Sugar Tech (July-Aug 2019) 21(4):569–577
https://doi.org/10.1007/s12355-018-0655-6

RESEARCH ARTICLE

Elicitation of Stevia Glycosides Using Salicylic Acid and Silver Nanoparticles Under Callus Culture

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Received: 6 May 2018 / Accepted: 1 August 2018 / Published online: 20 August 2018

Abstract Development of biosynthesis of phytochemicals, especially medicinal products, is highly important due to their broad bioactivity properties. In this study, optimization of callus growth was initially carried out using various combinations of plant growth regulators. Callus with the highest fresh weight was produced on Murashige and Skoog (MS) medium containing 1 (mg/l) naphthalene acetic acid + 0.5 (mg/l) benzyl aminopurine. The effect of different concentrations of salicylic acid (SA) (0.25, 0.5 and 0.75 mg/l) and silver nanoparticles (Ag NPs) (15, 30, 45 and 60 mg/l) on callus growth as well as the possibility of stevia glycosides (SGs) production in callus culture was subsequently evaluated. The SA elicitation, at a concentration of 0.75 (mg/l), resulted in the highest level of callus growth rate (0.1 cm/day), callus diameter (0.79 cm) and relative callus fresh weight (0.085). Likewise, 45 (mg/l) of Ag NPs led to the highest amount of stevioside (32.34 mg/g dry weight callus). The addition of 0.25 (mg/l) of SA to the MS medium led to production of the highest amount of rebaudioside A (3.40 mg/g dry weight callus). The results of this study may enhance the commercial application of important glycosides prevalent in Stevia by highlighting that the nano-elicitors and SA should be utilized at optimized concentrations.

Keywords Callus · Elicitors · Growth · Secondary metabolites · Stevioside

Introduction

Secondary metabolites (SMs) are the unique sources of pharmaceuticals, food supplements and other industrial products requiring plant growth and development (Piasecka et al. 2015; Tiwari and Rana 2015). Increasing SM production and accumulation is considered a part of the defense reaction of plants (Piasecka et al. 2015), activated by adding various kinds of abiotic and biotic elicitors (Kang et al. 2004; Namdeo 2007). Nowadays, the plants containing beneficial bioactive compounds are increasingly employed in pharmaceutical and food industries (Bourgaud et al. 2001; Tiwari and Rana 2015). Plant cell culture is also considered an effective biotechnological method to produce high amounts of SMs (Rao and Ravishankar 2002) in a short period of time in large-scale production. Biotic (e.g., chitosan, chitin, yeast extract) and abiotic (e.g., sodium chloride and salicylic acid) stresses are known as the elicitors stimulating SM biosynthesis in various plant cell cultures (Gupta et al. 2014).

Salicylic acid (SA), one of the plant growth regulators (Kang et al. 2004), plays an important signaling role in the activation of various defense responses of plants (Yu et al. 2006). SA has widely been applied as an elicitor in SM production, including taxol in *Taxus chinensis* (Wang et al. 2007), tropane alkaloids in *Scopolia parviflora* (Kang et al. 2004), and jaceosidin and syringing in *Saussurea medusa* (Yu et al. 2006). In SM elicitation based on callus culture,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12355-018-0655-6) contains supplementary material, which is available to authorized users.

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the use of nanoparticles (NPs) is relatively new and needs further investigation (Fazal et al. 2016; Goswami et al. 2017). NPs, especially silver NPs, can produce SMs via stimulating the cell's defense mechanism (Marslin et al. 2017). Most recent studies carried out in the area have focused on the effect of $AgNO_3$ as a growth-promoting agent in plants (Kumar et al. 2009; Marslin et al. 2017), while few studies have so far been evaluated the effects of NPs on the enhancement of SMs (Vecerova et al. 2016; Javed et al. 2017) and antioxidant activities (Barbasz et al. 2016; Fazal et al. 2016; Javed et al. 2017).

