedo of both lemon and grapefruit (*Citrus paradisi* Macf.). The effect of postharvest heat treatment and plant growth regulators on citrus decay might be related to the modulation of endogenous disease resistance of fruit via influence on the changes of preformed antifungal materials such as citral.

Keywords: *Citrus limon; Citrus paradisi; postharvest; antifungal compounds; citral; disease resistance; gibberellin; 2,4-D; ethylene; heat treatment*

INTRODUCTION

The chemical basis of plant disease resistance is related to the presence of preformed and/or induced antimicrobial substances, lignification of wounds and sites of pathogen infection, and other substances such as pathogenesis-related proteins (Kuc, 1991). Preformed antifungal compounds found in various organs of plants belong to chemically diverse groups such as alkaloids, flavonoids, phenols, coumarins, and terpenoids (Kuc, 1991).

In different *Citrus* species, several compounds were isolated and identified as preformed antifungal materials (Ben-Yehoshua et al., 1988). In previous work, we (Ben-Yehoshua et al., 1992; Kim, 1992) identified citral, limettin, 5-geranoxy-7-methoxycoumarin, and isopimpinellin as preformed antifungal materials in lemon fruit exerting inhibitory activity against a postharvest pathogen, *Penicillium digitatum*. Of these compounds, only citral was present in the flavedo of lemon at a concentration sufficient to significantly influence decay development. Citral has been cited as having activity against various fungi (Asthana et al., 1988). Recently, Rodov et al. (1995) found a correlation between fruit age, citral concentration in the flavedo, and fruit resistance against decay. The relationship between preformed antifungal compounds, disease resistance of citrus fruits, and various postharvest treatments has been discussed recently (Ben-Yehoshua et al., 1994).

Heat treatment (34–36 °C) of sealed citrus fruit accelerated healing of fruit wounds, induced the activity of phenylalanine-ammonia lyase (PAL), and markedly reduced decay (Ben-Yehoshua et al., 1987). Ben-Yehoshua et al. (1988) showed that the mode of action of heat treatment in reducing decay of citrus fruit might be explained in part by the enhanced activity of preformed antifungal substances.

Preharvest or postharvest application of gibberellins delays the maturation and senescence of citrus fruits and extends the life of stored fruits (Coggins et al., 1969). Preharvest foliar application of GA alone or in combination with the cytokinin isopentenyladenine reduced the yellowing and decay of lettuce (Aharoni et al., 1975).

Ethylene is known to accelerate the maturation and senescence of citrus fruits. Early-season ethylene treatment is used commercially to degreen citrus fruit that has reached its maturity but still lacks desirable color. Ethylene-treated fruits are more susceptible to decay than untreated ones (Brown and Lee, 1993).

The objective of this work was to study the effects of the postharvest practices of application of the growth regulators gibberllic acid, 2,4-D, and ethylene and heat treatment on the level of preformed antifungal materials in citrus flavedo as related to fruit decay incidence during storage.

MATERIALS AND METHODS

General. Mature, light green lemons [*Citrus limon* (L.) Burm. cv. Eureka] and grapefruit (*Citrus paradisi* Macf. cv. Marsh) were obtained from orchards or packing houses before any postharvest treatment had been applied, unless specified differently. Samples of fruit of uniform size and appearance, originating in one orchard, were subjected to different treatments at random.

Most of the work was carried out on lemons since we have already studied the preformed antifungal compounds in lemon flavedo (Ben-Yehoshua, 1992; Kim, 1992).

Seal-packaging of Eureka lemon fruits in polyethylene film and their heat treatment (36 °C, 72 h) were done as described by Ben-Yehoshua et al. (1987).

