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ORIGINAL PAPER

GIBBERELLIN APPLICATION INFLUENCES ON *EX VITRO* GROWTH, FLOWERING AND STEVIOL GLYCOSIDE ACCUMULATION OF *STEVIA REBAUDIANA* BERTONI

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Abstract

Stevia (Stevia rebaudiana Bertoni) is a small perennial herb with incredible sweetening property. Stevia is known to contain steviol glycosides (SGs), which are about 300 times sweeter than sucrose at their concentration of 4% (w/v). However, the growth of stevia plants and their SGs production are known to be influenced by external and internal factors. In this study, an ex-vitro experiment was conducted to determine the effect of exogenous GA_3 application on the growth, flowering and steviol glycoside (stevioside and rebaudioside A) accumulation in stevia under Philippine condition. A previously established tissue culture-derived plant was used as mother plants and categorized into two groups: (1) non-flowering (NF) and (2) flowering (F). Stevia stem cuttings were sprayed with GA_3 solutions of various concentrations: 0, 50, 100 and 200 mg L^{-1} at 1-week interval for 5 weeks. NF plants sprayed with GA_3 showed significant difference in shoot length, number of shoot tips, length of longest primary branches compared to the control. NF plants produced more leaves than F at any GA level but without significant difference. Application of gibberellic acid (GA_3) neither delayed nor inhibits flowering of ex vitro stevia plant but significantly reduced the flowering intensity of the plant. Exogenously applied GA_3 also did not affect SG accumulation in the leaves and flowers.

Key words: Stevia rebaudiana Bertoni, gibberellin, steviol glycosides

INTRODUCTION

Stevia is a small perennial herb with incredible sweetening property. The plant which is native to Paraguay is known to contain steviol glycosides (SGs), which are about 300 times sweeter than sucrose at their concentration of 4% (w/v) (Kinghorn and Soejarto, 1985). However, productions of secondary metabolites (i.e SGs) are known to be influenced by external and internal factors. SG accumulation pattern in leaves varies with cultivar (Bondarev et al., 2003; Serfaty, 2013), phenological stage (Brandle and Rosa, 1992) and growth conditions like photoperiod (Ceunen and Geuns, 2013), temperature and available nutrients (Pal et al., 2013). Hence, manipulation of some known physical and chemical factors can be used as means of intervention to delay flowering and thus increase steviol glycoside production.

Plant growth regulators (PGR_s) have been reported to be among the main factors that influence plant growth and secondary metabolite synthesis (Hassanpouraghdam et al., 2011). Gibberellin (GA) is a prominent class of phytohormone that is known to control growth and flowering in plants (Taiz and Zeiger, 2010; Medina & Saavedra, 1999: Lang and Reinhard, 1961). GAs are known to stimulate cell elongation and cell division thus promoting growth in plants, manifested as increase in shoot length, internode-length, increased leaf size and change in leaf shape, etc.

Flowering is said to be inhibited by GA in short-day plants under long day photoperiods (Brian, 1958). On the other hand, exogenous application of GA_3 has been found to induce flower formation in many plants under long-day suggesting that GA may be limiting under short days.

Scientific studies have shown significant effect of gibberellins on leaf yield of some medicinal plants (Hassanpouraghdam et al., 2011). GA_3 application reportedly affected leaf fresh and dry weights of the medicinal plant *Coleus amboinicus* Lour. (Pablo Morales-Payan, 2005). In stevia, foliar spraying with GA also showed maximum amount of stevioside content in treated plants on dry weight basis (Modi et al., 2011). The uptake of GA using foliar spray may increase its amount in the cell leading to shutting down of GA process and the pathway is diverted to steviol glycoside accumulation. On the other hand, *in vitro* studies on stevia revealed that GA_3 fed to cell suspension cultures of *S. rebaudiana* showed fast conversion to stevioside (Striedner et al., 1991). This may be related to the observation that SGs and gibberellins may share a common biosynthesis pathway (Brandle and Telmer, 2007).

