JARQ 29, 179-185 (1995)

Simultaneous Measurement of ABA, IAA and Gas in Citrus – Role of ABA in Relation to Sink Ability

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Abstract

Simultaneous measurement of concentrations of ABA, IAA and Gas from one extract was investigated. A simply assay of total Gas content could be obtained as only the early effluent on HPLC from ether and ethyl acetate extracts was collected, diluted gradually and applied for the bioassay together. This method was found to be suitable for application to citrus fruits and seedlings. Next, the distribution and changes in ABA and IAA concentrations in Valencia orange fruits were analyzed. Seeds showed a large peak of ABA (21 nmol/g fresh weight). In the central vascular axis, the IAA concentration reached a peak 119 days after full bloom. It is suggested that ABA in the seed and IAA in the central vascular axis play a role in the accumulation of assimilates. The effects of exogenous ABA on the changes in fruit drop, ABA catabolism, and the contents of sugars and organic acids of citrus fruits were evaluated *in vivo* by injection. Exogenously applied ABA was catabolized to about 10% in the fruits within 4 days. Injections of ABA resulted in the increase in the glucose and fructose concentrations, but not in that of the organic acids in juice. ABA appears to play a role in the increase of the sugar concentration in juice.

Discipline: Experimental apparatus and methods/Horticulture
Additional key words: ABA catabolism, bioassay, fruit drop, rootstock, Valencia orange

Introduction

Fruits grow relatively more rapidly than vegetative tissues in woody species. Mobilization of assimilates to the growing fruit often proceeds at a faster rate than that of the capacity of leaves to supply photosynthates[19]. Thus, the physiological status of fruit is drastically altered and is considered to be controlled by phytohormones. At least, abscisic acid (ABA), indole-3-acetic acid (IAA) and gibberellins (GAs) are involved in this process.

ABA, which was originally discovered as an abscission-promoting hormone, has been reported to be a growth-inhibitory substance[1,21,25]. However, a positive relationship between ABA level and the growth rate has also been reported in seeds of beans[10], peas[4], soybeans[9] and in spears of asparagus[15]. Moreover, it is known that in the Japanese citrus industry, cultivation methods which induce water deficiency in plants resulted in the production of sweeter fruits: e.g. growth in a container, growth in a greenhouse, control of root area using a sheet, covering of ground with waterproof sheet, etc. Generally, ABA content in plants remarkably increases by water deficiency[25]. Thus, ABA may play a role in the accumulation of assimilates by strengthening the sink activity[3].

To analyze the role of phytohormones, it is necessary to determine the level of endogenous hormones. The measurement of phytohormones using whole seeds[9] and whole fruits[11,12,16] masked the changes in the hormonal level in the component parts. Thus, to minimize sample quantity for analysis, it is important that the concentrations of ABA, IAA and GAs are determined from the same sample.

Most of the investigations have involved separate measurements of the individual phytohormones and there are few studies in which two or more classes of hormonal compounds have been determined from one extract. In recent years, simultaneous measurement of the concentrations of ABA and IAA has been reported[8,6,24]. However, there is no report on the simultaneous measurement of the concentrations

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of ABA, IAA and GAs from the same fruit.

As for the GAs assay, coexisting inhibitory substances, such as chlorogenic acid and ABA sometimes suppressed or masked the GAs activity in plant extracts[18]. To reduce the effects of inhibitory substances, a method in which the products were separated into a small fraction after thin layer or column chromatography has been used. Recently, the sensitivity of the micro-drop assay to GAs using dwarf rice has increased considerably by treatment with an inhibitor of GA biosynthesis[20].

Procedure for simultaneous measurement of ABA, IAA and GAs[14]

1) Extraction, purification and fractionation of phytohormones

Fig. 1 shows the flow diagram of purification, fractionation and determination of plant hormones. To the tissues of each fruit and its parts (2–3 g fresh weight) 10,000 dpm ^{3}H-ABA (purchased from Amersham, Buckinghamshire, England) and 200 pmol ^{13}C-IAA (purchased from MSD Isotopes, Montreal, Canada) were added as internal standards. Whole fruit tissues were homogenized in 50 ml of 80% ethanol with 0.3 g of soluble polyvinylpyrrolidone (PVP, K-30). After the filtrates were concentrated *in vacuo* to the aqueous phase, the aqueous phase was adjusted to pH 2.5 with H_3PO_4. The acidified

