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Effects of Gibberellic Acid and Gibberellin Biosynthesis Retardants on Ethylene Production, Batatasins, and Free Sugars in Dormant Tubers of Chinese Yam

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ABSTRACT: Gibberellic acid did not affect ethylene production, whereas gibberellin biosynthesis inhibitors triggered ethylene production in dormant tubers. Gibberellic acid did not induce sprouting of dormant tubers, however, treatment of gibberellin biosynthesis retardants enhanced sprouting rates. Sprouting rate in ancymidol-treated tubers was highest among gibberellin biosynthesis retardants. Sprouting rate of tubers treated with ancymidol increased to 91.4%. Batatasin-III content in GA_3 treated tuber was increased in the highest concentration (30 μg l^{-1}). Tubers treated with mepiquat chloride, Batatasin-I was increased steadily, but contents of Batatasin-III and V showed dramatic decrease at the 1,000 μg l^{-1} concentration. This infers that gibberellin biosynthesis retardants play key roles in promoting breaking dormancy on dormant tubers of Chinese yam.

Keywords: Chinese yam, gibberellic acid, mepiquat chloride, ancymidol, trinexapac-ethyl, ethylene, batatasins, sugars

The storage of Chinese yam (*Dioscorea* spp.) tubers is dependent on their endogenous dormancy (Okagami & Nagao, 1971). Provided that tubers are damaged and disease-free, they can be stored satisfactorily throughout the dormant period. Once dormancy has broken and sprouting occurs, stored tubers rapidly senesce, carbohydrate is quickly broken down and accompanied by tuber weight loss and increase of sucrose concentration. It was reported that the dormant period of *Dioscorea alata* L. tubers is about 16 weeks. The tubers of *Dioscorea rotundata* Poir. also maintain dormant for about 14 weeks (Adesuyi, 1973), while that of *Dioscorea esculenta* (Lour.) Burk is around eight weeks (Passam, 1978).

Apart from Chinese yams, dormancy period of *Dioscorea opposita* (Thunb.) are relatively much longer than tropical Chinese yams, and their, tubers and bulbils remain dormant for about 22 weeks. For these reasons, considerable economic benefit may be obtained if the dormancy of these tubers could be shortened by just few weeks. Consequently, an early harvesting results from early planting allowed by shortening dormancy periods in the Chinese yam.

Batatasins (batatasin-I, batatasin-II and batatasin-III) has been first identified and isolated in yam bulbils (Hashimoto *et al.*, 1972). These compounds, which are produced *de novo* or show increased production on elicitation, together with batatasins I to V have been implicated as dormancy inducing compounds in *D. opposita* (Ei-Olemy & Reisch 1979; Hashimoto *et al.*, 1972). In the Chinese yam, in particular, batatasin-III is closely related to dormancy-inducing compound (Passam & Ireland, 1985).

Dormancy of the Chinese yam, is induced by exogenous gibberellins (GA_4, GA_3, and GA_1) or endogenous gibberellins (Okagami & Nakao, 1971). However, exogenously applied gibberellic acid (GA_3) was found to extend bulbil dormancy and to inhibit sprouting in bulbils of temperate *Dioscorea* species, and it was suggested that endogenous GA_3 also plays some physiological roles in bulbil and tuber dormancy.

Dormancy of yam is not broken by gibberellin (specially, GA_3) and is re-induced when stratified bulbils and tubers are treated with gibberellins. This action of gibberellin is inconsistent with the dormancy-breaking activity of this growth promoter in seeds or buds of many plant species (Okagami & Nagao, 1971). Naturally occurring growth inhibitors, termed batatasins, discovered in *Dioscorea opposita*, have been found to increase during bulbil development and to be associated with dormancy (Hasegawa & Hashimoto, 1972, 1974). For that case, we supposed that the treatment of gibberellin biosynthesis retardants to dormant tubers may be involved in breaking the dormancy or shortening the dormancy period, resulting in an early sprouting of dormant tubers in Chinese yam. After harvesting the tubers, sprouting rate was promoted by storeing in airtight sealing-bags as compared to the tubers stored in a temperature-controlled room (unpublished data). There are several growth retar-

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dants were reported to inhibit gibberellin biosynthesis, and four types of such inhibitors are known: (1) onium compounds, such as chlormequat chloride, mepiquat chloride (MC), chlorphonium, and AMO-1618, (2) nitrogen-containing heterocyclic comounds, ancymidol (AN), flurprimidol, tetcyclasis, paclobutrazol, uniconazole-P and inabenfide; (3) acylcyclohexanediones, prohexadione-calcium, trinexapac-ethyl (TNE) and daminozide, and (4) 16,17-dihydro-GA_5 and related structures (Rademacher, 2000).