Considering these reports, the effect of GA_3 on the growth, flowering response and SG accumulation in stevia from stem cuttings under greenhouse condition was then determined under Philippine condition.

MATERIALS AND METHODS

Establishment of plant material

Previously established tissue culture-derived plants were used as mother plants in this study. These mother plants were categorized into two groups: (1) non-flowering (NF) and (2) flowering (F). NF plants were maintained under 15-h photoperiod while F plants were kept under short-day condition. Both groups of mother plants were ratooned to allow new shoots to develop. Once the plants had developed sufficient number of shoots, shoot tip cuttings with three nodes each were excised. The cuttings were planted in small polyethylene bags (250 cm^3) with potting medium composed of coconut coir dust: burnt rice hull: garden soil (1:1:1 v/v/v), then covered with clear plastic to avoid dehydration. These cuttings were maintained under 15-h photoperiod until sufficient roots have developed before transplanting to bigger polyethylene bags (4,500 cm^3) with the same potting medium. After transplanting, the plants were brought to the greenhouse for the experiment. Henceforth, plants derived from these rooted cuttings were referred to as F and NF plants accordingly.

Application of gibberellic acid (GA_3)

Established F and NF plants were sprayed with GA_3 solutions of various concentrations (0, 50, 100 and 200 mg L^{-1}) at 1-week interval for 5 weeks. The solution was sprayed on whole plants up to dripping wet. The plants were maintained in the greenhouse under natural environmental condition until the termination of the experiment (10 weeks).

Measurement of growth and evaluation of flowering response

The number of days to first sighting of floral bud and flower opening were recorded. Vegetative growth of the plants was assessed by recording the shoot length, number of shoots, number of branches and root length. Percentage of flowering and total weight of plants was also taken at termination of the experiment. There were three replications per treatment, with 10 samples per replicate for both plant groups, NF and F. All data were analyzed by ANOVA for Completely Randomized Design (CRD) using computer software Statistical Tool for Agricultural Research (STAR) 2013. HSD test was applied for means separation.

Steviol glycoside levels in stevia applied with of GA_3

Above shoots of N and NF potted stevia were cut and washed with water to remove the soil. The shoots of the plants obtained from each treatment were separately weighed and placed in brown paper bags for air-drying for 24-hours. Then, paper bags containing the stevia samples were subjected to oven-drying for 16-hours at 50^0C. The

dried samples were pulverized using mortar and pestle, then placed in sealed polyethylene bags and stored in a refrigerator at 0-4^{0}C until use.

The powdered plant samples per replicate from the experiment were mixed and about 1.0 g was submitted for analysis at the Philippine Institute of Pure and Applied Chemistry (PIPAC), Ateneo de Manila University through HPLC analysis using the protocol established by the FAO (2010). Extraction was done following the method of Kolb et al. (2001) with 70% ethanol (w/w) as the solvent. Stevioside and rebaudioside-A from *ex vitro* stevia were calculated from the samples through HPLC chromatograms and compared with known standard of both glycosides.

RESULTS AND DISCUSSION

Growth response

Stevia sprayed with GA_3 produced small, narrow pale green leaves and elongated internodes at the upper part of shoots. After a month of spraying, it was observed that some of the leaves developed in alternate position on the stem. This response was more common at higher GA_3 levels (100 and 200 mg L^{-1}). The secondary branches also had thin internodes and narrow leaves.

Plants sprayed with GA_3 showed significant difference in shoot length compared to the control, however, no significant differences were observed among GA_3-treated plants (Figure 1). The shoots of the NF plants exhibited increased elongation rate compared to the F plants which showed reduced elongation at 200 mg L^{-1}. Both NF and F produced longer shoots at 100 mg L^{-1} but was comparable to other GA_3-treated plants, the shortest was at zero GA_3 (control).

