Article

Changes in the content of phenolic compounds, steviosides and level of methylation in *Stevia rebaudiana* elicited

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Abstract

The application of elicitors induces a response in the level of secondary metabolites and in the DNA methylation of plants. The objective of this work was to apply two elicitors to stevia plants and evaluate the content of several phenolic compounds and steviol glucosides; as well as the methylation level in elicited and not elicited plants. The phenolics and glucosides were determined by HPLC and spectrophotometry while methylation by 5-mC DNA ELISA. As a result of elicitation, a slight increase in chlorogenic and ferulic acids was observed. Total phenols were increased seven days after the application of the elicitors, without showing any increase at 15 and 21 days. In contrast, flavonoids and tannins increased significantly at 7, 15 and 21 days after application. The content of stevioside and rebaudiosides A and C increased 9.5, 5.3 and 3.3 times more than the control group. Throughout the experiment, rebaudioside C was higher in its concentration than the sum of steviol and rebaudioside A. It was observed that elicited plants had a lower level of methylation than those not elicited. Due to its high content of rebaudioside C, stevia creole, here used, can produce a less bitter sweetener than other materials. The elicitation of stevia can be a strategy to increase the yield of sweeteners.

Keywords: *S. rebaudiana*, elicitors, phenolic.

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Introduction

Stevia (*Stevia rebaudiana*) is a plant used as a source of sweeteners. Of the more than 154 species of the genus, *S. rebaudiana* is the only one with sweetening properties thanks to the presence of steviol glycosides (GsE) (Yadav *et al*., 2011; Herrera-Cedano *et al*., 2012). At least fifteen GsE are known, of which stevioside and rebaudioside A (reb-A) and C (reb-C) are the most important; these compounds are up to 300 times sweeter than sugar, but stevioside and reb-A are bitter (Molina-Calle *et al*., 2017). The stevia leaf is also consumed to reduce blood glucose levels, protect from kidney and liver damage and increase insulin levels (Jeppesen *et al*., 2000; Momtazi-Borojeni *et al*., 2016). In addition to GsE, stevia contains phenolic compounds (CF) such as flavonoids, anthocyanins, tannins and phenolic acids (Sytar *et al*., 2016; Molina-Calle *et al*., 2017).

On the other hand, as the human body generates reactive species (ER) of oxygen (ROS) and nitrogen (RNS), among others, a balance between ER and antioxidant compounds is required to ensure an appropriate physiological function. If ERs exceed the human body's ability to regulate them, oxidative stress occurs causing damage to lipids, proteins and DNA (Lobo *et al*., 2010). To avoid these damages, it is necessary to count and consume external sources with the highest possible content of antioxidant compounds, such as CF (Tapas *et al*., 2008). There are several factors that allow to increase secondary metabolites such as temperature, light and CO_2. Another strategy is the application of elicitors such as jasmonic acid, chitosan or salicylates which induce physiological changes in plants, affecting their metabolism and increasing the synthesis of CF and other secondary metabolites (Nieves-Baenas *et al.*, 2014; García-Mier *et al*., 2015). Hao *et al.* (2015) used a mixture of methyl jasmonate and salicylic acid (AS) to increase the content of tanshinone, an active diterpene present in the roots of *Salvia miltiorrhiza*, which is widely used in the treatment of cardiovascular diseases. It was also reported that the application of AS increases the anti-inflammatory activity of *Aloe vera* by the increase of anthraquinones (Lee *et al*., 2013).

Elicitors reprogram the expression of genes related to secondary metabolites and manifest as differential patterns of the level of chemical compounds between elicited and non-elicited plants (Lukens and Zhan, 2007; Chinnusamy and Zhu, 2009; Mejia-Teniente *et al*., 2013). The reprogramming of gene expression can be measured by determining the epigenetic level; that is, the level of methylation of the chromatin in the histone or in the DNA which may or may not be hereditary (Eichten *et al*. 2014; Avramova, 2015). It is known that DNA methylation is the most important epigenetic alteration in eukaryotes and consists in the post-synthesis addition of a methyl group in the 5' position of the pyrimidine ring of cytosine (5-mC) (Vanyushin and Ashapkin, 2011).

