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EFFECTS OF BA AND CPPU ON POLYAMINE CONTENT, SETTING AND DEVELOPMENT OF SEEDLESS GRAPES INDUCED BY GIBBERELLIN A_3

S. Shiozaki*, R. Sakurai, M. Hotta and T. Ogata[1]

Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan
Corresponding Author email: ssgvo67@plant.osakafu-u.ac.jp

ABSTRACT

The effects of N^6-benzyladenine (BA) and N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) in combination with gibberellic acid (GA_3, 100 mg l^{-1}) on berry setting and development and on the endogenous level of polyamines (PAs) were investigated in order to elucidate the role of PAs in the setting and development of GA_3-induced seedless grape berries affected by cytokinins. BA stimulated berry development, but not berry setting, while CPPU significantly stimulated berry setting. The PA synthesis inhibitors DL-α-difluoromethyl-arginine and methylglyoxal-bis(guanyl-hydrazone) inhibited the berry development induced by GA_3 + BA at 11 days after full bloom. However, they did not inhibit the increased berry-setting rate induced by GA_3 + CPPU. Application of BA in combination with GA_3 greatly increased the levels of free putrescine and spermidine. CPPU had almost no effect on the levels of free PAs, but significantly increased conjugated PAs. The effect of the addition of BA and CPPU on levels of bound PAs was small compared with the effect on free and conjugated PAs.

Keywords: Berry set, Berry development, Cytokinin, Gibberellin, Polyamine, Seedless

INTRODUCTION

Gibberellins, especially gibberellic acid (GA_3), are used as plant growth regulators to increase the size of seedless berries and induce parthenocarpic fruit development in grapes (Gianfagna, 1995). Two applications of GA_3 are generally necessary to produce seedless berries in seeded cultivars: pre- and postbloom treatments to induce seedlessness and then to stimulate berry development. Cytokinins (CKs) such as N^6-benzyladenine (BA) and N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) are used with GA_3 to avoid poor berry set, which is caused by improper timing of prebloom GA_3 application (Dan, 1996; Motomura and Hori, 1978; Naito *et al*., 1974; Nickell, 1985). These CKs are also applied to stimulate the development of seedless grape berries (Motomura and Hori, 1978; Zabadal and Bukovac, 2006).

Polyamine (PA) is widely distributed in plants and regulates many physiological events including reproductive development (Egea-Cortines and Mizrahi, 1991). High PA levels at an early stage of fruit development have been reported in grapes (Shiozaki *et al*., 2000). Furthermore, there is strong evidence that exogenous PA stimulates fruit setting and development in many fruits (Bibi *et al*., 2010; Rugini and Mencuccini, 1985; Singh and Singh, 1995). Interestingly, CKs can stimulate PA synthesis and retard oxidative degradation (Mukhopadhyay *et al*., 1983; Palavan *et al*., 1984). This raises the possibility that PA and CKs interact in fruit setting and development.

This paper explores the relation between CKs (BA and CPPU) and PAs in the setting and development of seedless grape berries induced by GA_3. It focuses particularly on the effect of exogenous CKs in combination with GA_3 on flower and berry PA levels. The effect of the PA synthesis inhibitors DL-α-difluoromethyl-arginine (DFMA) and methylglyoxal-bis (guanyl-hydrazone) (MGBG) on berry setting and development stimulated by CKs and GA_3 was also investigated.

MATERIALS AND METHODS

Grapes and treatment: The experiment, conducted at a research field in Osaka Prefecture University in 2000, used 10-year-old ‘Delaware’ (*Vitis labrusca* L.) grapes. Fruiting was adjusted to two clusters per cane to determine berry setting and berry fresh weight, and adjusted to three clusters per cane for PA analysis. For PA analysis, 12 canes (36 clusters) were used for each treatment. A dipping treatment of clusters with chemicals took place 17 days before full bloom (DBB) of seeded berries; this period coincides with 14 DBB of seedless berries induced by GA_3 because GA_3 application accelerates flowering. Clusters on the same cane received the same treatment in all experiments. A water-soluble dosage of GA_3 (containing surfactant; Meiji Seika Pharma, Tokyo) was used. BA (chemical grade; Wako, Osaka) and CPPU (liquid dosage; Kyowa, Tokyo) were dissolved in a small amount of ethanol or distilled water and then mixed with the GA_3 solution. DFMA (Hoechst Marion Roussel, Inc., Cincinnati) and MGBG (Sigma-

Aldrich Japan, Tokyo) were dissolved in distilled water and then mixed with other chemical solutions.