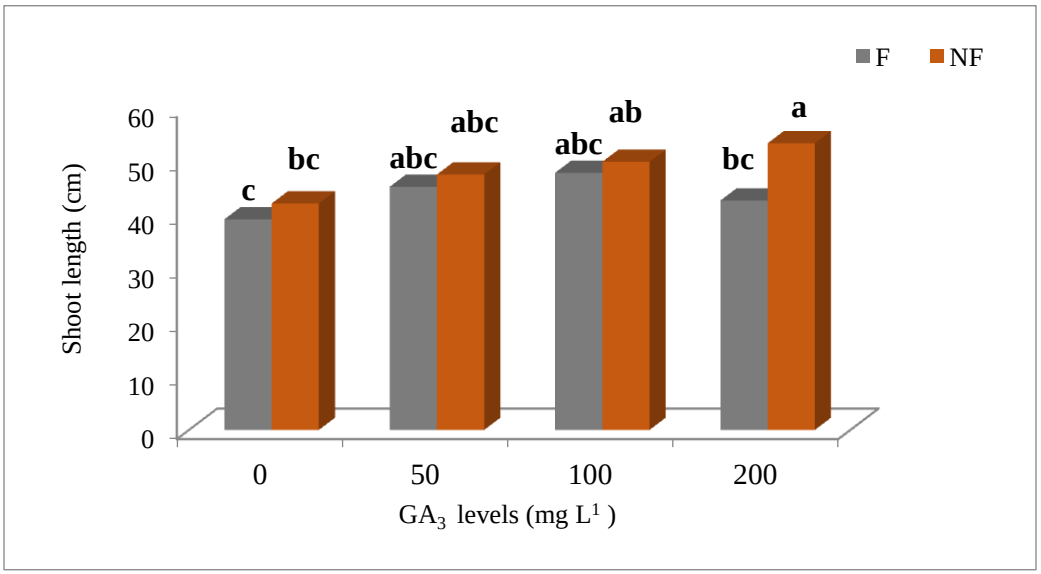

Figure 1. Shoot length of flowering and non-flowering stevia cuttings to different GA_3 levels after 10 weeks. Bars with common letter are not significantly different (HSD 5%).

The enhanced shoot elongation in GA_3-treated plants can be attributed to the established effect of the hormone in inducing cell division and cell elongation, resulting to hyper elongation of the internode.

All plants in both NF and F that were sprayed with GA_3 induced production of more leaves but were not statistically different with the other treatments (Table 1). Generally, NF plants produced more leaves than F at any GA_3 level.

Table 1. Growth response of non-flowering (NF) and flowering (F) stevia cuttings to different GA_3 levels after 10 weeks.

PLANT GROUP	GA_3 LEVELS (mg L^1)	NO. OF 1^0 BRANCH	NO. OF SHOOT TIPS	NO. OF LEAVES
F	0	6	26 d	289
	50	6	40 ab	451
	100	5	38 abc	554
	200	5	28 cd	421
NF	0	6	33 bcd	307
	50	6	36 abcd	527
	100	6	44 ab	550
	200	6	46 a	666
CV (%)		10.11	10.79	29.50

Means followed by the same letter are not significantly different (HSD 5%) test.

GA_3 application consistently induced shoot formation in stevia plants propagated from both non-flowering (NF) and flowering (F) stock plants compared to the control (Figure 2). NF plants produced increasing number of shoot tips as the concentration of GA_3 sprayed increased (200 mg L^{-1}) but was comparable with other GA_3 sprayed plants except in F plants at similar concentration (Figure 2). F plants that were not sprayed with GA_3 and those sprayed with highest GA_3 concentration showed the lowest number of shoot tips.

In both F and NF plants, application of GA_3 at concentration used in the experiment showed similar effects in terms of the plants vegetative growth. GA_3 was effective in NF at higher concentration unlike those from F plants. Brian (1958) had earlier suggested that internal levels of GAs increased in long-day photoperiods and such change in hormone balance in plants initiate the maintenance of vegetative phase in short-days plants. In addition, no difference was observed on the number of primary branches in both sources of stevia cuttings.