According to Razin and Cedar (1991) there is an inverse relationship between DNA methylation and the expression of genes, a hypomethylated genome will express a greater number of genes than a hypermethylated one. The objective of this work was to evaluate the effect of the application of elicitors on the level of antioxidant capacity, GsE and CF, as well as the level of DNA methylation in stevia. To our knowledge, this is the first time that elicitors have been applied to stevia plants to evaluate their effect on methylation and the synthesis of metabolites with biological activity.

Materials and methods

Stevia plants

The experiment was carried out in the greenhouse of medicinal plants of the Bajio Experimental Field and was established on September 5, 2017. Twenty-five stevia plants were planted in individual pots of a Paraguayan creole genotype. The fertilization formula used was 40-30-10 and was supplemented with foliar applications of Agro k-amino (3g L^{-1}). For the control of pests and diseases, products based on Neem, garlic, soy, chamomile, rue and copper were applied (Ramírez-Jaramillo, 2011).

Application of elicitors and biological material

Two commercial elicitors were applied: hydrogen peroxide (H_2O_2, 14 mM) and chitosan (670 μg ml^{-1}) in the vegetative stage of the plants. The chitosan was applied to the soil and the hydrogen peroxide to the leaves by spraying. Elicitors were applied at time 0 (immediately after harvesting leaves without any treatment) and 15 days later. Leaves were harvested from each of the 25 plants separately, at 7, 15 and 21 days after application. The harvest of leaves at 15 days was made before the second application of the elicitors. After harvest, the leaves were frozen at -80 °C, lyophilized and stored at -20 °C until analysis. The chemical analyzes were performed by plant independently (n= 25).

Determination of steviosides

The stevioside and reb-A and -C were determined with high resolution liquid chromatography and diode detector (HPLC-DAD) based on the method of (Giraldo *et al.*, 2005). The identification and quantification of the sweeteners was determined by comparing the retention time and spectrum of the peaks of the sample with the peaks of commercial standards (Sigma).

Phenolic acids

They were determined with a HPLC-DAD equipment according to Ramamurthy *et al*. (1992). The identification and quantification was carried out comparing the retention times and the absorption spectrum of the following commercial standards (SIGMA, Mexico City): acid caffeic, cinnamic, chlorogenic, ellagic, ferulic, gallic, 2-hydroxycinnamic, p-hydroxybenzoic, protocateic and vanillic, as well as epicatechin, eriocitrin, catechin, hesperidin, kaemferol, myricetin, naringenin, naringin and quercetin.

Total phenols

The quantification of total phenols was carried out by the technique proposed by Singleton *et al*. (1999) with the Folin-Ciocalteu reagent. The content of total phenols was expressed as mg equivalents of gallic acid per 100 grams of dry sample (bs).

Flavonoids

The flavonoid content was quantified according to Dewanto *et al*. (2002) and were reported as mg routine equivalents per 100 grams of dry sample (bs).

Anthocyanins

These compounds were determined according to the technique described by Lee *et al*. (2005) by pH difference and were expressed as mg equivalents of cyanidin 3-glucoside per 100 g of dry sample.

Tannins

The condensed tannins were quantified according to the methodology described by Deshpande and Cheryan (1985) and were reported as mg equivalents of (+)-catechin per 100 g of sample.

Statistic analysis

The results of all the chemical compounds were carried out through a multivariate AnovA and a multiple variable analysis (n= 25) and a comparison of means by the Tukey method (0.05).