The objective of this study was focuses on possible effect of gibberellin biosynthesis retardants to promote the sprouting of tubers, changes of endogenous batatasins content, ethylene production, and sugar content during dormant tubers in the Chinese yam.

MATERIALS AND METHODS

Plant material and sampling

Dioscorea opposita Thunb. cv. Tsukune were planted at the experimental field of the Institute of Bioresources Research in Gyeongbuk Provincial Agricultural Technology Administration on June 15, 2001. The Chinese plants were grown as common cultural manner, and the tubers were harvested on November 22, 2001, and the whole tubers (approximately 70 - 80 g, fresh weight) were stored in a temperature-controlled in the dark at 4 ± 0.5 °C at a relative humidity of 85 ± 1% for two months. The concentration of endogenous batatasins, ethylene, and free sugars in the tuber were analyzed. Tubers were immediately frozen with liquid nitrogen and stored at -70 °C until analysis of batatasins and free sugars.

Application of GA_3 and GA biosynthesis retardants

Intact tubers stored in a controlled environment chamber were washed in distilled water and soaked in GA_3 (0.3, 3, and 30 μg l^{-1}) and plant growth retardants following ancymidol (AN: 1, 10 and 100 μg l^{-1}), mepiquat chloride (MC: 10, 100 and 1,000 μg l^{-1}), trinexapac-ethyl (TNE: 5, 50 and 500 μg l^{-1}) at 25 °C for 24 hr in the dark. After soaking, tubers were placed into 1.5l-sealed vessels (5 tuber per vessel, fifteen replicates per chemical treatment), and then incubated at 25 °C in the dark until analysis of ethylene, batatasins, and free sugars.

Measurement of ethylene

Ethylene concentration was determined in the tubers the which were treated with GA_3 and gibberellin biosynthesis retardants and stored for two months. Ethylene gas were determined by removing 1 m*l* samples of gas from the vessels and analyzed by gas chromatography (Hewlett Packard 6890 Series). Poropak Q (80 - 100 mesh) was used in a glass column (2500 mm × 6.5 mm). The temperature of the column, injector, and flame ionization detector were 70, 100 and 160°C respectively The ethylene peaks were identified by a retention time, flow rates were 20 m*l* min^{-1} for He, 40 min^{-1} for H_2. The identification of ethylene peak was separately confirmed on other samples by repeating the injection after exposing the vessel atmosphere to potassium permanganate solution (0.1 M) as an ethylene absorber. Data were recorded as μ*l* of C_2H_4 kg^{-1} h^{-1}.

Analysis of batatasins

For the analysis of batatasins by GC-MS-SIM (Hasegawa & Hashimoto, 1975; Wallstedt *et al.*, 1997). The lyophilized tubers (1 g, DW) were homogenized in 50 m*l* of cold acetone and filtered. The acetone extract was evaporated to dryness and chromatographed on TLC plates [Silica gel : chloroform-methanol (97 : 7, v/v)] and the band corresponding to each of batatasin I, III and V were scrapped and eluted with ethylacetate. Each fraction was evaporated to dryness, derivatized at 70 °C for 30 min with 30 μ*l* of BSTFA in 25 μ*l* of pyridine containing 10 ng μl^{-1} of 1,3-dihydroxynaphthalene as an internal standard.

Finnigan GCQ Mat equipped with a fused silica DB-5 column (30 m × 0.25 mm, 0.25 μm, J & Scientific) was used for the quantification of each batatasin in the samples The column temperature was initially 150 °C with a linear increase of 7 °C min^{-1} to 285 °C. Helium gas was used as carrier gas at a flow rate of 38 cm sec^{-1}. The each sample was injected to 1 μ*l* Identification of each batatasin I, III, and V was determined with retention time and ion spectrum (356, 388, 360 *m/z* for B-I, B-III, and B-V) with external standards (a obtained from Professor, Tohru Hashimoto, Dept. of Life Sciences, Kobe Women's University, Kobe, Japan). For quantification of these compounds, a standard calibration curve was drawn by plotting the area ratio between batatasins I, III, and V and 1, 3-dihydroxynaphthalene for eight different concentrations of (10 - 600 ng μl^{-1}).

Analysis of sugars

For the extraction of free sugars (sucrose, fructose, and glucose) by some modification (Sinniah *et al*, 1998), the lyophilized tubers (1 g, DW) were homogenized in 50 m*l* of 80% ethanol and sonicated at 30 °C for 1 h. Homogenate was filtered and supernatant was passed through a Sep-Pak C_{18} (Waters, Co, USA), the few drops were was discarded and the 2.5 m*l* was collected. A standard calibration curve

was prepared by injecting 2, 6, 10, 14 and 18 μl injected of the standard solution. The 30 μl of each aliquot was subjected to HPLC analysis by RI detector (Waters). The separation of sugars was achieved by carbohydrates column (4.6 mm × 250 mm), heated to 80 °C. The mobile phase was a solution (acetonitrile : water, 75 : 25, v/v). Flow rate of mobile phase was 1.2 ml min^{-1}. Identification of sugars was by comparison of retention times with known standards (Sigma Chemical Co.).

