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SHORT COMMUNICATION

Plant Growth Regulators as Potential Elicitors to Increase the Contents of Phenolic Compounds and Antioxidant Capacity in Stevia Plants

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Abstract *Stevia rebaudiana* Bertoni is mainly used as a sweetener in many countries without any reported negative effects on health. This species is also abundant in phenolic compounds, which makes this plant a good candidate to satisfy the growing demand of natural sources for sweeteners and antioxidant compounds. Exogenously applied plant growth regulators (PGRs), acting as elicitors, influence the biosynthetic pathways of secondary metabolites. In this context, methyl jasmonate (MeJa), spermidine (SPD), salicylic acid (SA), and paclobutrazol (PBZ) (100 µM) were applied to stevia plants growing in a hydroponic system to assess their effects on temporary changes (at 24, 48, and 96 h) of antioxidant capacity and phenolic compounds (total soluble phenols, flavonoids, and hydroxycinnamic acids) levels. Overall, it was observed that MeJa increased total soluble phenolic content and antioxidant activity in extracts from Stevia leaves. By contrast, the addition of SPD, SA, and PBZ did not show a significant increase in any of the evaluated parameters. The results also showed a positive and strong correlation between phenolic compounds contents and antioxidant capacity. In short, results suggest that the application of MeJa could be a feasible way to enhance the biosynthesis of high added value phytochemicals that have notable antioxidant properties, and consequently potential health benefits, in stevia plants.

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Medicinal plants constitute an important source of natural antioxidant compounds which can play a very important role in disease prevention and treatment (Dey et al. 2013). In this respect, *Stevia rebaudiana*, an Asteraceae (Compositae) species native to Paraguay and Brazil, deserves attention (Lemus-Mondaca et al. 2012) due to the accumulation of not only low-calorie sweeteners, but also compounds with marked ROS scavenging activity (Bender et al. 2015). The leaves of stevia produce more than 30 steviol glycosides (SGs) responsible for the sweetening properties of this herb. Although SGs have been reported to display antioxidant properties (Hajihashemi and Geuns 2013), it seems that these compounds are not the main contributors to antioxidant capacity in stevia extracts (Álvarez-Robles et al. 2016). Other phytochemicals, such as flavonoids, alkaloids, hydroxycinnamic acids, vitamins, phytosterols, and essential oils, have been described in stevia (Karaköse et al. 2011). Concerning antioxidant capacity, the most commonly used tests for its determination indicate a good correlation between total soluble phenolic compounds (and also flavonoid) contents and antioxidant capacity of stevia extracts (Kim et al. 2011). Phenolics have been recognized to be stress markers due to the increased levels observed when plant materials are under unfavorable conditions.

In fact, biotic and abiotic stresses induce changes in both primary and secondary plant metabolisms, leading to the production of phenols and other valuable compounds. We know now that some physical and chemical agents

(elicitors) can stimulate specific biosynthetic pathway and hence increase the levels of stress metabolites. These elicitors can trigger a network of complex responses, at the molecular, biochemical, and physiological levels, aiming at acclimatization and adaptation to the environment (Giri and Zaheer 2016). Several types of compounds are involved in stress signaling and response in plant cells. Plant hormones, like methyl jasmonate (MeJa) and salicylic acid (SA), are molecules known to act as efficient elicitors for the production of secondary metabolites in diverse plant materials (Pérez-Tortosa et al. 2012; Rodrigues-Brandão et al. 2014). Other PGRs are also related to stress tolerance in plants, and hence they can be considered good candidates to be used as elicitors. In this respect, some studies have highlighted the importance of polyamines, mainly spermidine (SPD) (Li et al. 2016). Synthetic plant growth retardants can modify hormonal balances in cells, thus affecting metabolite profiles. Paclobutrazol (PBZ) is an inhibitor of gibberellin synthesis whose influence on the synthesis of secondary metabolites is also being explored, mainly because this growth retardant could alleviate damages caused by stress and additionally induce an increase in plant antioxidant capacity (Karimi et al. 2014).

In this sense, the present work was undertaken to examine the evolution of antioxidant capacity and phenolic compounds levels in ethanolic extracts of *Stevia rebaudiana* leaves in order to assess whether MeJa, SPD, SA, and PBZ treatments are able to increase the production of these compounds. The main goal of the work is to check the validity of this PGRs elicitation approach for being included in productive schemes aimed to exploit antioxidant compounds from stevia plants.