Obtaining DNA and methylation analysis

Of the 25 plants under study, three random groups of eight plants each were formed. Leaves of the three groups of plants were harvested, separately, before applying the elicitors (time 0) and 7 days after the first application. Leaves were not harvested at 15 or 21 days after the application of elicitors. The leaves of each group were mixed perfectly separately. The DNA of the six samples was obtained following the extraction protocol with CTAB (Doyle, 1987). Detection and quantitation of the levels of DNA methylation was performed with the reagent pack 5-mC DNA ELISA kit (D5325 and D5326, Zymo Research Corp) containing a monoclonal antibody specific for 5-mC. The reading of the reaction was carried out an absorbance of 405-450 nm in a spectrophotometer (Thermo Scientific model Multiskan GO) and the 5-mC was quantified according to the protocol of the kit. The data were tested by Anova using the GraphPad PRISM 6 program for Windows 10. A multiple Dunett comparison was also carried out, with a significance level of 0.05%.

Results and discussion

Phenolic acids

Chlorogenic, caffeic and ferulic acids were identified in stevia plants not elicited (0 days) and elicited (Table 1). The chlorogenic and ferulic acids consistently presented the highest concentrations at 21 days, with a chlorogenic acid increase of 16% at seven and 15 days and then a new increase of 22%, compared to the control (time 0). Regarding ferulic acid, it decreased by almost 1 μg seven days after elicitation. After 15 days, a recovery in the content

of this acid was detected and at 21 days a growth of 24% was observed in comparison with the plants without eliciting (0 days). With respect to caffeic acid, the plants showed an 18% decrease at 21 days compared to non-elicited plants (0 days). It is not known if, after 21 days, both the colorgenic and ferulic acids continue to increase and the brown acid decreases. These data suggest that, if it is desired to have a higher concentration of chlorogenic and ferulic acids in the stevia plant, the leaf should be harvested at least 21 days after the two applications of the elicitors.

Table 1. Content of phenolic acids (µg g^{-1}, bs) in elicited stevia.

Days [&]	Chlorogenic	Caffeic	Ferulic
0	3.91 ±0.18 c	0.264 ±0.014 b	4.72 ±0.09 b
7	4.68 ±0.29 b	0.305 ±0.023 a	3.68 ±0.16 c
15	4.59 ±0.27 b	0.267 ±0.03 ab	4.71 ±0.17 b
21	5.03 ±0.28 a	0.217 ±0.012 c	6.23 ±0.13 a

[&]= leaf was harvested on day 0, without eliciting (control) and at 7, 15, 21 days after the elicitation. The harvest at 15 days was made before the second application of the elicitors. Averages with similar letters are not statistically different (Tukey, 0.05).

Due to the lack of reports in the literature on the effect of the application of elicitors on stevia, it is not possible to make a comparative discussion of these results. However, reports of the application of jasmonate in bell pepper increase the content of phenolic compounds (García-Mier *et al*., 2015). On the other hand, it is known that when chlorogenic acid and ferulic acid are present in higher concentrations, they are responsible for a high antioxidant capacity that results in the reduction of harmful free radicals (Kikuzaki *et al*., 2002; Lan, 2007; Jeszka-Skowron *et al*., 2016) and therefore, elicited stevia is expected to contribute to the prevention of different diseases related to oxidative stress (Yashin *et al*., 2017).

Phenolic compounds

In response to the elicitation, stevia increased the content of total phenols, flavonoids and 65%, 66% and 13% condensed tannins, respectively at seven days after treatment (Figure 1A, B and C). However, at 15 and 21 days the content of total phenols and flavonoids was not increased, statistically speaking; This was despite the fact that the flavonoid content at 21 days was 5393.1 mg 100 g^{-1}, compared to 4523.1 mg 100 g^{-1} detected at seven days (Figure 1). This behavior can be explained due to the high variability in the content of this compound that was observed between plants (high standard deviation). On the contrary, the tannins continued to increase after 15 days, without presenting statistical differences with the content after seven days; however, 21 days after the application of the elicitors, the tannin content had increased 25% compared to the content of the plants, at the beginning of the experiment. The data presented here suggest that, if it is desired to have a high content of phenolic compounds, the leaves should be harvested at 21 days after the elicitation.

