Formation of *n*-Hexanal from Rind Tissues as Related to Aging and Disorder of Rind of Naruto (*Citrus medioglobosa* hort. ex Tanaka)

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Summary

n-Hexanal was found in macerated rind tissues of Naruto (*Citrus medioglobosa* hort. ex Tanaka). Hexanal formation was thought to be catalyzed by enzyme system. In this reaction, optimum pH was about 4.5.

Rates of hexanal formation were closely associated with the degree of degreening of the fruit rind: their order was CEPA-treated fruits > outside fruits > inside or GA-treated fruits. There was a high rate of the hexanal formation in the fruit rind affected by 'rind yellow spot', a physiological rind disorder of Naruto, compared to healthy fruit rind. The hexanal formation was influenced by ambient temperature to which fruits had been exposed. A higher rate was observed on fruits held at 20°C than those held at 5°C. After transfer of fruits from 5°C to 20°C, the rate increased within 3—4 hours.

Possible physiological importance of the hexanal formation was discussed in relation to aging of the rind and 'rind yellow spot' which occurs in accordance with aging of the rind.

Introduction

The presence of volatile aldehydes and alcohols in macerated tissues has been known in a wide range of plants. They are produced by fatty acid breakdown on the physical disruption of the tissues. *n*-Hexanal, one of the volatile aldehydes, is formed mainly from linoleic acid via its hydroperoxide intermediates (4, 5, 13). In the course of studies of 'rind yellow spot', a physiological rind disorder of Naruto (*Citrus medioglobosa* hort. ex Tanaka), it was found that affected fruits produced a higher amount of *n*-hexanal compared to healthy fruits on macerating the rind tissue. Lipid hydroperoxidation is known to damage biological membranes and considered to be a significant factor in aging (5). Since 'rind yellow spot' occurs in accordance with maturation and senescence of the rind (8), the present work was undertaken to investigate hexanal formation in the Naruto rind as related to

aging and disorder of the rind.

Materials and methods

Naruto fruits used in this study were selected only from the outside position on the tree to avoid positional effects within the tree, unless otherwise noted. An aqueous solution containing 100 ppm gibberellin (GA) or 500 ppm ethephon (CEPA) was sprayed to an individual fruit in early October of 1978, using one tree which was planted in Awaji experiment station. In addition, unsprayed control treatment was used. The 3 treatments were randomly allocated within a tree. These fruits were picked at intervals from late November to mid-March during maturation period. For comparison, untreated fruits from the inside (shaded) position on the tree were picked. During the period of occurrence of 'rind yellow spot' (late January to mid-March), both affected and healthy fruits were picked from trees growing in the experiment station and pomology orchards at Tsuna-cho, Awaji island. Picked fruits were kept overnight at 20°C in a polyeth-

Received for publication July 9, 1979.

ylene bag and used for measuring hexanal. Hexanal and hydroperoxides formed from added linoleic acid were measured for some of the fruits. Samples of 10 fruits were used for each determination.

In order to determine hexanal formed in the macerated rind tissues, portions of the rind (40 g) were peeled from fruits and albedo tissues were trimmed away with a razor blade. The remaining flavedo tissues were homogenized for 2 min with 100 ml distilled water in a 200 ml conical flask sealed with a rubber stopper at room temperature, and held in water bath at 40°C for 15 min. When hexanal formed from added linoleic acid was measured, 0.5 ml of 25% linoleic acid (obtained from Nakarai Chemical Co.) in ethanol was added to the homogenate before blending. *n*-Hexanal was determined by injecting 2 ml samples of headspace vapor into GLC. The chromatograph was operated under the following conditions: at 100°C isothermal; 3 m by 3 mm column with 10% FFAP on silanized Chromosob W; nitrogen flow 40 ml/min (Fig. 1).