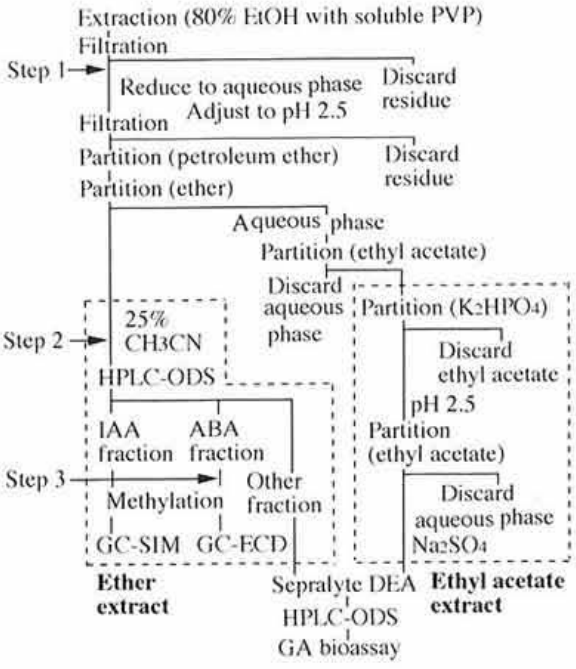

Fig. 1. Flow diagram of purification, fractionation and determination of phytohormones in citrus

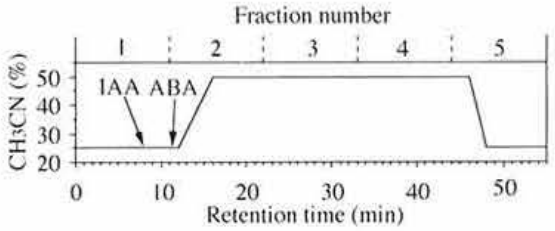

Fig. 2. Changes in acetonitrile concentration in HPLC eluent, and designation of fraction number

phase was filtered through Millipore filters (0.2 mm pore size, Nihon Millipore, Yonezawa, Japan). The aqueous filtrate (ca. 50 ml) was partitioned three times against 50 ml of petroleum ether and the organic layer was discarded. The aqueous phase was partitioned three times against 50 ml of diethyl ether and the organic layer was evaporated to dryness.

Dried extracts were dissolved in 0.2 ml of 25% acetonitrile, and were fractionated with an HPLC system equipped with a UV and a fluorescence detector. The column used for HPLC was Inertsil ODS-2 (150 × 6.0 mm, GL Sciences Inc. Tokyo). The column temperature was maintained at 40°C. The sample was eluted with a mixture of 25 and 50% acetonitrile solution (20 mM acetic acid) at a flow rate of 1.6 ml/min as shown in Fig. 2. The fractions were collected every 11 min and designated as fractions 1–5, respectively. The effluent corresponding to the retention time of ABA and IAA was collected, respectively.

2) ABA analysis

The methylated sample was injected into a GC system equipped with a ^{63}Ni electron capture detector using a fused silica glass capillary column. A portion of the methylated sample was injected into the HPLC system and the methylated ABA fraction was collected. The radioactivity was measured in a scintillation counter. Data were corrected according to the recovery rates, respectively.

3) IAA analysis

Determination of IAA content was carried out according to the method of Cohen et al.[5]. The methylated sample was injected into a gas chromatograph equipped with mass spectrometry (QP-5000, Shimadzu Inc., Kyoto) using the split-less technique. The injection port was maintained at 250°C. A fused silica capillary column (CBP 1, 25 m × 0.22 mm i.d., 0.25 mm film thickness, Shimadzu Inc.)

was used. The oven was programmed from 2 min at 100 to 280°C at 30°C/min and then a temperature of 280°C was maintained for 15 min.

4) GAs analysis

The aqueous phase (ca. 50 ml) after partitioning against diethyl ether was partitioned two times against 50 ml of ethyl acetate and the aqueous phase was discarded. The ethyl acetate layer was partitioned two times against 50 ml of 0.2 M K_2HPO_4 and the organic layer was discarded. The aqueous phase was adjusted to pH 2.5 with H_3PO_4. The acidified phase was partitioned two times against 50 ml of ethyl acetate. The ethyl acetate layer was dried over anhydrous Na_2SO_4 overnight.