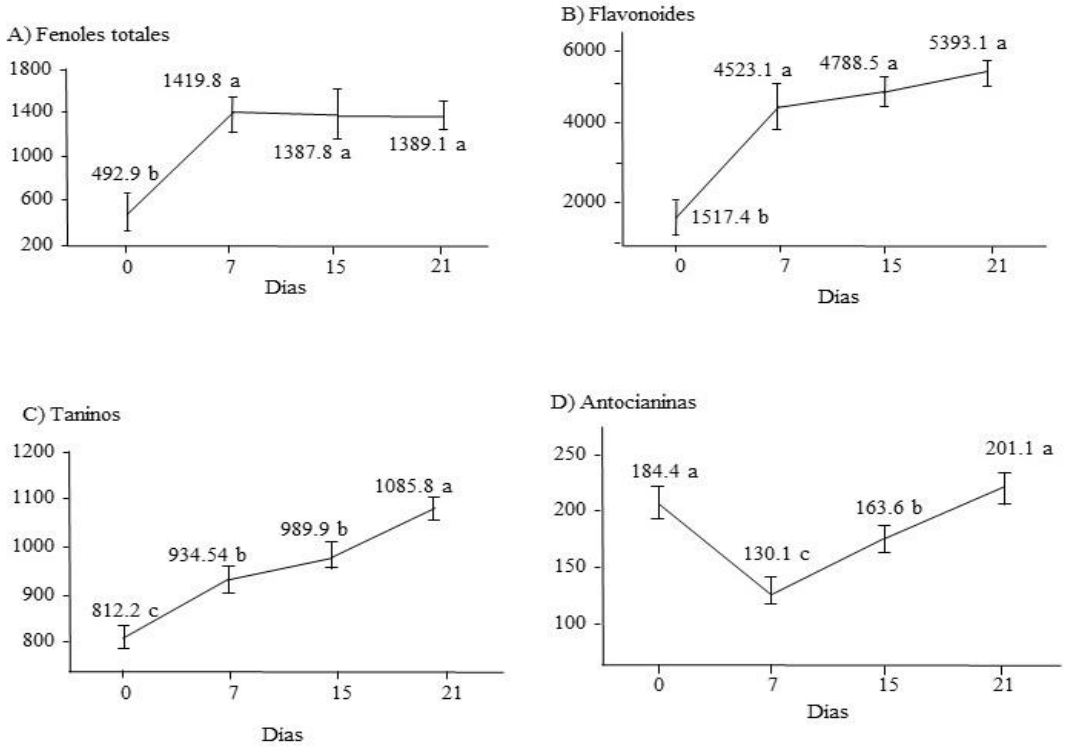

Figure 1. A) total phenols (mg EAG 100 g^{-1}); B) flavonoids (mg EAG 100 g^{-1}); C) tannins (mg EC 100 g^{-1}); and D) anthocyanins (EC3G 100 g^{-1}) in stevia not elicited (0 days) and at 7, 15 and 21 days after elicitation. EAG= gallic acid equivalents; EC= catechin equivalents; EC3G= equivalents of cyanidin 3-glycoside.

Application reports of elicitors, such as those used in this work, induce stress by causing the plant to generate an immune defense response that activates the expression of different genes for the expression of secondary metabolites, including phenolic compounds (Jiménez-García *et al*., 2013). For example, when chitosan and hydrogen peroxide were applied to peppers, the flavonoid content increased 37% (García-Mier *et al*., 2015), this result was lower than the one found here (66%). reported 46% increase in the content of condensed tannins, greater than 13% reported in this work. The differences in the results of García-Mier *et al*. (2015) and those of this work, are due to the metabolism and genetic response of each species.