The period in which about 80% of flowers in each cluster were open was taken as full bloom. All clusters in the experiment used to determine berry setting and development received 100 ppm GA_3 11 days after full bloom (DAB) except for those sampled on that day (11 DAB).

Evaluation of berry setting and berry development: The number of flowers per cluster was counted 11 DBB, and ranged from 200 to 250. By full bloom, no flower abscission was observed in any of the treatments. At 11 DAB, one cluster per cane (five clusters in total) was sampled from each treatment. After the number of berries in each cluster had been counted, 20% of the berries in each cluster were randomly sampled and each of them weighed. These berries were then cut open with a razor blade to check for seedlessness, and the fresh weight data of any berries containing seeds were deleted from the analysis. The number of berries per cluster was also determined for the remaining clusters. There was no difference in the number of berries on the clusters 11 DAB and at harvest (55 DAB). Consequently, the berry-setting rate in each treatment, which was determined from an average of 10 clusters per treatment, could be deduced from the data from the sampled clusters and from those left on the shoot at 11 DAB. At harvest, the proportion of seedless berries in each treatment was determined from the remaining five clusters. Seeded berries could be distinguished from seedless berries because they were still green, whereas seedless berries had ripened to have pale-red skins. The berry fresh weight was also determined in 20% of seedless berries in each cluster at harvest.

Sampling of flowers and berries for PAs: Before full bloom, the flowers were collected just before treatment (17 DBB; seeded), at 12 DBB and at 5 DBB. On and after full bloom, the berries were collected at full bloom and at 3, 6 and 11 DAB. One cluster was sampled from each of six canes in the above two treatments. Samples were respectively taken from the first, second and third clusters for the three sampling dates for each treatment. The samples were frozen and ground to a fine powder under liquid nitrogen and stored at –30°C until PA analysis.

PA extraction and analysis: Samples were homogenized in cold 5% perchloric acid (PCA) (0.1 g sample ml^{-1} PCA) using a glass homogenizer, and the homogenate was maintained at 4°C for 30 min. Extracts were centrifuged for 10 min at 25,000 *g*, and the supernatant was used for the determination of free PA and PCA-soluble conjugated PA. The pellet was used for the determination of PCA-insoluble bound PA; it was washed in 5 ml of PCA, centrifuged for 10 min at 25,000 *g* and then resuspended in the original volume of PCA by vortexing. The pellet suspension and the original supernatant (0.2 ml each) were hydrolyzed for 20 h with 0.2 ml of 12 N HCl at 110°C in a reaction vial. The hydrolysate was centrifuged and a 0.1 ml aliquot of the supernatant was dried *in vacuo* at 60°C and then dissolved in 0.1 ml PCA. The soluble conjugated PA was estimated as the concentration of PA in the hydrolysate of the original supernatant less that of the free PA. The PA samples were dansylated and analyzed by reverse-phase HPLC with a fluorescence detector (Shiozaki *et al*., 2000). Each determination was performed in triplicate.

Statistical analysis: The data were analyzed by analysis of variance and means were compared by Tukey–Kramer test, with significance set at $P < 0.05$, with StatView 5.0 (SAS Institute Inc.). Percent data of berry set were arcsine transformed before analysis.