The dried ethyl acetate layer and the fractions except for ABA and IAA fractions in the ethyl extract were combined and purified by Sepralyte DEA (40 μm, Analytichem International, Harbor City, Ca., USA) according to the modified method of Goto et al.[7]. The column of Sepralyte DEA (0.3 g) was washed with 2 ml of methanol. The combined sample was dissolved in 1 ml of methanol, loaded onto the column and washed with 1.3 ml of methanol. The column was eluted with 6 ml of 0.5% acetic acid in methanol and the eluate was evaporated. The purified extract was fractionated by HPLC as previously described.

Fraction 1 plus 2 of the ethyl plus the ethyl acetate extracts from fruit parts was used for the GAs assay. The bioassay procedure used was similar to the 'modified micro-drop bioassay'[20]. Seeds of dwarf rice (*Oryza sativa* L., cv. Tan-ginbozu) were sterilized and soaked in water containing S-3307 (20 mg/l, Sumitomo Chemical Co., Takarazuka, Hyogo, Japan) in darkness for 24 h at 30°C. Then the seeds were rinsed and germinated in water under the same conditions. When the coleoptiles were ca. 4 mm long, seedlings were planted on 0.8% (w/v) agar and incubated for 72 h at 30°C, under continuous illumination with cool white fluorescent lamps (5,500 lx). Standard (GA_3) and samples were dissolved in 50% (v/v) aqueous acetone. One μl of the test solution was applied with a microsyringe to the region between the coleoptile and the first leaf of a seedling. Three days later, the length of the second leaf sheath was measured.

Results of simultaneous measurement of ABA, IAA and GAs

1) Development of method[14]

When both ether and ethyl acetate extracts were chromatographed on HPLC, almost all the GAs activity was recovered in fractions 1 and 2 (Table 1). Thus, the collection of the effluent from 0 to 22 min retention time on HPLC is suitable for a simple measurement of the total GAs activity. As these fractions were gradually diluted with 50% acetone for the GAs assay, the GAs activity value tended

Table 1. GAs activity in HPLC fractions of ether and ethyl acetate extracts from whole fruits (50 g fresh weight)

Dilution 50% acetone (μl)	Threshold (ng)	GAs activity (ng GA_3 eq.) Fraction number 1	2	3	4	5
Ether extract						
20	0.2				BL (87)	BL (78)
100	1	2 (118)	13 (151)	BL (85)	BL (79)	BL (75)
500	5	9 (113)	12 (122)	BL (94)	BL (75)	BL (71)
2500	25	BL (99)	40 (113)	BL (93)		
12500	125	BL (87)	BL (93)	BL (86)		
Ethyl acetate extract						
20	0.2				1 (141)	0.2 (111)
100	1			1 (110)	BL (78)	BL (79)
500	5	BL (96)	20 (122)	BL (90)	BL (75)	BL (72)
2500	25	28 (101)	30 (104)	BL (90)		
12500	125	BL (72)	BL (73)			

The fractions were diluted with 50% acetone for the GAs assay.
The threshold is based on the sensitivity of the rice seedlings treated with 0.01 ng of GA_3.
BL: Content below the threshold for measurement of GAs activity.
Values in parentheses represent the percentage of the length of the second leaf sheath treated with 0.01 ng GA_3.

to increase until the threshold for measurement of GAs activity was reached, presumably, because the inhibitory substances on the elongation of the rice seedlings were diluted. Thus it is considered that a more reliable value of GAs activity can be obtained if the sample is diluted in more minute steps to reach the threshold for the measurement of GAs activity. Consequently, when only the early effluent on HPLC from ether and ethyl acetate extracts was collected, diluted gradually and applied to the bioassay together, a simple assay of total GAs content was possible. Furthermore, by determining the GAs activity in HPLC fractions, the method could be applied to a broad range of plant tissues.

2) Phytohormone distribution

Using the procedure developed, the distribution of ABA, IAA concentrations and GAs activity within the citrus fruit 60 days after full bloom (DAB) was analyzed[14] (Table 2). ABA and IAA were present in the pulp and GAs activity in the axis as highest concentrations.