With respect to anthocyanin content, a differential behavior was observed; for example, at seven days, the level of anthocyanins decreased from 184 (time 0) to 130.1 EC3G 100 g^{-1}; subsequently an increase was presented to 163.6 EC3G 100 g^{-1} at 15 days at 201.1 EC3G 100 g^{-1} at the end of the experiment (Figure 1D). However, the level of anthocyanins detected at 21 days after application was not statistically different from that found in plants at time 0 (before applying the elicitors). Apparently, chitosan and hydrogen peroxide do not elicit any response that modifies the synthesis of anthocyanins in stevia.

Esteviosides

In control plants (without elicitation, 0 days), the detected proportions of stevioside and reb-A and -C were 5.29 mg g^{-1}, 10.52 mg g^{-1} and 59.56 mg 100 g^{-1}, respectively (Table 2). When applying the elicitors (seven and 15 days) the amounts were significantly increased in all of them compared to the control plants; however, 21 days after application, the differences between the amounts of stevioside (49.49 mg g^{-1}) and reb-A 55.98 mg g^{-1}) were shortened, being only 13%. In the case of reb-C (198.61 mg g^{-1}) it was potentiated, detecting a difference of 400% with respect to the stevioside (Table 2).

Table 2. Content of sweetening compounds (mg g^{-1}, bs) in elicited stevia.

Days [&]	Estevioside	Rebaudioside	
		A	C
0	5.29 ±2.31 c	10.52 ±1.2 c	59.56 ±2.07 d
7	11.89 ±4.51 c	15.15 ±3.02 b	107.59 ±23.75 c
15	27.17 ±3.86 b	39.49 ±2.06 a	145.35 ±10.87 b
21	49.49 ±7.76 a	55.98 ±2.71 a	198.61 ±30.59 a

[&]= leaf was harvested on day 0, without eliciting (control) and at 7, 15, 21 days after the elicitation. The harvest at 15 days was made before the second application of the elicitors. Averages with similar letters are not statistically different (Tukey, 0.05).

For the specific case of stevioside, a significant increase of almost 10 times was detected during the experiment compared to non-elicited plants (Table 2). The data suggest that stevioside most likely continues to accumulate after 21 days. However, it is necessary to confirm this behavior. With respect to reb-A, the content of this compound was increased 3.8 and 5.3 times more at 15 and 21 days after the application of the elicitors, respectively, compared to the control treatment (at 0 days) (Table 2). On the other hand, the content of the reb-C doubled seven days after the application of the elicitors, compared with the control group. At 15 and 21 days it had increased almost two and three times more than the control group (Table 2).

It has been reported that the content of the GsE in stevia is very variable and that it depends on the genotype and the production environment (Brandle and Telmer, 2007). The mixture of steviosides in a sample will determine the sensory characteristics of the sweetener. It is known that stevioside is 143 times sweeter than sucrose, while reb-A is 242, but that the taste of reb-C is better because it is less bitter than stevioside and reb-A (Nicklasson *et al*., 2018). In this sense, the low concentration of stevioside and reb-A compared to reb-C, suggest that the batch of plants used here will produce a less bitter sweetener and, consequently, with less aftertaste characteristic of this plant.

On the other hand, Badran *et al*. (2015) showed that drought, induced with ethylene glycol, in *Stevia rebaudiana* plants significantly increases the content of stevioside. However, these authors detected up to 40 times less concentration (12.6 mg g^{-1}) than reported in this work since 49.49 mg g^{-1} was detected after 21 days of treatment (Table 2). This difference can be explained by the use

of elicitor and the plant material used. These authors did not determine any rebaudioside. Bayraktar *et al*. (2016) by applying elicitors *in vitro* culture model of stevia, steviosides increased from 1.56 mg g^{-1} dry weight to 14.69 mg g^{-1} dry weight.