In order to determine hydroperoxide, fla-

vedo tissues were homogenized in the presence of linoleic acid for 2 min as described above. The homogenate was mixed with 2 volumes of $\text{CH}_3\text{Cl-MeOH}(2:1)$ and shaken vigorously. The mixture was filtered through 2 layers of gauze and residue was washed with a volume of $\text{CH}_3\text{Cl-MeOH}(2:1)$. Lower layer of the filtrate was dried over anhydrous Na_2SO_4 and taken to dryness at 45°C under reduced pressure. The residue was dissolved in a small volume of *n*-hexan. An aliquot of the hexan extract was applied to silica gel TLC plate and developed in a solvent of ethyl-ether/hexan/acetic acid, 60/40/1. Hydroperoxide products were detected after spraying the mixture of 1 ml of 4% KI and 4 ml of acetic acid followed by a spray of 1% starch solution(12). Areas of plate bearing hydroperoxides are stained purple. These portions were scraped into vials and extracted with $\text{CH}_3\text{Cl-MeOH}(2:1)$. Aliquots of the extract were taken and after removing the solvent, the residue was dissolved in 2 ml of 50% ethanol. Hydroperoxides were measured with a xylenole orange according to a method of Michaels and Hunt(4). The reagent consists of $2 \times 10^{-4}\text{M}$ Mohr's salt, $5 \times 10^{-4}\text{M}$ xylenole orange and 0.05 N H_2SO_4 . To 2 ml of sample solution was added 4 ml of the reagent. After mixing, the color was allowed to develop for 1 hour and absorbance was measured at 450 nm.

Results

The fruit color began to change from green to yellow orange with the onset of November. On November 22, at the first sampling date, CEPA-treated fruits became yellow orange in color. Outside (untreated) fruits showed yellow green, while inside (untreated) or GA-treated fruits remained green. Although the color differences among treatments became less obvious as the season advanced, they persisted until the third or final sampling date. Small green areas were still present on the rind surface of inside and GA-treated fruits.

Table 1 shows *n*-hexanal formation from macerated rind tissues in the fruits given CEPA or GA, and untreated furits from the outside and inside positions on the tree.

Fig. 1. Gas chromatogram of volatiles from macerated rind tissues of Naruto. Chromatographic conditions given in materials and methods.

Table 1. Evolution of *n*-hexanal from outside and inside fruits, and CEPA- and GA-treated fruits.

Date	Outside	Inside	GA	CEPA
Nov. 24	0.11*	0.08	0.08	0.17
Jan. 18	0.11	0.05	0.06	0.21
Mar. 5	0.19	0.05	0.07	0.22
Mar. 22	0.18	—	0.06	—

* : nl/ml sample of headspace vapor.

 Table 2. Formation of *n*-hexanal from added linoleic acid (Sampled on 22nd of March).

	Linoleic acid	<i>n</i> -Hexanal*
Outside fruits	Without	0.18
	With	1.71
GA-treated fruits	Without	0.06
	With	0.57

* : nl/ml sample of headspace vapor.

Fig. 2. Detection of hydroperoxide products by thin layer chromatography. 1 : Linoleic acid; 2 : hydroperoxide of linoleic acid ; 3 & 4 : macerated rind tissues from outside and GA-treated fruits ; 5 & 6 : those from outside and GA fruits after heating.

The pH of the macerated rind tissues was about 5.5. Large amount of hexanal was found in the order: CEPA-treated fruits >

Table 3. Formation of hydroperoxide products from added linoleic acid (Sampled on 22nd of March).

	Condition	Hydroperoxide*
Outside fruits	No heating	0.109
	Heating**	0.034
GA-treated fruits	No heating	0.051
	Heating	0.031

* : μg equivalent of $\text{H}_2\text{O}_2/\text{g}$ fresh rinds.

** : For 10 min at 60°C before blending.

 Table 4. Evolution of *n*-hexanal from healthy and injured fruits.

Date	Site	<i>n</i> -Hexanal*	
		Healthy	Injured
Jan. 26	Okamura's	0.22	0.28
Mar. 5	Okamura's	0.11	0.23
	Hiraoka's	0.14	0.20
Mar. 22	Exp. Sta.	0.09[0.69]**	0.24[1.23]

* : nl/ml sample of headspace vapor.

** : with added linoleic acid.

outside fruits > inside or GA-treated fruits. It seems clear that the rates of hexanal formation were related to the degree of degreening throughout the experimental period.