Next, the concentrations of ABA, IAA and GAs in seedlings of the rootstock variety were analyzed[17] (Table 3). IAA concentration of roots in Shiikuwasha was about three times higher than in other varieties, suggesting that the high content of IAA suppressed root growth. GAs concentration of roots in trifoliate orange was about three times higher than in other varieties, suggesting that GAs produced in roots translocate to the shoots and promote the shoot growth.

Distribution of ABA and IAA within orange fruit[12]

Bain[2] divided the fruit development of Valencia orange into three stages: stage I, corresponding to the period of cell division; stage II, to the period of rapid enlargement of cells; stage III is considered to correspond to the maturation period. Waynick[23] reported that maximum growth of Valencia orange fruit occurred approximately between September and December within stage II.

The seeds of Valencia orange showed a large peak of ABA concentration at 150 DAB (Fig. 3). Ackerson[1] suggested that ABA in developing seeds played a role in the suppression of precocious germination. However, ABA in developing seeds of

Table 2. Distribution of ABA, IAA and GAs concentrations within citrus fruits 60 days after full bloom

Part	Fresh weight (g/fruit)	ABA (ng/g FW)	IAA (ng/g FW)	GAs (ng GA_3 eq./g FW)
Axis	0.26	140 ± 5	15 ± 1	2.0
Pulp	2.30	800 ± 71	31 ± 4	1.2
Peel	7.39	130 ± 13	2 ± 1	1.3
Calyx	0.20	170 ± 34	29 ± 2	1.6
Total	10.15	284[a)]	10[a)]	1.3

All the values of ABA and IAA concentrations are means ± SE of 3 determinations.
a): Calculated values using part values.

Table 3. Height, fresh weight and concentrations of ABA, IAA and GAs in seedlings of three citrus varieties for rootstock

Variety	Height (mm)	Fresh weight (g)	ABA (ng/g FW)	IAA (ng/g FW)	GAs (ng GA_3 eq./g FW)
Shoot					
Trifoliate orange	107 ± 5	2.93 ± 0.11	98 ± 1	1.5 ± 0.1	0.12
Hiryo	68 ± 3	1.41 ± 0.06	63 ± 1	1.6 ± 0.2	—[a)]
Shiikuwasha	67 ± 3	1.68 ± 0.21	40 ± 1	1.4 ± 0.1	0.11
Root					
Trifoliate orange		2.71 ± 0.19	17 ± 1	1.1 ± 0.1	0.99
Hiryo		1.71 ± 0.07	26 ± 2	1.2 ± 0.1	0.36
Shiikuwasha		1.22 ± 0.07	15 ± 1	3.6 ± 0.3	0.37

Height (n = 10), fresh weight (n = 5), and concentrations of ABA and IAA (3 determinations) are means ± SE.
a): less than 0.1 ng GA_3 eq./g FW.

Valencia orange in stage II seemed to play different roles, because the ABA concentration of the seed reached a peak and decreased conspicuously at 188 DAB. Brenner[3] stated that ABA promotes unloading of assimilates from the phloem into the sink as well as sink activity. If this assumption is valid, ABA may promote the sink activity in the seeds of Valencia orange during stage II.

The central axis region of a citrus fruit, which includes axial vascular bundles with the phloem connected to the developing seed, contained a large amount of IAA (Fig. 4). In tomato fruit, the axis region showed a low concentration of IAA[11], but a high concentration of ABA[16] during the cell enlargement stage. Thus the high concentration of IAA in the central axis region may be ascribed to the fact that it contains the axial vascular bundles which feed the developing seed.

Effects of ABA application on sugars and organic acids of fruit juice[13]

In citrus fruit, the effects of ABA can be analyzed by injection of the compound. As the central axis region contained spongy parenchyma cells[22], it absorbed the liquid injected. The advantages of the injection are that a definite amount of ABA can be applied to the fruit flesh through the hard skin and that the isomerization into trans-ABA and breakdown by sunlight can be avoided.