Methylation analysis

This analysis was carried out once statistically significant changes were found in the accumulation of GsE in the stevia leaves to which the elicitors were applied, in comparison with those not elicited. Considering that these changes in the concentration of GE reflect changes in the metabolism of elicited plants and that this may be of epigenetic origin, the percentage of 5-mC present in elicited and non-elicited plants was measured. The results obtained indicate statistically significant differences in% 5-mC among the treatments evaluated (Figure 2). In this figure, a clear trend of hypomethylation can be observed in the elicited plants compared to the non-elicited plants.

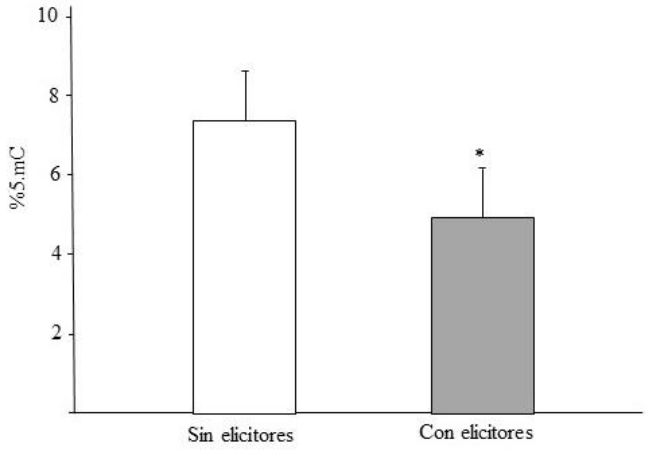

Figure 2. Percentage of 5-mC presented by two genomic DNAs of *Stevia rebaudiana* plants; SE= plants of the experimental variety of the INIFAP without application of elicitors and CE= plants of the experimental variety of the INIFAP with application of elicitors; *= significant at 0.05%.

The levels of % 5-mC found in this work range from 7.45% in plants not elicited to 4.88% in the elicited plants, values that are considered in the low ranges of methylation obtained in plants, since the methylation reports until now published, ranging from 6% to 14% depending on the method used for its evaluation in Arabidopsis, up to 25% in maize (Kakutani *et al*., 1999; Papa *et al*., 2001; Capuano *et al*., 2014). These differences can occur if it is considered that DNA methylation in plants is specific to species, tissues, organelles and age, as well as to the effect of the elicitor and to the sensitivity of the measurement method used (Vanyushin and Ashapkin, 2011).

The most important result is the hypomethylation of plants elicited with peroxide and chitosan, compared to those not elicited. There are reports that DNA methylation can be altered in response to abiotic stress, causing a new genetic regulation (Lukens and Zhan, 2007; Chinnusamy and Zhu, 2009), which is reflected in changes in the patterns of expression caused by a different number of

genes that are being expressed and therefore there is an inverse relationship between DNA methylation and the expression of genes, so that a hypomethylated genome comparatively with another hypermethylated one will have an expression of a greater number of genes (Razin and Cedar, 1991). The typical consequence of methylation in a genomic region is the repression of nearby genes, therefore, a high degree of methylation is associated with gene silencing (Bird, 2002). The results of this work suggest a relationship between the stress caused by the application of elicitors and epigenetic changes in the genome of the plant. Epigenetics is an area of opportunity that can be used to improve current production systems.

Conclusions

The application of hydrogen peroxide and chitosan modify the levels of compounds with biological activity present in stevia. The synthesis of steviosides and phenolic compounds differs within the same batch of stevia plants. While the application of elicitors decreases the content of the stevioside, but it increases the levels of rebaudioside A and of three phenolic acids evaluated (chlorogenic, caffeic and ferulic). It is necessary, not to suspend the application of elicitors if you want to maintain the high level of rebaudioside A and most likely of ferulic acid. The application of elicitors seems to be a promising strategy to increase metabolites of interest for human health. In the stevia plants to which the elicitors were applied, an increase in GE production and a decrease in DNA methylation was detected. In the absence of factors that induce mutations in the DNA sequence, it is very likely that changes in metabolism are due to changes in DNA methylation.

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