RESULTS

Effect of GA_3, BA, CPPU and PA synthesis inhibitors DFMA and MGBG on seedlessness, berry setting and seedless berry development: None of the treatments affected the seedlessness induced by GA_3 (Table I). The treatment with GA_3 + CPPU induced a higher rate of berry setting than GA_3 alone, with the rate equivalent to that of seeded berries. GA_3 + BA had no effect on the berry setting compared with GA_3 alone. In the GA_3 + BA and GA_3 + CPPU treatments, the addition of DFMA or MGBG did not affect berry setting. GA_3 + BA increased the fresh weight of the seedless berries by 1.8 times that in the treatment with GA_3 alone 11 DAB. GA_3 + CPPU had no effect on the berry fresh weight 11 DAB. At harvest, the berry fresh weight after the GA_3 + CPPU treatment was slightly higher than that after treatment with GA_3 alone. On the other hand, no significant difference was found in berry fresh weight between treatment with GA_3 + BA and GA_3 alone.

Effect of GA_3 and CKs on PA levels in flowers and berries: The levels of spermine (Spm) were lowest in all fractions (free, PCA-soluble conjugated and PCA-insoluble bound) compared with the levels of putrescine (Put) and spermidine (Spd) (Figs I, II and III). In free and bound fractions, the levels of Spd were higher than those of Put, whereas in the conjugated fraction, Put was the predominant PA in most samples. At 2 days after treatment (12 DBB), GA_3 + BA had significantly increased free Put and Spd levels: those levels in the treated flowers with GA_3 + BA were respectively 2.0 and 1.6 times those treated with GA_3 alone. CPPU had little effect on the levels of free PAs 2 days after treatment. Nine days after treatment (5 DBB), free PA levels were similar across the treatments. On and after full bloom, the levels of free Put and Spd in the berries were higher after treatment with GA_3 alone. The level of PCA-soluble

Table 1. Effects of prebloom treatment of GA,. And GA, combined with BA, CPPU and Put, on seedlessness, berry setting and seedless berry fresh weight of Delaware' grapes

Treatment	Seedless berry [b] (%)	Berry set [b] (%)	Berry fresh weight [b,c]	
			11 DAB (mg)	At harvest (g)
None (Seeded)	—	48 ab	54(93) c	1.5(52) a
GA_3	98 a	38 bc	75(66) bc	1.3(51) c
GA_3 + BA	82 a	35 c	139 (79) a	1.4(49) bc
GA_3 + BA + DFMA	93 a	34 c	101(75) b	1.3(54) bc
GA_3 + BA + MGBG	91 a	36 c	82(84) bc	1.4(53) ab
GA_3 + CPPU	95 a	50 a	68(114) c	1.4(50) ab
GA_3 + CPPU + DFMA	91 a	44 abc	100(99) b	1.4(51) ab
GA_3 + CPPU + MGBG	94 a	53 a	77(116) bc	1.3(51) bc

a Concentration of treatments is as follows: GA,: 100 mg I-1, CPPU: 5 mg i-1, dfma: 3 mM, MGBG: 3 mM.
b The values followed by the same letter show no significant differences in each GA, + CK treatment ($P<0.05$).
c The data in parentheses show the number of berries for the data.