RESULTS

Effects of GA_3 and GA biosynthesis retardants on ethylene production and sprouting in dormant tubers

Ethylene production in dormant tubers treated with GA_3 and GA biosynthesis inhibitors were evaluated for five days after incubation in continuous dark (Table 1) Ethylene was not detected during two days of incubation GA_3 did not affected ethylene production, whereas ethylene production in two gibberellin biosynthesis retardants, ancymidol and trinexapac-ethyl, were higher than those of GA_3 and control. GA_3 treatment showed no effect on sprouting of tubers. While, MC, AN, and TNE affected to ethylene production and promotion of sprouting rate in tubers Ethylene production in AN treated tubers (at 1 μg l^{-1}) was highest to 21 9 μl and TNE-treated tubers were also highest at 5 μg l^{-1}, in the 3 days after incubation MC treated tubers were lowest among three GA biosynthesis retardants Ethylene production was enhanced in the order of AN > TNE > MC > GA_3 = control.

We also investigated effects of GA_3 and GA biosynthesis retardants on sprouting of fully dormant tubers (Table 2) Sprouting rate was checked at 5 DAI, which showed highest level in ethylene production (Table 1). GA_3 did not induce sprouting of dormant tubers, however, sprouting rate of MC and TNE treated tubers was lower than that of AN treated tubers. Sprouting rate in AN treated tubers was increased to 91.4%.

Table 2. Effects of GA_3 and GA biosynthesis retardants on sprouting rates in dormant tubers of *Dioscorea opposita* cv Tsukune

Chemicals [1]	Treatments (μg l^{-1})	Sprouting rate (%) after 5 days
Control		–
GA_3	0 3	–
	3	–
	30	–
MC	10	32.6 e
	100	36.1 e
	1000	37.5 e
AN	1	91.4 a
	10	62 8 b
	100	35.0 e
TNE	5	53 2 c
	50	42 8 d
	500	32 1 e

[1] GA_3, gibberellic acid; MC, mepiquat chloride, AN, ancymidol ; TNE, trinexapac-ethyl
The same letters in each column were not significantly different at the 5% level by DMRT.

Table 1. Effect of GA_3 and GA biosynthesis retardants on ethylene production for 3, 4, and 5 days after incubation in dormant tubers of *Dioscorea opposita* cv Tsukune

Chemicals [1]	Treatments (μg l^{-1})	Ethylene Concentration (μl, kg^{-1} h^{-1})		
		3 DAI [1]	4 DAI	5 DAI
Control		8.2 b	8 7	8 8 j
GA_3	0 3	9.2 b	8.7 h	10 4 i
	3	8 9 b	9.3 h	9 6 j
	30	9 3 b	8 6 h	8 9 j
MC	10	9.5 b	499 1 d	564 3 e
	100	7 3	143 7 f	162 5 g
	1000	1 9 d	1 7 i	2 2 k
AN	1	21 9 a	1615 a	1822 5 a
	10	20.8 a	630 9 c	673 2 d
	100	3 9 c	111 5 g	135 4 h
TNE	5	11 9 b	692 8 b	707 5 b
	50	10 7 b	632 8 c	645 1 c
	500	3 6 c	269 0 e	283 5 f

GA_3, gibberellic acid, MC, mepiquat chloride, AN, ancymidol, TNE, trinexapac-ethyl
[1] days after incubation into vessel The same letters in each column were not significantly different at the 5% level by DMRT

Effects of GA_3 and GA biosynthesis retardants on batatasin content and sugars in dormant tubers

Table 3 shows quantitative changes of three major batatasins in the stored tubers as treated with GA_3 and gibberellin biosynthesis retardants. GA_3 did not increase in batatasin I and V as compared to control. Whereas, batatasin-III content in GA_3 treated tuber was increased at the highest concentration (30 μg l^{-1}). In MC treated tubers, batatasin-I content increased as increasing the MC concentrations. Contents of batatasin-β decreased oppositely as increasing concentration. We also examined the quantitative changes of sugars in stored tubers as MC treated with GA_3 and gibberellin biosynthesis retardants (Table 4). Gibberellin biosynthesis retardants did not affect the glucose and fructose contents compared to the GA_3, although glucose and fructose contents were more increased than control. However, sucrose content in tubers treated with gibberellin biosynthesis retardants was remarkably increased compared to the GA_3 and control. In particular, sucrose content in An treated tubers was higher than that of MC and TNE treated tubers.