Lemons (cv. Eureka) were given the following additional treatments: (1) dipping fruit in water for 2 min (control); (2) dipping fruit in 50 or 100 ppm of gibberellic acid (GA) for 2 min; (3) dipping fruit in 200 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) for 2 min; (4) keeping fruit in an atmosphere of 50 ppm of ethylene for 3 days at 25 °C. Each treatment

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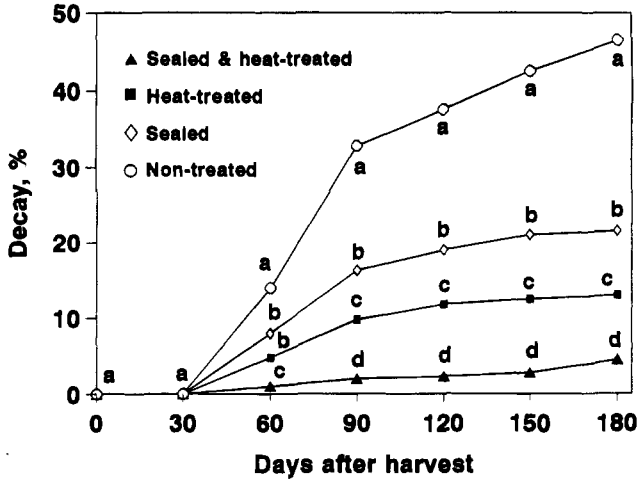

Figure 1. Effect of heat treatment on the percentage of *Penicillium*-infected fruit during storage of Eureka lemon. Fruit was kept at 36 °C for 72 h and afterward stored at 17 °C and 85% RH. The control fruit was kept at 17 °C and 85% RH throughout the experiment. Here and in further figures different letters indicate the statistically significant difference between treatments as determined by Duncan's multiple-range test ($p = 0.05$).

consisted of 150 fruits with 5 replications. Fruits were stored at 17 °C and 85–90% relative humidity (RH) for 4 months. Additionally, the regular commercial ethylene degreening treatment (25 ppm for 3 days at 25 °C) was applied to grapefruit (cv. Marsh).

The preparation of crude extract of flavedo tissue was carried out according to the method of Ben-Yehoshua et al. (1992).

Analytical. Flavedo tissues were extracted with petroleum ether as described by Kim et al. (1991) and Ben-Yehoshua et al. (1992).

Concentrations of monoterpenes in flavedo extracts were quantified by gas chromatography (GC) using the method of external standardization. Authentic samples of citral (64% geranial and 32% neral, Sigma Chemical Co., St. Louis, MO) were used as external standards. The GC analysis was carried out on a Vega 6000 GC instrument (Carlo Erba Strumentazione, Milano, Italy) equipped with a flame ionization detector and fitted with an OV-17, Chrom W-HP steel column (6 ft length, 1/4 in. o.d.). Nitrogen was used as the carrier gas; flow rate was 80 mL min^{-1}. The initial temperature of 105 °C was held for 5 min, and then the temperature was increased to 130 °C at 1 °C min^{-1} and held at 130 °C for an additional 5 min. The temperature of the injector was 250 °C and of the detector 220 °C.

Measurement of Antifungal Activity of Crude Extracts. The antifungal activity of crude extracts from the flavedo of lemon and grapefruit was evaluated by quantitative bioassay with *P. digitatum* spores (Kim et al., 1991). The antifungal activity was measured by the inhibition of germ-tube elongation in a spore suspension containing 1000 ppm of crude extract, 0.5% sucrose, 0.5% orange juice, and 5% ethanol, as compared with control spores growing in the same conditions but without a crude extract.

Statistical analysis was done by ANOVA, and means were separated by Duncan's multiple-range test.

RESULTS

Effect of Heat Treatment. Heat treatment of individually sealed Eureka lemons markedly reduced their decay. After 180 days at 17 °C, sealed and heat-treated fruit had only 4.5% decay as compared to 46.5% in nontreated fruit (Figure 1). The nontreated sealed fruit had 21.0% and nonsealed and heat-treated fruit had 13.0% decay.