To determine the threshold of exogenously applied ABA which does not cause abscission of fruits during a period of about two weeks, ABA was injected at three levels into 100 fruits for each level. The larger the amount of ABA, the higher the incidence of fruit drop after ABA injection (Fig. 5). Ninety-two percent of the fruits remained 16 days after the injection of 0.2 μmol ABA, while only 81% remained after the injection of 1.2 μmol ABA. Next, to determine the period during which injected ABA is catabolized, both ABA (0.2 μmol) and radiolabelled ABA were injected into the fruits. Radioactivity in step 1 (Fig. 1) remained at an almost constant level, while in step 2 it gradually decreased (Fig. 6). In step 3, the radioactivity decreased more rapidly to 10% four days after the injection. Thus, it was demonstrated that repeated injection of exogenous ABA is required to induce a hormonal effect.

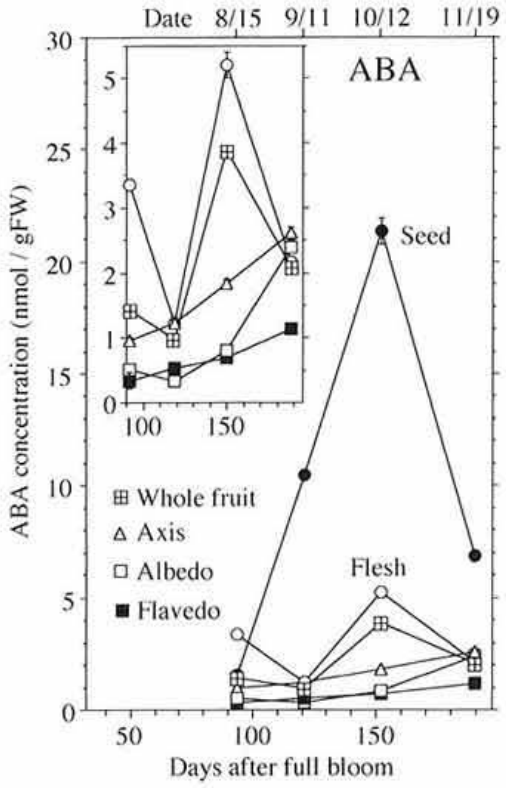

Fig. 3. Changes in ABA concentrations of the developing Valencia orange fruit and its parts
Means of 3 determinations and their SE are shown (n = 3).
The whole fruit value was calculated using the part values.

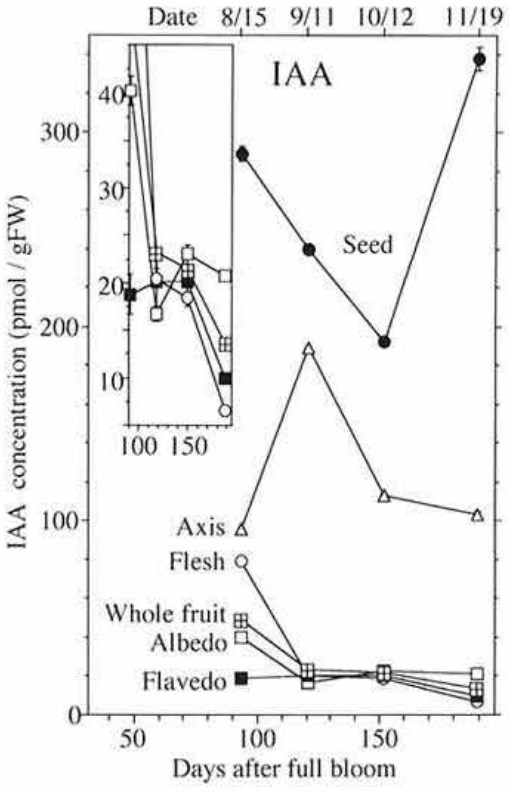

Fig. 4. Changes in IAA concentration of the developing Valencia orange fruit and its parts

Based on previous results of the experiments on fruit drop and ABA catabolism, the amount of ABA for injection per fruit was 0.2 μmol and the interval between the injections was three or four days. ABA did not affect the fruit weight and organic acid content in both injections 1 and 2 (Table 4). However, ABA application increased significantly the glucose and fructose concentrations in both injections 1 and 2, and sucrose injection 2, respectively.