Preparation of plants for elicitor treatments was carried out as described by Lucho et al. (2018). Five treatments were applied: Control (T1) and four elicitor treatments, MeJa (T2), SPD (T3), SA (T4), and PBZ (T5), all at a concentration of 100 µM. Leaves were harvested over a period of 4 days at 24, 48, and 96 h. Then, leaves were stored in liquid nitrogen until lyophilization of the samples, about 3 days later. Lyophilized samples (0.15 g) were homogenized in 3 mL 70% ethanol using an ultrasonic bath set at 80 °C for 30 min. Subsequently, centrifugation was carried out at $15{,}000 \times g_{max}$, 4 °C, for 5 min, and the supernatants obtained were stored at − 20 °C until further determinations.

Total soluble phenol content (TPC) was determined according to the Folin–Ciocalteu method (López-Orenes et al. 2013b) using gallic acid (0–3000 µM) as the standard. (The standard curve equation and the regression coefficient obtained were $y = 0.0004x + 0.0589$ and $r^2 = 0.9953$, respectively.) Total flavonoid content (FLA) was determined according to Kim et al. (2003). A standard curve in the range 0–3000 µM of rutin was used for calibration ($y = 0.0003x + 0.0439$; $r^2 = 0.9993$). Hydroxycinnamic acids (HCA) were determined using the Arnow's reagent as described by Álvarez-Robles et al. (2016). A standard curve using caffeic acid concentrations in the range 0–7000 µM was constructed ($y = 0.0001x + 0.0262$; $r^2 = 0.9923$).

The ABTS radical was measured using the method of Katalinic et al. (2006). Caffeic acid (0–1500 µM) was used as a standard to quantify antioxidant capacity ($y = 0.0009x + 0.0078$; $r^2 = 0.9785$). DPPH radical-scavenging activity was determined according to Pérez-Tortosa et al. (2012). The absorbance readings were compared to a calibration curve constructed using caffeic acid (0–1500 µM) as a standard ($y = 0.0010x + 0.0376$; $r^2 = 0.9937$). The FRAP assay was carried out according to Katalinic et al. (2006). A standard curve in the range 0–7000 µM of $FeSO_4 \cdot 7H_2O$ was used for calibration. For calculating purposes, the standard curve equation was applied and the regression coefficients obtained were $y = 0.0002x + 0.1906$ and $r^2 = 0.9964$, respectively. The experiment was arranged in a completely randomized 5 × 3 factorial design, with four elicitor agents and a control (without elicitors) and three periods of exposure to the corresponding treatments. For each combination, three biological repetitions were performed, of which each consisted of one pot with five plants. Results correspond to mean ± standard deviation. For statistical analyses, ANOVA and Duncan's multiple range tests were performed at $P < 0.05$ to calculate significant differences in treatments and exposure times, using the R software. Pearson correlation was used in the correlation evaluations.

The variations in TPC, FLA, and HCA contents after the application of elicitors are shown in Fig. 1. The results seem to indicate a differential sensitivity of the parameters evaluated to each of the elicitors used, although no statistically significant differences between treatments were observed before 96 h. At that time, SPD treatment resulted in the lowest levels of TPC, FLA, and HCA, whereas with MeJa treatment, plants showed the highest levels of these three families of compounds, although values found were significantly different from those of control only for TPC. Evolution with time of phenolic compounds within each treatment showed marked differences. Control and SA treatments did not provoke significant changes in leaf TPC after 96 h; conversely, TPC levels at 96 h were higher than those at 24 h for MeJa-treated plants, whereas lower values of this parameter were found at 96 h in leaves of plants treated with SPD (Fig. 1a). The analysis of variations with time of FLA and HCA contents in leaf extracts revealed differences between elicitors that could account for the changes observed in TPC. Thus, reduced levels of total soluble phenolics in SPD-treated plants at 96 h seemed to