Even at higher GA_3 application (200 mg L^1), F plants decreased in number of shoot tips, since these plants were derived from other plants previously maintained under short-day photoperiods which is inductive to stevia flowering. The F plants could be physiologically closer to the flowering phase than the NF plants. It was noted that stevia plants that were about to flower tend to assume the rosette habit of growth such as shorter internodes and smaller leaves.

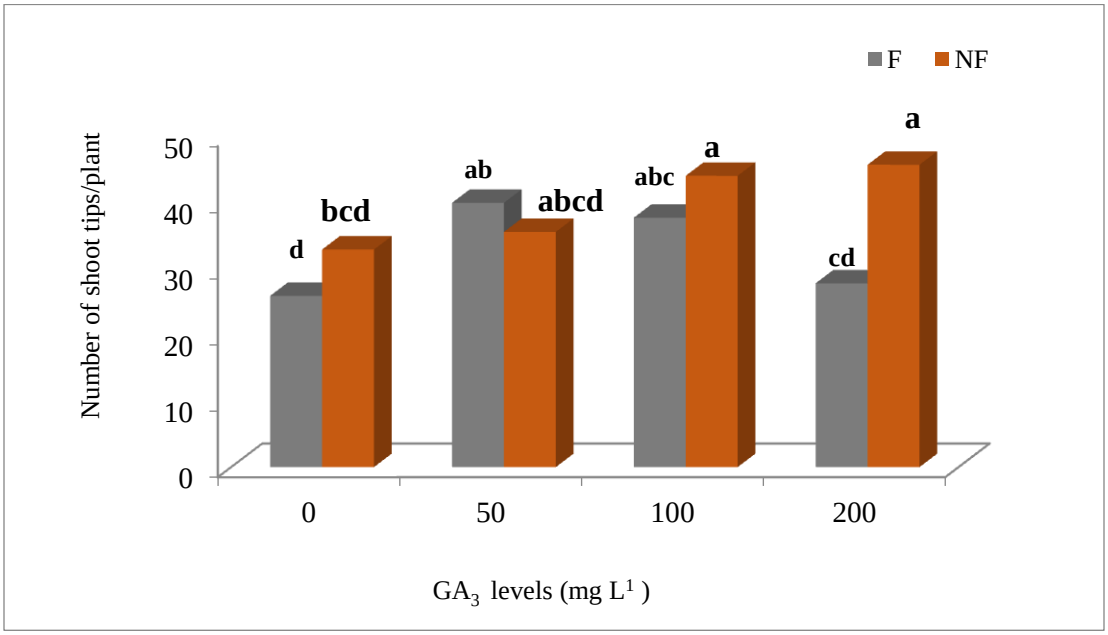

Figure 2. Shoot tips from flowering (F) and non-flowering (NF) stevia sprayed with GA_3 after 10 weeks. Bars with common letter are not significantly different (HSD 5%).

To assess further the effect of exogenously applied GA_3 on growth, the extent of growth of the longest primary branch of the plants which were found at the 3rd node from the base of the main stem was evaluated (Table 3). Significant differences were observed in shoot length, number of secondary branches and number of leaves. Internodes of most NF plants tend to be longer than those of the F plants but their number of nodes was comparable. The NF plants also had a greater number of shoot tips and leaves. Even without GA_3 treatment (control), after 10 weeks of growth, NF plants, produced more branches and more leaves compared to F plants.

As GA_3 concentration sprayed on NF plants increased, vegetative growth of stevia increased compared to F. Overall, the NF plants appeared to be more predisposed to vegetative growth than the F plants.

GA_3 treatments did not influence the fresh and dry weight of shoot and leaves of both plants. No significant difference was also observed on root length, root fresh and dry weights of both F and NF plants. According to Brian (1958) treatment with GA_3 does not stimulate growth of intact roots.

Table 3. Extent of growth of the longest primary branch in F and NF stevia plants 10 weeks after spraying with GA_3.