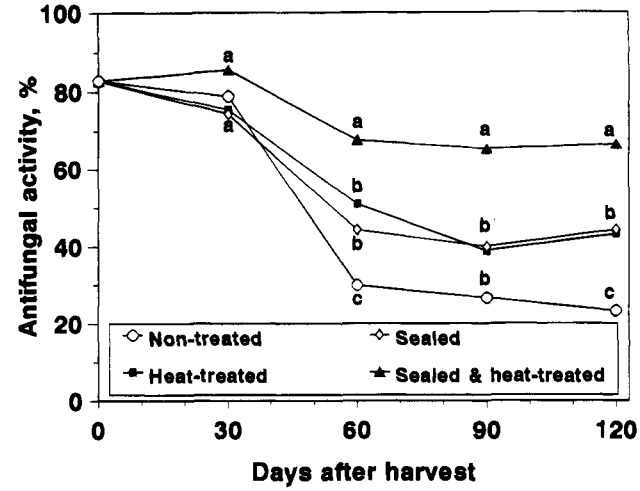

Figure 2. Effect of heat treatment on the antifungal activity of the crude extracts from lemon fruits. The antifungal activity of the crude extracts was expressed as percent inhibition of germ tube elongation of *P. digitatum* spores as compared with that of the control treatment containing 0.5% sucrose, 0.5% orange juice, and 5% ethanol. The assay was done in triplicate.

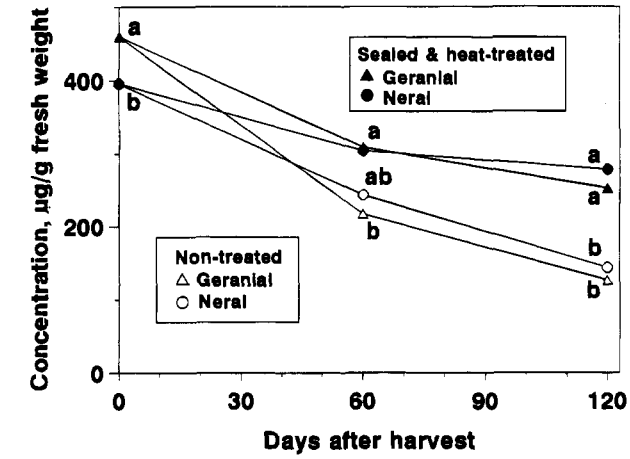

Figure 3. Effect of heat treatment on the changes of content of the two isomers of citral in Eureka lemon fruit.

The effects of heat treatment in reducing decay were correlated with its effect on inhibiting the decline of the antifungal activity of the crude extract of flavedo tissues of the lemon fruit (Figure 2). This decline in the nontreated fruit was significantly more rapid than that in the sealed and heat-treated fruit. It is interesting that sealing or heating per se also had an independent effect in delaying this decline of antifungal activity.

The decline in resistance against decay of lemon fruit during storage correlated with the reduction of both the antifungal activity (Figure 2) and of the citral level (Figure 3) of the crude extract of the flavedo tissue. Figure 3 shows that heat treatment, 3 days at 36 °C, also delayed the decline of the levels of geranial and neral, the two isomers of citral.

Effect of GA. Postharvest application of GA was very effective in delaying the decline of antifungal materials in lemon fruit. In nontreated fruit, the decline of citral concentration (Figure 4) and antifungal activity of flavedo extract (Figure 5) started within the first month of storage and continued progressively during the whole 3-month storage period. This significant decline of antifungal materials occurred along with the rise of decay development during storage (Figure 6).