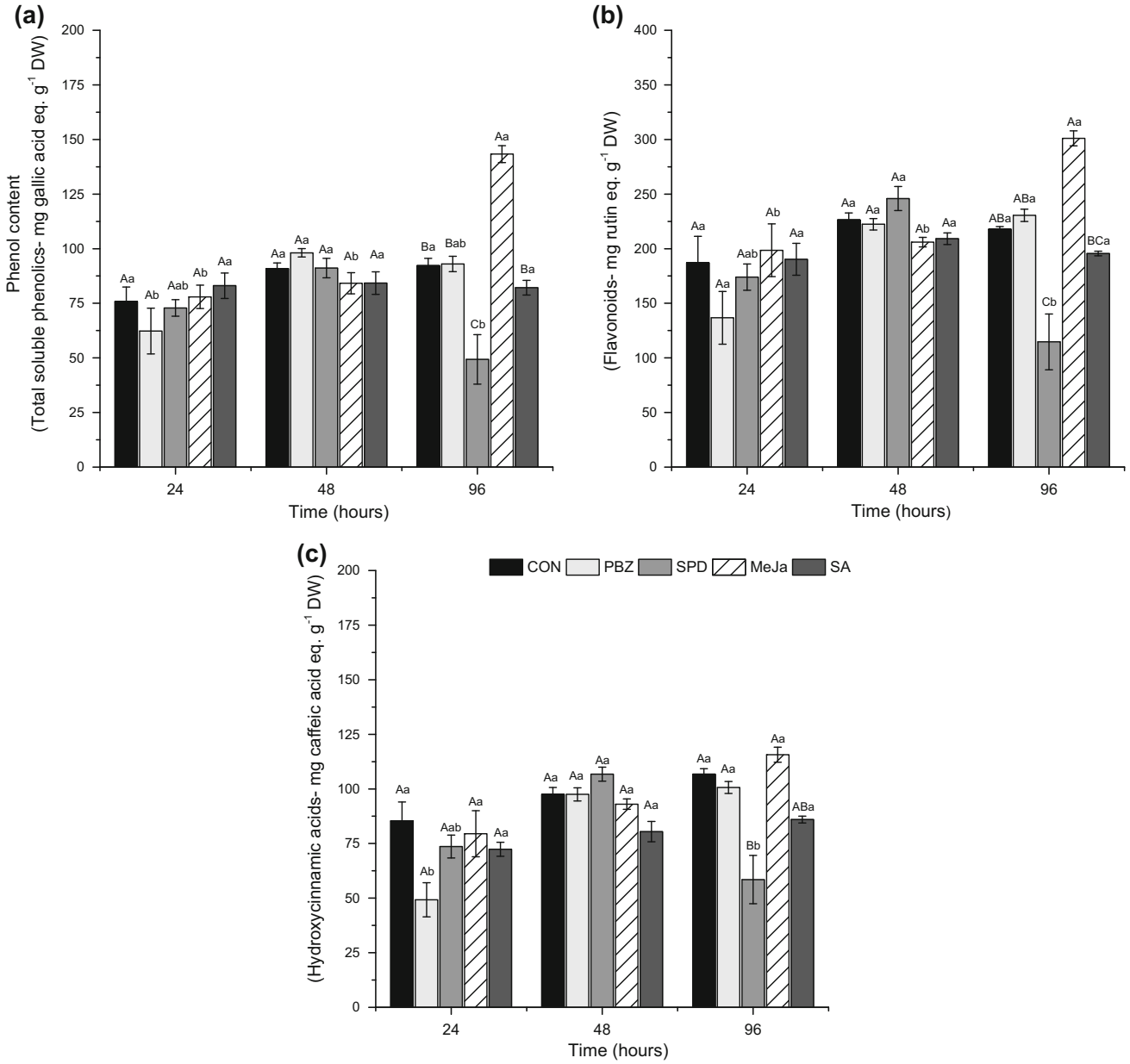

Fig. 1 Phenolic compounds contents (total soluble phenolics, flavonoids, and hydroxycinnamic acids) in extracts from *Stevia rebaudiana* under the effect of elicitors paclobutrazol (PBZ), spermidine (SPD), methyl jasmonate (MeJa), and salicylic acid (SA) after three exposure times (24, 48, and 96 h). Error bars represent mean ± SD. Columns with different uppercase letters indicate differences in treatments for the same time, while different lowercase letters indicate differences in time for the same treatment. Significant differences based on ANOVA followed by Duncan's test $P \leq 0.05$

be related to an overall decrease in soluble phenolic compounds since this treatment also resulted in decreased concentrations of both FLA and HCA. Interestingly, although both PBZ and MeJa treatments resulted in increased TPC, the families of phenolic compounds responsible for these increases seem to be different for each treatment. In this way, PBZ provoked an increase in HCA levels at 96 h, whereas no significant changes in FLA were observed at this time (Fig. 1b, c). The opposite trend was observed when plants were treated with MeJa, suggesting a differential effect of these two elicitors on phenol biosynthetic pathways.