PLANT GROUP	GA_3 CONCENTRATION (mg L^1)	LONGEST PRIMARY BRANCH		
		Length (cm)	No. of shoot tips	No. of leaves
F	0	28.56 c	8 b	81.1 c
	50	34.06 abc	11 a	152.4 abc
	100	35.39 abc	11 a	180.4 abc
	200	32.35 bc	10 ab	143.6 abc
NF	0	31.73 bc	10 ab	120.5 bc
	50	34.24 abc	10 ab	164.3 abc
	100	37.29 ab	11 a	221.5 ab
	200	40.13 a	12 a	234.8 a
CV (%)		7.64	10.67	23.99

Means followed by the same letter are not significantly different (HSD 5%).

Flowering

After spraying with different concentrations of GA_3, some of the F plants did not flower in the highest GA_3 (200 mg L^{-1}) treatment (Table 4). In addition, the results showed a decreasing occurrence of flowering as the concentration of GA_3 increased from 50 to 200 mg L^1. These finding indicate that spraying higher GA_3 (100 to 200 mg L^{-1}) lower the incidence of flowering in stevia plants propagated from plants that previously flowered. These results can be advantageous to the growers since most of the stem cuttings used for propagation were taken from plants which are already flowering. At 200 mg L^{-1} GA_3, NF plants decreased flowering percentage already.

Overall, there were indications that exogenous application of GA_3 may reduce flowering in stevia. However, since difference among treatments in the two-plant group was found not significant, this would need to be validated.

Table 4. Percentage of flowering of F and NF plants 10 weeks after spraying with different levels of GA_3.

GA_3 CONCENTRATION (mg L^{-1})	Flowering (%)	
	F	NF
0	96.97	100.00
50	99.51	99.97
100	94.91	95.56
200	88.13	100.00
Average	94.88	98.88

Onset of flowering, or days taken for the first floral bud sighting, did not differ significantly among the treatments in both F and NF plants. On the average, it took about 24-30 days from planting to start of flowering and 36-41 days to flower opening (data not shown).

The result showed that even with exogenous GA_3 application, flowering was observed in plants maintained under natural inductive condition (short day). This is in contrast to reports that flowering will be inhibited in short-day plants with its application. Cid, as cited by Cardoso et al. (2012) suggested that gibberellins exogenously

applied promote the flowering induction and development in plants that normally require long days under the conditions of short days, however, the reverse does not occur, although there are exceptions.

Intensity of flowering

In NF plants, significant difference was found in number of flowers in the untreated plants and those sprayed with GA_3 (Figure 3). The number of flowers in all GA_3 sprayed plants did not significantly differ from each other, but F plants at 200mg L^1 GA_3 was significantly lower than that of the untreated plants.

It was observed that as GA_3 treatment increased, the number of flowers per plant developed decreased. The highest number of flowers was obtained in untreated plants (350 flowers) and this was significantly higher in GA_3treated plants. Highest GA_3 concentration (200 mg L^{-1}), gave the lowest number of flowers (88) in F plants. Application of GAs reduce the number of flowers produced by the plant specifically in previously flowering source of plants.