On blending tissues with added linoleic acid, hexanal developed markedly. Since its formation was prevented partly by heating tissues at 60°C for 10 min, hexanal might be generated enzymically. Furthermore, optimum pH of the reaction was about 4.5. The rates of hexanal formation from added linoleic acid by the macerated rind tissues was compared between GA-treated and outside fruits. As shown in Table 2 the rates are higher in outside fruits than in GA-treated fruits.

The presence of hydroperoxide intermediates in the course of hexanal formation from linoleic acid was reported(4,13). In the present experiment, the hydroperoxide was detected by TLC(Fig.2), the amount being larger in outside fruits than in GA-treated fruits (Table 3).

Table 4 presents results of hexanal determinations from the rind tissues of the fruits with and without symptoms of 'rind yellow spot'. From this table, it was apparent that the rate of hexanal formation was high in the affected fruits compared to the healthy fruits. However, there was no difference

Table 5. Evolution of *n*-hexanal from healthy and injured portions of the rind within injured fruits.

Date	Site	<i>n</i> -Hexanal*	
		Healthy	Injured
Jan. 26	Okamura's	0.27	0.26
Mar. 22	Exp. Sta.	0.25[1.33]**	0.22[1.39]

* : nI/ml sample of headspace vapor.

** : with added linoleic acid.

Table 6. Effect of temperature during storage on the subsequent *n*-hexanal formation (Sampled on 22 nd of March).

Conditions unders storage		Linoleic acid	
		Without	With
For 20 hours at 20°C	Outside fruits	0.18*	1.71
	GA-treated fruits	0.06	0.57
For 5 days at 5°C	Outside fruits	0.04	0.21
	GA-treated fruits	0.03	0.18
For 5 days at 5°C, then for 4 hours at 20°C	Outside fruits	0.16	0.16
	GA-treated fruits	0.09	0.09
For 5 days at 5°C, then for 24 hours at 20°C	Outside fruits	—	—
	GA-treated fruits	—	—

* : nI/ml sample of headspace vapor.

between the rates of hexanal formation from areas showing visible and no symptoms within the affected fruits (Table 5).

The rates of hexanal formation were found to vary significantly according to ambient temperatures to which fruits had been exposed. As indicated in Table 6, the rates are much higher in the fruits held at 20°C than those held at 5°C. This is not due to a difference in the tissue temperature during blending. When the fruits held at 5°C was transferred to 20°C, the rate increased within 3–4 hours.

Discussion

GA delays certain aspects of aging of rind tissues of citrus fruits when applied at pre-harvest(1). Because of this, GA is used for reducing rind disorders that appear during maturation period (1). But, unfortunately GA application results in greener-colored fruit at harvest, and such undesirable effect on the fruit color cancels desirable effect on the disorder(3). This is equally true of 'rind yellow spot', a physiological rind disorder of Naruto. Therefore, an improved method of reducing the disorder is needed. However, little is known about the

mechanism of the antisenescence action of GA.

Coggins et al (2) studied the relationship between GA-delayed senescence and changes of essential oils in the navel orange rind, and found that concentrations of certain oil constituents such as valencene varied with the degree of senescence. In the course of studies of 'rind yellow spot', it was found that *n*-hexanal, one of the volatile aldehydes, was generated upon macerating rind tissues of Naruto. Volatile aldehydes have been known as major components of the characteristic flavor in several plants which are produced on physical disruption of their tissues(5). They are also known as secondary products formed as the results of lipid peroxidation(4, 5, 13) which has been considered to be a significant factor in aging(5). Thus an attempt was made to see relations between hexanal formation and aging of the rind and 'rind yellow spot' which occurs in accordance with aging of the rind.