Previous reports indicate that stevia extracts contain relatively high levels of total phenolic compounds and flavonoids (Tadhani et al. 2007). In our study, the TPC determined in leaves from *S. rebaudiana* ranged from 49.30 to 143.31 mg gallic acid eq. g^{-1} dry weight, when treated for 96 h with SPD and MeJa, respectively. Shukla et al. (2009) reported TPC values in both ethanolic and aqueous extracts from stevia leaves of 61.50 and 56.73 mg

gallic acid g^{-1} dry weight, respectively. These values are slightly lower than those obtained in our study for almost all treatments and exposure times, and considerably lower than the values determined in extracts from plants treated for 96 h with MeJa, thus reinforcing the positive effect of this elicitor on phenolics accumulation. According to Martínez-Esplá et al. (2017), the positive effect of MeJa on phenolic content could be attributed to its role in the activation of the phenylpropanoid pathway, which is one of the inducible defense responses leading to FLA and HCA accumulation. MeJa-treated plants owned the highest FLA content at 96 h, with an increase of 28% over control plants (although no statistically significant differences were observed between treatments), whereas SPD-treated plants had the lowest FLA content (Fig. 1b). The accumulation of HCA was also increased at 96 h in MeJa-treated plants, although the value found did not differ statistically from that determined in control plants (Fig. 1c). Values reported for this family of phenolics in stevia plants are around 50 mg eq. caffeic acid g^{-1} dry weight (Karaköse et al. 2011). Therefore, MeJa could be considered as a good elicitor for HCA production since the value found at the longest incubation period was 115 mg eq. caffeic acid g^{-1} dry weight, approximately. This means that elicitation of stevia plants with MeJa could be a potential technology for stimulating the biosynthesis of these phytochemicals that have potential health-promoting properties.

Figure 2 shows the results obtained after the application of three assays (ABTS, DPPH, and FRAP) to determine antioxidant capacity of ethanolic extracts from stevia leaves. In the ABTS assay, significant differences among the effect of elicitors within each exposure time were only observed at both 24 and 96 h (Fig. 2a). At 24 h, all elicitors assayed showed a decrease in the levels of antioxidant capacity in relation to control plants, although only statistically significant for PBZ and SA treatments. Significant differences between control and the rest of treatments could not be determined at 96 h of elicitor exposure. However, PBZ- and MeJa-treated plants showed antioxidant capacities significantly higher than those determined in extracts from SPD- and SA-treated plants. In Fig. 2a, it can also be observed the evolution with time of antioxidant capacity determined using the ABTS test. PBZ and MeJa treatments resulted in significant changes at 96 h in relation to the values obtained at 24 h, with increases of 75% and 49%, respectively. DPPH test gave similar results to those described for ABTS (Fig. 2b). Differences among treatments were only observed at both 24 h and 96 h, although at 24 h PBZ was the only treatment that resulted in a statistically significant different antioxidant capacity (47% lower) in relation to control treatment. At 96 h, MeJa-treated plants showed the highest levels of antioxidant capacity with a net increase (44%) after 96 h with respect to initial values (24 h). The same was observed for PBZ treatment, although in this case the net increase was even higher (124%). No significant changes with time could be observed for the rest of treatments.