The increase in the sugar level induced by ABA can be ascribed to three factors as follows: (1) ABA reduces the juice content and concentrates sugars. This assumption is unlikely, because there was no difference in the fruit weight between the control and ABA-injected fruits. (2) ABA may also release sugars from storage carbohydrates and/or cell materials in segment membrane and fruit peel. (3) ABA injection mobilizes sugars from leaves and/or other fruits via the phloem. Thus further studies are required to investigate the changes in the carbohydrate components of the segment membrane and fruit peel, and the changes in the phloem transport after ABA treatment.

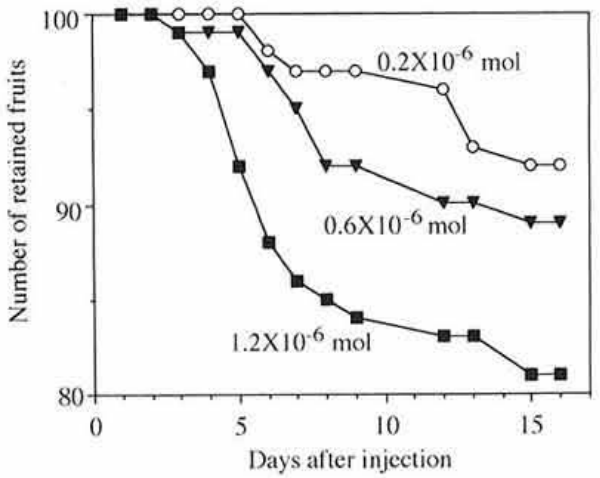

Fig. 5. Changes in the number of fruits retained during 16 days after ABA injection
Each amount of ABA was injected into 100 fruits.

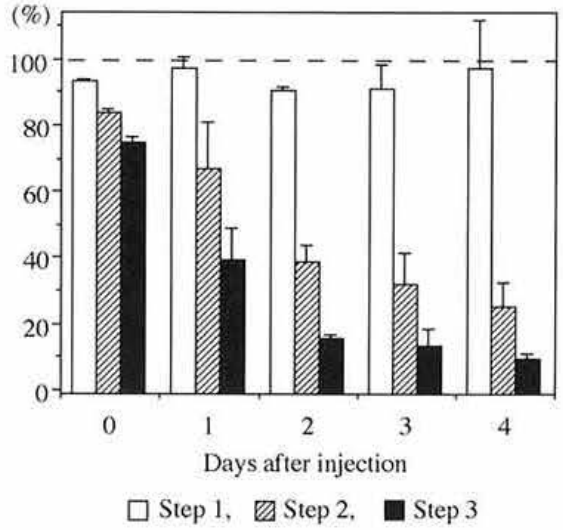

Fig. 6. Changes in the radioactivity of the injected ^{14}C-ABA in 3 purification steps from citrus fruits during 4 days
Data are expressed as the percentage of the total radioactivity injected on the treatment day. Step 1 refers to the crude extract, step 2 refers to the organic layer after solvent partitioning, and step 3 to the fraction corresponding to the retention time of ABA on HPLC (Fig. 1).

Table 4. Effects of ABA injection on the concentrations of sugar components and organic acids of fruit juice of citrus

Treatment[a]	Weight (g/fruit)	Sugar[b] (%)			Organic acid (% citric acid eq.)
		Sucrose	Glucose	Fructose	
Injection 1					
Control	151 ± 5	2.14 ± 0.07 (100)	1.12 ± 0.04 (100)	1.19 ± 0.04 (100)	2.33 ± 0.07
ABA	152 ± 5	2.37 ± 0.11 (111)	1.30 ± 0.05* (116)	1.37 ± 0.06* (115)	2.23 ± 0.06
Injection 2					
Control	304 ± 7	3.36 ± 0.19 (100)	1.36 ± 0.08 (100)	1.48 ± 0.09 (100)	1.73 ± 0.03
ABA	315 ± 6	4.14 ± 0.14* (123)	1.68 ± 0.08* (126)	1.83 ± 0.09* (124)	1.70 ± 0.07

Injection 1 refers to the experiment conducted during 11 days from Sept. 3, and injection 2 to the experiment conducted during 9 days from Oct. 27.
* Significant difference from the data of control at $P \leq 0.05$.
a): Injection 1; n = 10, Injection 2; n = 18.
b): Values in parentheses indicate percentages of controls.

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(Received for publication, Dec. 5, 1994)