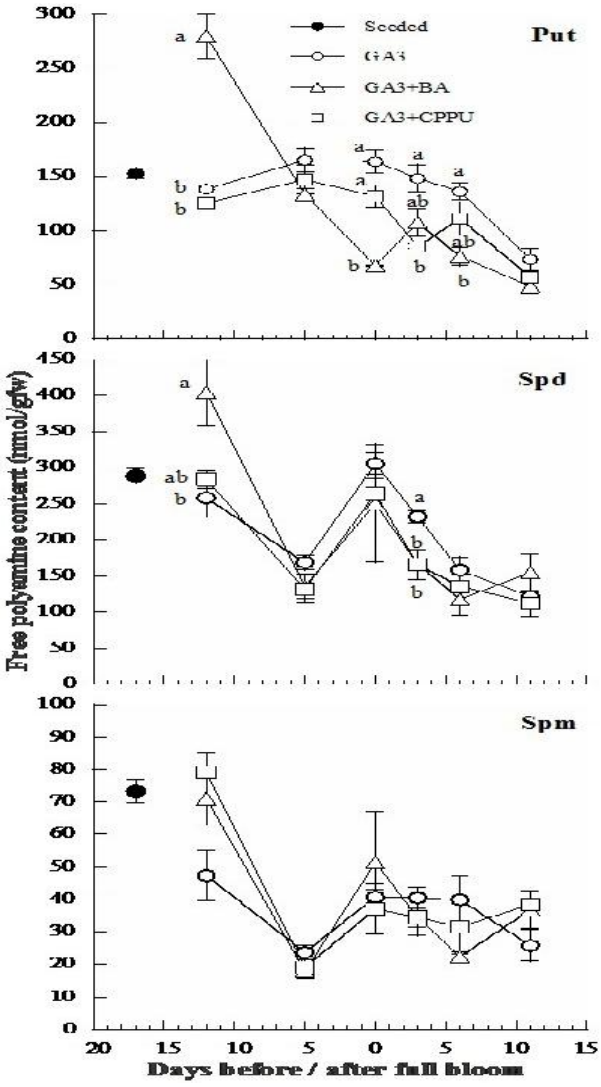

Figure I Changes in levels of free PAs in 'Delaware' grapes treated with CKs and GA_3. Treatment was conducted at 17 days before bloom of seeded berries, corresponding to 14 days before bloom of seedless berries.

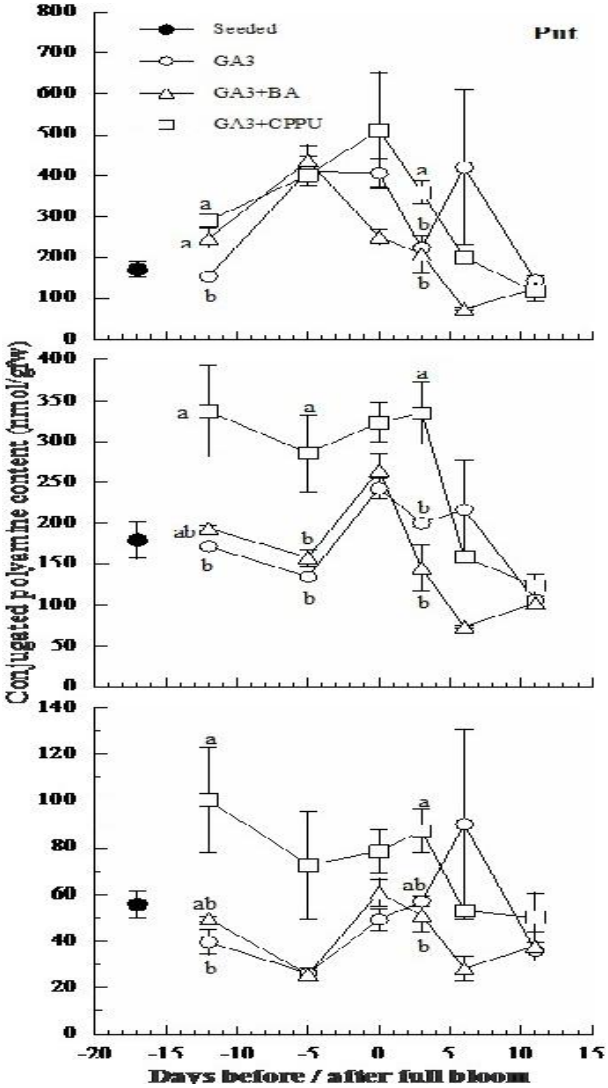

Figure II Changes in levels of conjugated PAs in 'Delaware' grapes treated with CKs and GA_3. Treatment was conducted at 17 days before bloom of seeded berries, corresponding to 14 days before bloom of seedless berries.

conjugated Put was higher in the GA_3 + BA and GA_3 + CPPU treatments than in GA_3 alone at 2 days after treatment. The most striking effect on the levels of Spd and Spm conjugates was found in the GA_3 + CPPU treatment: the conjugated Spd levels in the treatment were significantly higher than with GA_3 alone at 12 DBB, 5 DBB and 3 DAB. The fluctuation of conjugated Spm levels was similar to that of conjugated Spd, although the levels were less than 30% of the conjugated Spd. The highest levels of conjugated Spm were found with GA_3 + CPPU at 2 days after treatment (12 DBB) and 3 DAB. In bound PAs, BA and CPPU had no effect on the bound Put level at 2 days after treatment (Fig. III).