FRAP values remained relatively stable among treatments for shorter exposure times and varied considerably between treatments at 96 h exposure time. The highest values of antioxidant capacity were observed in MeJa-treated plants, although these values were not significantly higher than those obtained in the control treatment (Fig. 2c). MeJa was the only elicitor that provoked a significant increase in antioxidant capacity determined using the FRAP assay at the end of the incubation period with respect to the initial value (46%). SPD treatment resulted in a transient increase at 48 h with the lowest FRAP value being determined at 96 h. The rest of treatments did not provoke significant changes with time. At 96 h of elicitor exposure, DPPH scavenging assay showed that MeJa application resulted in significantly improved antioxidant capacity, reaching 1079.11 ± 23.41 μmol caffeic acid g^{-1} dry weight (Fig. 2b) possibly this increase being due to the induction of compounds with free radical scavenging capacity. Information about the effect of MeJa treatments on the antioxidant capacity of stevia plants grown in hydroponic conditions is scant. However, the benefits of exogenous application of MeJa were also demonstrated in other species using different cultivation systems (Martínez-Esplá et al. 2017; Wang et al. 2009).

Overall, the results of the three antioxidant assays showed that SPD was not able to increase the antioxidant capacity in stevia leaves, although it cannot be ruled out that other assay conditions would have a positive effect on antioxidants production. Plants treated with SA did not present an increase in the antioxidant capacity either. Results obtained by using this elicitor were similar to those found by López-Orenes et al. (2013a) in *Cistus heterophyllus*, who found that low SA doses did not promote an increase in antioxidant capacity when measured by both the DPPH and FRAP methods. In our study, antioxidant capacity values determined in MeJa-treated plants were significantly higher than those of control plants only for the DPPH assay. These results point to the fact that the increase observed in antioxidant capacity was due to a higher TPC, in general, and to the specific accumulation of certain types of phenolics, in particular. Based on this, the next objective of this work was to verify whether correlations exist between the parameters evaluated.

Correlation analysis was performed through Pearson's test. Table 1 shows that as can be expected, positive and strong relationship were found between FLA and FRAP (r^2 = 0.959), DPPH (r^2 = 0.833), and ABTS (r^2 = 0.702) assays. In a similar way, a clear relationship was also found between TPC and FRAP assay (r^2 = 0.896), although