Both GA doses practically prevented the decrease of antifungal activity in the flavedo for 90 days (Figure 5)

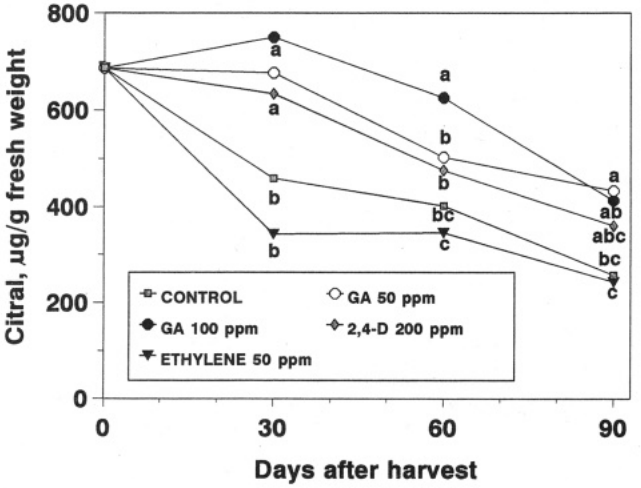

Figure 4. Effect of treatments with GA, 2,4-D, and ethylene on citral content of lemon fruit.

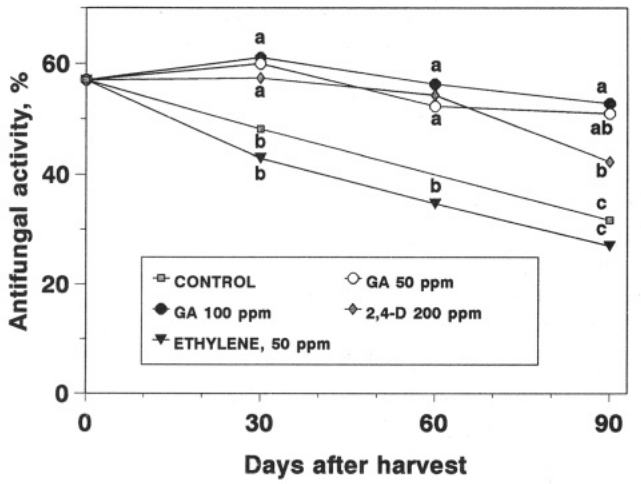

Figure 5. Effect of treatments with GA, 2,4-D, and ethylene on antifungal activity of the flavedo of lemon fruit.

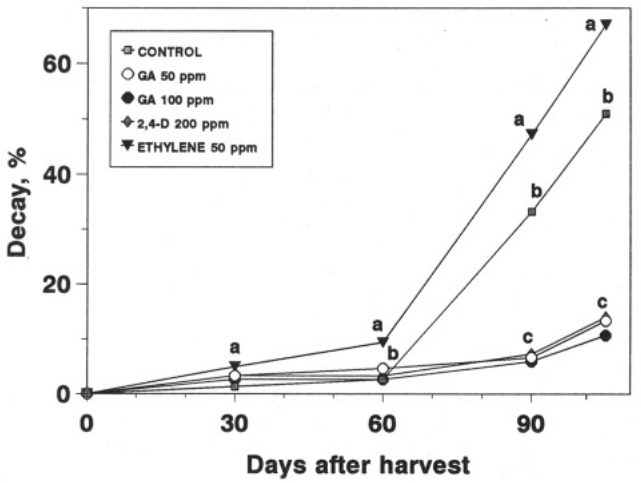

Figure 6. Effect of treatments with GA, 2,4-D, and ethylene on the percentage of infected fruit during storage of Eureka lemon.

and markedly inhibited the decline of citral concentration (Figure 4). Moreover, during the first 30 days of storage, the citral level kept constant in fruit treated with 50 ppm of GA and even demonstrated a slight but statistically significant increase in fruit treated with 100 ppm of GA. Accordingly, GA applications markedly inhibited lemon decay during storage (Figure 6).