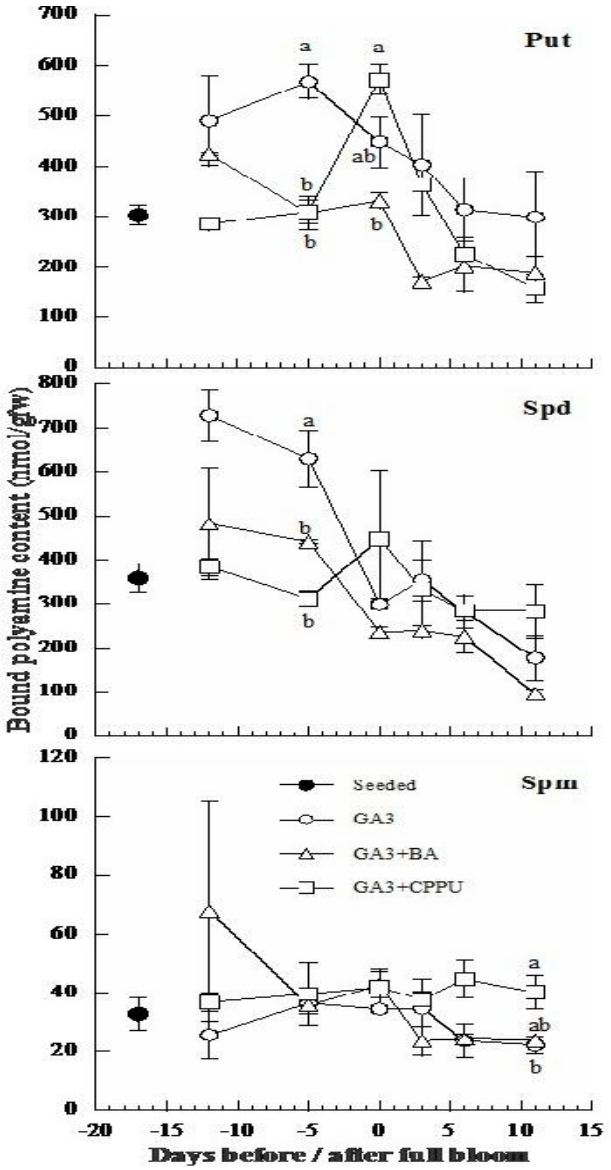

Figure III Changes in bound PA levels in 'Delaware' grapes treated with CKs and GA_3. Treatment was conducted at 17 days before bloom of seeded berries, corresponding to 14 days before bloom of seedless berries.

DISCUSSION

BA and CPPU (in combination with GA_3) have distinct effects on the setting and development of seedless berries. BA stimulated berry development by increasing the levels of free PAs, whereas CPPU stimulated berry setting by increasing the levels of conjugated PAs (Table I, Figs I and II). The involvement in plant physiological functions of an adenine-type CK such as BA and a urea-type CK such as CPPU has been discussed elsewhere (Mok and Mok, 2001). BA and its derivatives, which were originally considered only to be synthetic CKs, have since been found to be endogenous CKs in some plants (Mok and Mok, 2001). Although CPPU is a synthetic CK, it has intrinsic CK properties and acts by interaction with a CK receptor that is also sensitive to native CKs of adenine type (Shudo, 1994). Consequently, BA and urea CKs may play similar roles in physiological mechanisms in plants, as confirmed by the prevention of oxidative degradation of endogenous CKs (Laloue and Fox, 1989). However, different or opposing physiological effects have also been found between adenine- and urea-type CKs. Adenine-type CKs including BA can stimulate endogenous CK synthesis (Redig *et al*., 1997). In contrast, levels of endogenous CKs were reduced by the application of CPPU in the fruit of kiwifruit (Lewis *et al*., 1996). Differences in the changes in the levels of free and conjugated PAs caused by the addition of BA and of CPPU imply differences in the effects of BA and CPPU on PA biosynthesis and metabolism.