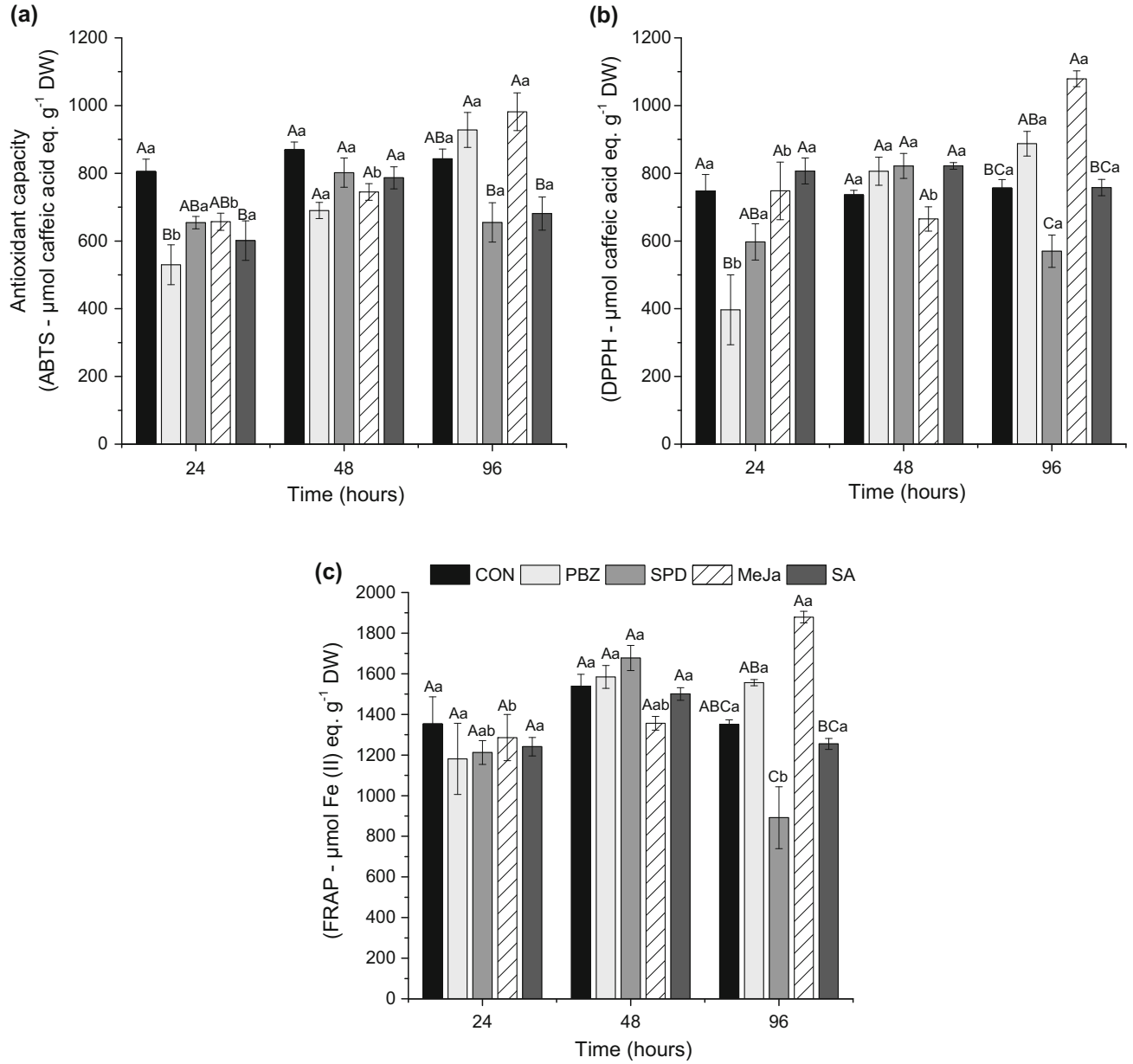

Fig. 2 Antioxidant capacity evaluated by ABTS, DPPH, and FRAP assays in extracts from *Stevia rebaudiana* under the effect of elicitors paclobutrazol (PBZ), spermidine (SPD), methyl jasmonate (MeJa) and salicylic acid (SA) after three exposure times (24, 48, and 96 h). Error bars represent mean ± SD. Columns with different uppercase letters indicate differences in treatments for the same time, while different lowercase letters indicate differences in time for the same treatment. Significant differences based on ANOVA followed by Duncan's test $P \leq 0.05$

correlations between TPC and the DPPH and ABTS assays were lower ($r^2 = 0.799$ and 0.657, respectively). Such a correlation between TPC and antioxidant activity was also observed in previous works with stevia plants (Karimi et al. 2015) and with other medicinal plants (Pérez-Tortosa et al. 2012; López-Orenes et al. 2013a). Therefore, our results agree with the view that phenolic compounds are the major contributors to antioxidant capacity not only in stevia plants, but also in other plant species and that MeJa treatment of plants results in higher levels of total soluble phenolic compounds what in turn provide higher antioxidant potentialities. As far as we know, the present study is the first to evaluate the potential of the elicitors MeJa, SPD, and SA for bioactive compounds accumulation in stevia plants grown in hydroponic conditions. Results obtained provide scope for further research with these or other elicitors by varying concentrations, application forms, and exposure times.

Table 1 Correlation coefficients between antioxidant capacity (determined by DPPH, FRAP, and ABTS assays) and total soluble phenol content (TPC), flavonoids (FLA) or hydrocinnamic acids (HCA) on *Stevia rebaudiana* leaf extracts under the effect of the elicitors paclobutrazol, spermidine, methyl jasmonate, and salicylic acid after three exposure times (24, 48, and 96 h)

	TPC	FLA	HCAs	DPPH	FRAP	ABTS
TPC		0.918**	0.872**	0.799**	0.896**	0.657**
FLA			0.943**	0.833**	0.959**	0.702**
HCAs				0.788**	0.916**	0.693**
DPPH					0.792**	0.669**
FRAP						0.664**
ABTS						

DPPH 2,2-diphenyl-1-picrylhydrazyl; *FRAP* ferric reducing antioxidant power; *ABTS* 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
Significance level at $^{**}P < 0.01$

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Author's Contributions SRL, AAC, AMK, and EJBB planned and designed the experiments. SRL, MNA, LA, ALO, and AMK did experimental work and analyzed the data. SRL, EJBB, AAC, MAF, and VJB wrote the manuscript. All authors have contributed, seen, and approved the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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