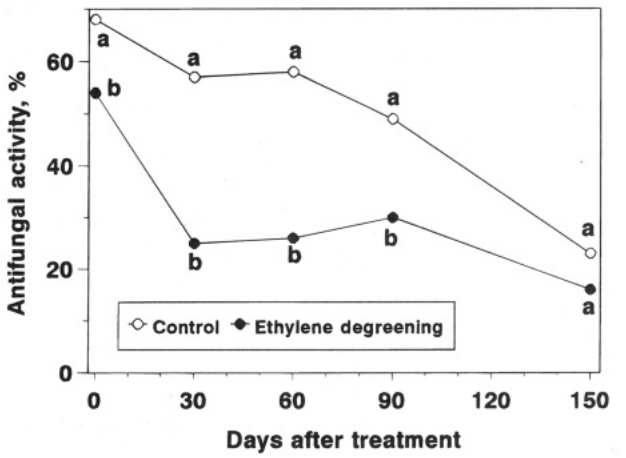

Figure 7. Effect of ethylene degreening treatment (3 days, 25 ppm) on the antifungal activity of the flavedo of grapefruit.

Effect of 2,4-D. A dip in 200 ppm of 2,4-D resulted in a similar effect to that of 50 ppm of GA. Citral content was very similar to that of GA-treated fruit and significantly higher than that of the control (Figure 4). The antifungal activity of the 2,4-D-dipped fruit was again similar to that of the two GA treatments and significantly larger than that of nontreated fruit. Only at 90 days were both antifungal activity and citral level lower than those of the 100 ppm of GA but similar statistically ($p = 0.05$) to the 50 ppm of GA treatment (Figure 5). The incidence of decay in 2,4-D-treated fruit was similar to the two doses of GA and much lower than that of the control fruit (Figure 6).

Effect of Ethylene. Ethylene enhanced the decline of citral content in lemon fruit (Figure 4). Similarly, the ethylene application enhanced the decline of antifungal activity of crude extract (Figure 5). This enhancing effect of the decline of antifungal activity had the same trend in two experiments. However, in one experiment the difference was significant at 5% compared with the nontreated fruit, but in another experiment this difference was not significant, probably because of sample size or too few replications. In all cases the differences from the GA- and 2,4-D-treated fruit were significant. Ethylene application accelerated decay development (Figure 6) in a significant way.

The commercial ethylene degreening treatment with 25 ppm for 3 days markedly and significantly reduced the antifungal activity of crude extract from the flavedo of grapefruit (Figure 7).

DISCUSSION

The important findings in this work are the marked effects that various postharvest treatments have on the level of the preformed antifungal materials and decay incidence of lemon fruit. Ethylene degreening accelerates the decline of antifungal materials in the flavedo during fruit storage, while heat treatment, 2,4-D, or gibberellin applications markedly inhibit this decline. Accordingly, ethylene enhances and heat, 2,4-D, or gibberellin reduce decay incidence of the fruit during its storage. The effect of these postharvest treatments on citrus decay might be related to the modulation of endogenous disease resistance of fruit by influencing the changes of preformed antifungal materials.

Heat treatment was shown to drastically reduce decay of citrus fruits (Ben-Yehoshua et al., 1987, 1989) and to inhibit markedly the decline of the preformed antifungal activity of citrus fruits (Kim et al., 1992). The

present work demonstrates that the latter effect may be related in lemon to the level of preformed antifungal material citral. The mechanism of heat effect on citral level is still uninvestigated. Heat shock is known to induce various changes in cell ultrastructural and biochemical functions which lead in certain cases to enhancement of disease resistance [see Nover (1990)]. The inhibited decline of preformed antifungal materials may contribute to the decay reduction in lemon together with other heat-shock effects such as induced lignification (Ben-Yehoshua et al., 1988) and synthesis of stress proteins (Nover, 1990) as well as with direct thermal inhibition of the pathogen. The relative contribution of each of these mechanisms cannot yet be evaluated.