It is known that BA increases free PA levels (Cho, 1983; Mukhopadhyay *et al*., 1983) by stimulating the activity of an enzyme related to PA biosynthesis (Palavan *et al*., 1984). The PA biosynthesis inhibitors DFMA and MGBG reduced the seedless berry fresh weight induced by GA_3 + BA at 11 DAB (Table I). MGBG is a potent inhibitor of S-adenosylmethionine decarboxylase, which is involved in the synthesis of Spd and Spm, and DFMA is an irreversible inhibitor of arginine decarboxylase which catalyzes the synthetic pathway of arginine to Put (Slocum, 1991). The levels of free Spm were not affected by BA when combined with GA_3 (Fig I). Therefore, with GA_3 + BA, *de novo* synthesized free Put and Spd seem to play an important role in ovary and berry development of seedless grapes. Cell division following the exogenous application of BA has been found in some fruit species (Kinet *et al*., 1985; Takeno *et al*., 1992). PA levels are generally strongly correlated with cell division in plants. In a few cases, exogenous PAs stimulate cell division in tissue culture (Evans and Malmberg, 1989). Stimulation of berry development by BA may be the result of a synergistic effect of BA and free PAs on cell division in the ovary. At harvest, however, no effects of BA on berry fresh weight were found. The final berry size would be more

affected by the GA_3 treatment 11 DAB, which is conducted to induce berry development.

A positive effect of exogenous free PAs on fruit setting has been reported in many species (Bibi *et al*., 2010; Rugini and Mencuccini, 1985; Singh and Singh, 1995). However, the application of Put + GA_3 had no effect on berry setting of seedless grapes (data not shown). BA dramatically increased the level of free Put and Spd but did not affect setting, whereas CPPU stimulated setting by increasing the levels of conjugated Spd and Spm from 12 DBB (2 days after treatment) to 3 DAB. Therefore, unlike in other fruit species, conjugated PAs, but not free PAs, seem to be involved in berry setting in seedless grape berries. Conjugated PAs (PCA-soluble fraction) are mostly covalently bound with low-molecular-weight compounds such as hydroxycinnamic acids, forming the hydroxycinnamic amides (HCAs). HCAs play a role in flowering in numerous plant species, given that the levels of HCAs were found to be correlated with floral initiation and development (Flores and Martin-Tanguy, 1991). Stimulation of berry setting might be an important function of HCAs besides flowering, at least in grapes. Neither DFMA nor MGBG affected the berry setting stimulated by CPPU combined with GA_3. This suggests, but in no way proves, that the increase in conjugated PAs induced by CPPU + GA_3 is not the result of *de novo* synthesis of free PAs followed by formation of the conjugation. Interconversion of free, conjugated and bound PAs might be implicated in the changes in the levels of conjugated PAs induced by CPPU + GA_3. The effect of CPPU on PA biosynthesis and the regulation of PA metabolism needs to be elucidated.

As for the bound form of PAs, which is a covalent linkage of PAs with high-molecular-weight compounds, a regulatory role in cell division has been proposed (Kaur-Sawhney *et al*., 1986). In the present study, however, levels of bound PAs were relatively low following the addition of BA. Therefore, levels of bound PAs may not correlate with cell division in seedless grapes. The same seems to be true for the correlation between levels of bound PAs and the effect of GA_3 + CPPU. The low levels of bound PAs following GA_3 + CPPU treatment may suggest that they have no involvement in berry setting.

In conclusion, BA and CPPU had distinct effects on the levels of PAs, berry setting and the development of seedless grapes induced by GA_3. BA stimulated ovary development and increased free PA levels in the flowers, which suggests the involvement of free PAs in BA-stimulated ovary development. On the other hand, CPPU stimulated berry setting by increasing the levels of conjugated PAs. Thus, conjugated PAs should be taken into consideration when examining the effect of CPPU on seedless berry setting.

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