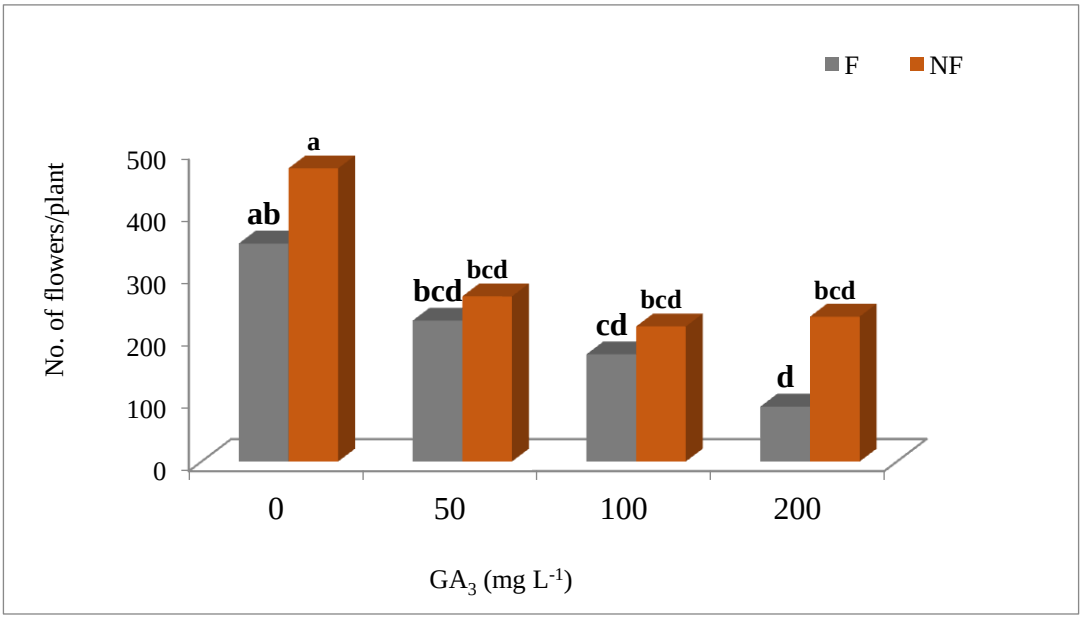

Figure 3. Flowers of stevia stem cuttings from F and F stock plants. Bars with common letter are not significantly different (HSD 5%).

Steviol glycoside accumulation as affected by exogenous GA

Exogenous application of GA_3 showed enhancing effect on SG accumulation in stevia flowers but not in the leaves when plants are grown from stem cuttings of flowering stock plants (Table 6). The results showed that in F plants, SG content of leaves did not change with GA_3 application. However, SG content of flowers appeared to be slightly higher when plants were sprayed with GA_3 either at 100 or 200 mg L^{-1} but not at lower level. Plants from NF showed that GA_3 spraying did not affect SG accumulation in either the flowers or the leaves.

Stevioside content in flowers was lower compared to the leaves in agreement with previous reports (Ramesh et al., 2006; Ceunen and Geuns, 2012). In plants grown from stem cuttings of flowering (F) stock plants, an increasing SG content on the flowers was observed as the applied GA_3 increased. Highest SG was achieved in 200 mg L^{-1} GA_3 and lowest at the untreated plants. While in NF, highest stevioside in flowers was achieved in 50 mg L^{-1} GA_3 while the highest Reb-A was recorded on the highest GA_3 applied. However, differences in SG content were not significant.

Table 6 . Steviol glycoside content of stevia plants derived from stem cuttings of flowering and non-flowering stock plants 10 weeks after spraying with GA_3.

SOURCE OF CUTTINGS	GIBBERELLIN LEVEL (mg L^1)	STEVIOSIDE (%)		REBAUDIOSIDE A (%)	
		LEAVES	FLOWERS	LEAVES	FLOWERS
Flowering	0	2.72± 0.10	1.21±0.10	2.64±0.40	1.40±0.40
	50	2.93±0.20	1.24±0.30	2.61±0.10	1.44±0.20
	100	2.46±0.90	2.06±0.80	2.54±0.50	1.90±0.60
	200	2.71±0.90	2.25±0.80	2.34±0.70	1.82±0.90
Non-flowering	0	2.63±0.10	1.38±0.10	2.54±0.60	1.04±0.10
	50	2.99 ±0.10	1.97 ±0.20	2.71 ±0.20	1.15 ±0.10
	100	3.19 ±0.10	1.84 ±0.10	2.85 ±0.10	1.12 ±0.10
	200	2.96 ±1.00	1.91 ±0.20	2.59 ±0.90	1.35 ±0.30

NS-No significant difference.

CONCLUSIONS

Application of gibberellic acid (GA_3) appeared to reduce the number of flowers produced by the plant specifically in previously flowering source of plants. However, its application neither delayed nor inhibits flowering of *ex vitro* stevia plants. Exogenously applied GA_3 also did not affect SG accumulation in the leaves and flowers.

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