Results presented in Table 1 indicates that rates of hexanal formation closely corresponded with the degree of rind senescence. GA treatment lowers the rate, whereas CEPA treatment enhances it. Hexanal is formed mainly from linoleic acid and lipoxygenase may initiate its formation(4, 5, 13). In the present experiment, the rates of hexanal formation were increased by the addition of linoleic acid; reduced by heating rind tissues before blending; affected by pH during blending; influenced by the temperature to which fruits had been exposed. Furthermore, hydroperoxide intermediate products were detected, amounts being related to those of formed hexanal. These lines of evidence suggest that the hexanal formation in the macerated rind tissues is catalyzed by enzyme systems and that lipoxygenase may be involved in this reaction. In tomato amounts of volatile aldehydes were related to the stage of maturity(10, 11). In ripening apple, an increase in lipoxygenase activity was observed during the climacteric period(15). Recently, Grossman and Leshem(6) have reported that lipoxygenase activity was significantly reduced by cytokinin-treated pea leaves. Thus it seems probable that GA

acts as a senescence retardant at least in part by lowering potential of the rind for the hexanal formation.

As shown in Table 4, the rind from the affected fruits had a high rate of hexanal formation compared to that of the healthy fruits. Whether this difference implies the physiological importance for development of the disorder remains unknown. However, the possibility exists that the elevated rates of hexanal formation might be associated with development of the disorder. The affected fruit evolved higher amount of ethylene than the healthy fruit as reported in the previous paper(9). Wooltorton et al(15) showed that in ripening apple an increase in lipoxygenase activity was accompanied by increase in ethylene production, suggesting the involvement of lipoxygenase in ethylene production.

Further evidence in support of the above mentioned possibility was shown in data of temperature effects on the hexanal formation. Incidence of the disorder is severe in warm areas and warm years during maturation period. High incidence has been observed when fruits were grown in glasshouse (7). Good evidence thus indicates that the fruits exposed to higher temperature in the daytime develop more incidence. As shown in Table 6, the rate of hexanal formation markedly increased with a rise in temperature to which fruits had been exposed. This may again correspond with the previous findings(9) that chilled affected fruits repeated peaks of ethylene evolution upon transfer from 5°C to 20°C. Although the evidence was based on the observations of detached fruits, similar results may be obtainable on attached fruits on the tree. If this is the case, high day temperatures during maturation period result in high potential for the hexanal formation which in turn could be responsible for the development of the disorder.

Finally, we reported that the degree of susceptibility to the disorder varied significantly according to age of the rind: the order of the susceptibility was CEPA-treated fruits > outside fruits > inside or GA-treated fruits. This coincides closely with the rates

of hexanal formation by these fruits. Since the rates among the fruits had already differed before any sign of the disorder was evident, one may reasonably assume that low potential for the hexanal formation is associated with increase in resistance to the disorder. Further work is needed to clarify causal relationship between the hexanal formation, and the aging and disorder of the rind of Naruto.

Acknowledgment

The authors are particularly indebted to Dr. M. Nataka, Professor of Kobe University for his useful suggestions during the work and for his help in the preparation of this paper.

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ナルトミカンの果皮組織における *n*-ヘキサナールの生成 特に果皮の老化と生理障害（果皮黄斑症）に関連して

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摘 要

ナルトミカンの果皮組織（フラベド）の磨碎物中に *n*-ヘキサナールの生成が認められた。ヘキサナールの生成は酵素反応によるものと思われ、その至適 pH は 4.5 付近にあった。

果実の成熟期の 11 月下旬～3 月中旬に、ジベレリン (GA) 処理果、エセホン (CEPA) 処理果、外なり及び内なり果を採取し、果皮組織のヘキサナール生成量を比較した。生成量は、CEPA 処理 > 外なり > 内なりまたは GA 処理の順に多く、果皮の着色または老化の程度と密接な関係がみられた。

ナルトミカンの生理障害である果皮黄斑症の発生期の 1 月下旬～3 月中旬に、障害果及び健全果を採取し、ヘ

キサナール生成量を比較した。生成量は障害果の果皮の方が多かったが、障害果の果皮の肉眼的異常部分と正常部分との間では差がなかった。

ヘキサナール生成量は、測定前の果実が置かれていた温度によって異なり、20℃ に置いた果実の果皮は 5℃ のものに比して著しく多かった。また、5℃ から 20℃ に果実を移すと、3～4 時間で生成量が増加した。

以上、果皮組織の *n*-ヘキサナール生成能は、果皮の老化の進展、高温などによって高まり、その増大は老化に伴って起こる果皮黄斑症の発生にも関係しているように思われる。