Table 3. Effects of GA_3 and GA biosynthesis retardants on batatasin content in dormant tubers of *Dioscorea opposita* cv Tsukune.

Chemicals [1]	Treatments (μg l^{-1})	Batatasin Concentration (mg g^{-1}, D W) [2]		
		batatasin I	batatasin III	batatasin V
Control		7.9 d	11 4 e	5.9 f
GA_3	0.3	9 9 c	19.3 c	7.8 e
	3	11.5 c	23.2 b	10.1 d
	30	10 2 c	34 8 a	9.8 d
MC	10	20 5 b	14.1 d	17.4 b
	100	28.3 a	12.5 de	20 8 a
	1000	29 0 a	7.9 g	18 1 b
AN	1	34.9 a	2 4 g	22 4 a
	10	28 1 a	8.5 f	15 5 c
	100	29.0 a	10 3 e	17 4 b
TNE	5	33 1 a	10.1 e	11 2 d
	50	29 7 a	9 8 e	13.3 d
	500	30 6 a	11 6 e	10.0 d

[1] GA_3, gibberellic acid, MC, mepiquat chloride; AN, ancymidol, TNE, trinexapac-ethyl [2] Batatasin content were determined at five days after vessel incubation The same letters in each column were not significantly different at the 5% level by DMRT

Table 4. Effects of GA_3 and GA biosynthesis retardants on free sugar content in tubers of *Dioscorea opposita* cv. Tsukune.

Chemicals [1]	Treatments (μg l^{-1})	Free sugar content (mg g^{-1}, D W) [2]		
		sucrose	glucose	fructose
Control		42 3 d	22 7 d	32.3 d
GA_3	0 3	41 5 d	43 6 b	47 0 b
	3	35 9 e	40 9 c	42.5 c
	30	34 3 e	40 2 c	36 8 d
MC	10	60.1 c	51.5 b	54 3 a
	100	70 3 b	58 7 a	49 9 a
	1000	76 5 b	56 5 a	50 3 a
AN	1	93 4 a	45 2 b	47 4 b
	10	86 3 a	47 6 b	48 7 b
	100	70 6 b	49 9 b	51 2 a
TNE	5	61 4 c	48 7 b	49 1 a
	50	56 2 c	46 5 b	48 3 b
	500	53 0 cd	45 3 b	46 0 b

[1] GA_3, gibberellic acid, MC, mepiquat chloride, AN, ancymidol, TNE, trinexapac-ethyl [2] Sugar content was determined at five days after vessel incubation The same letters in each column were not significantly different at the 5% level by DMRT

DISCUSSION

The involvement of endogenous ethylene in tuber dormancy regulation is uncertain in Chinese yam. The growth and development of higher plants, from the earliest to the most advanced stages of the life cycle, are regulated by phytohormones including, such as ethylene (Dolan, 1997) Rosa (1925) reported an effect of ethylene on shortening the natural periods of potato tuber dormancy. Application of an ethylene-releasing agent, alone or in combination with GA_3, to dormant tubers has been reported to stimulate sprouting (Shashirekha & Narasimham, 1988).

Ethylene was not detected during two days of incubation in each chemical treatment. Three days after incubation, ethylene was produced in all chemicals including the control, suggesting on set of sprouting. As a plant growth retardant, chlorocholine chloride promotes the spouting of stratified bulbils in the Chinese yams, and it also accelerates the sprouting of bulbils applied with gibberellic acid (Okagami & Nagao, 1971) GA_3 treatment reduced the ethylene production in dormant tubers. However, MC, AN, and TNE affected to ethylene production and promotion of sprouting rate in tubers It suggests that the decrease of ethylene production by gibberellic acid may be resulted in lower sprouting rate of tubers.

It is believed that batatasins are closely involved in dormancy inducing compounds with exogenous gibberellins (Okagami & Nagao, 1971). In our experiments, treatment of gibberellic acid in dormant tubers was resulted in no sprouting tubers. In this state, batatasin-III content by gibberellic acid was much more increased than that of control and gibberellin biosynthesis retardants. It was suggested that exogenous gibberellin causes the increase of batatasins, and gibberellin biosynthesis retardants may delay or inhibit batatasin biosynthesis in dormant tubers of Chinese yam.

Treatment of gibberellin biosynthesis retardants did not influence the glucose and fructose content. However, sucrose content in dormant tubers by gibberellin biosynthesis retardants was remarkably enhanced the free sugars contents. In these results, ethylene production may induce by gibberellin biosynthesis retardants rather than by gibberellic acid. Furthermore, the shortening of dormancy in stored tubers could be enhanced by gibberellin biosynthesis retardants.

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