Reducing decay of lemon and other citrus fruits by pre- and postharvest applications of GA and 2,4-D is attributed usually to the known effects of these phytohormones on maintaining juvenility and delaying senescence of plant tissues. The inhibitory effect of GA and 2,4-D on the degradation of antifungal materials such as citral provides additional more specific explanation for the mode of action of these phytohormones in decay reduction.

The effect of preharvest gibberellin spray on citral was reported by Coggins et al. (1969), who checked the effect of GA on essential oil composition in navel orange rind. They found that GA treatment of fruit on the tree maintained the level of geranial for 34 weeks, while the concentration of geranial in nontreated fruit started decreasing just 18 weeks after spraying. This suggests that preharvest spray has a highly persistent effect on inhibiting the decline of citral level similar to the effect of postharvest dip.

Recently, McDonald (1994) reported that preharvest GA application resulted in an overall significant increase in peel oil in the flavedo tissue of Marsh grapefruit. This observation is in agreement with earlier findings that exogenous application of phytohormones (gibberellic acid, auxins, or cytokinins) can enhance monoterpene biosynthesis (El-Keltawi and Croteau, 1987) and, accordingly, increase the yield of essential oils in different plant species (Sharma et al., 1988; El-Keltawi and Croteau, 1987; El-Khateeb, 1989).

Recent reports showed that GA application reduced decay in persimmon (Perez et al., 1993) and celery (Afek et al., 1994). Similar to our observations, postharvest GA application on celery delayed the endogenous decline of the antifungal material marmesin (Afek et al., 1994). However, gibberellic acid, indoleacetic acid, or kinetin had either no significant effect on or decreased the level of defensive terpenoid aldehydes such as gossypol and hemigossypolone in root-knot nematode-infected cotton plants (Khoshkhoo et al., 1993). The content of terpenoid aldehydes in cotton roots was increased by a foliar spray of salicylic acid or a mixture of cytokinins (commercial name Burst), showing that different plant species vary in their major antifungal compounds and in their response to phytohormones.

Interestingly, the effect of ethylene is just the opposite to that of GA, that is, ethylene treatment enhanced the decline of antifungal activity and citral level in both lemons and grapefruit and increased the decay incidence. An alternate explanation for decay stimulation by ethylene was its known effect in enhancing senescence of plant tissues. This work opens a new role for ethylene in accelerating the degradation of the antifungal materials such as citral. Ben-Yehoshua et al. (1990) found that ethylene at temperatures beyond 30 °C caused destruction of many oil glands of pomelo fruit. It is possible that some of this destructive effect took place in our experiments at lower temperatures. This hypothesis is corroborated by early data of Norman and Craft (1968) that ethylene degreening enhances the evolution of citral and many other essential oil compounds from lemon to the atmosphere. This phenomenon might be a result of altered selectivity of the oil gland membranes enabling essential oil compounds to diffuse from their glands.

Recently, Brown and Lee (1993) reported that ethylene degreening treatment stimulated the stem-end rot of Valencia orange fruit caused by the pathogen *Diplodia natalensis* Pole-Evans. These authors explained their observations by direct ethylene influence on the pathogen. Our data regarding the effect of ethylene on the level of preformed antifungal materials in citrus fruit provide an alternative explanation. The enhanced pathogen growth could elicit the production of phytoalexin scoparone in ethylene-treated fruit observed by Brown and Lee (1993). However, this defensive response might be too late to prevent decay in this case of a weakened first line of fruit defense represented by the preformed antifungal materials (Ben-Yehoshua et al., 1992). The relation between preformed and induced factors of citrus disease resistance awaits investigation.

Although our data consistently show that ethylene treatment enhances decay of citrus fruit, the possibility exists that applying ethylene in certain cases may reduce disease by inducing the synthesis of various enzymes related to plant defense from pathogens (Ecker and Davis, 1987; Yoshikawa et al., 1990).

It is important to study the effects of other ongoing horticultural practices on these important preformed antifungal materials with the hope of optimizing these effects to enhance the natural disease resistance of fruit.

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