Stevia rebaudiana is a member of Asteraceae family and is considered an important plant used in pharmaceutical and food industries as a natural sweetener (Mathur and Shekhawat 2013; Mahmud et al. 2014). The leaves of Stevia are the source of glycosides and diterpene steviol, estimated to be 300–400 times sweeter than sucrose in the concentrations of 4% w/v (Geuns 2003), having non-toxic and non-mutagenic properties (Mathur and Shekhawat 2013). These compounds can successfully be used as possible sugar substitutes for diabetes and other diseases involving carbohydrate metabolism disorders (Mathur and Shekhawat 2013). Stevia glycosides (SGs) are glycosylated derivatives of the diterpenoid steviol (Geuns 2003). Stevioside (ST) and rebaudioside A (RebA) are considered the most prevalent SGs in the Stevia (Brandle and Telmer 2007; Mahmud et al. 2014). However, owing to the low yield of biomass in Stevia, further improvement in the production of SGs, by means of plant cell cultures, based on an effective regulation of SG biosynthesis in Stevia cells, seems to be essential. Few reports have been released focusing on the effects of different elicitors, such as salt stress (Gupta et al. 2014), plant growth regulators (Janarthanam et al. 2010) and photoperiod (Ahmad et al. 2016), on SG and SM production by mean of Stevia callus culture. Reviewing the related literature, the authors found out that the effects of NPs and SA elicitors on the improvement of major SGs have not thoroughly been investigated (ST and Reb A). Indeed, considering the new advances in the area of nanotechnology and various bio-interaction properties of NPs, the potentiality of NP, as a nano-elicitor, requires further investigation. Accordingly, this study was carried out (1) to optimize the callus production as a perquisite for successful production of secondary metabolites and (2) to investigate the effects of different elicitors (salicylic acid and Ag NPs) on ST and Reb A contents of Stevia using callus culture.

Materials and Methods

Explant Preparation and Callus Induction

Leaf explants were taken from 4 weeks old aseptically grown shoots on Murashige and Skoog's (MS) medium (1962) (Duchefa, Haarlem, The Netherlands), containing various degrees of macro- and microorganic elements and vitamins, supplemented with 4 mg/l kinetin (Gupta et al. 2014). The medium was solidified with 8 g/l agar (Merck) and supplemented (per liter) using 0.1 g/l myo-inositol (Merck Com.) and 30 g/l sucrose (Merck). The pH of the medium was then adjusted to 5.7 prior to autoclaving at 121 °C for 20 min. For callus induction, in vitro leaves were cultured on SM medium, supplemented with different combinations of plant growth regulators, including auxins (2,4 D and NAA) and cytokinins (BAP and Kin) (Table 1), 0.1 g/l myo-inositol (Merck Com.) and 30 g/l sucrose (Merck), and gelled with 8 g/l agar (Merck). Then, for the second time, the pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 30 min. The cultures were incubated inside a growth chamber with 16/8 light/dark photoperiod, relative humidity of 55%, and light intensity of 30 µmol m^{-2} s^{-1} provided by cool-white fluorescent tube lights (20 W), at 24±2 °C.

Elicitation Assay

Silver nano-powder was purchased from the US Research Nanomaterials Inc., Houston, TX, USA. According to the instructions of the manufacturer, the average size and purity of Ag NPs were considered as 30–50 nm and 99.99%, respectively. The effects of different

Table 1 Different treatments of auxins and cytokinins used for callus induction in *Stevia*

MS media	Plant growth regulations	Callus morphology
MS_1	MS+0.5 mg/l (2,4 D)+1 mg/l (Kin)	Soft and yellow–green
MS_2	MS+1 mg/l (2,4 D)+1 mg/l (Kin)	Friable and green
MS_3	MS+0.5 mg/l (2,4 D)+1 mg/l (Kin)	No callus appearance
MS_4	MS+1 mg/l (2,4 D)	No callus appearance
MS_5	MS+0.5 mg/l (NAA)+1 mg/l (BAP)	Friable and yellow
MS_6	MS+1 mg/l (NAA)+0.5 mg/l (BAP)	Friable and green

MS Murashige and Skoog; *2,4 D* 2,4-dichlorophenoxyacetic acid; *Kin* kinetin; *NAA* naphthalene acetic acid; *BAP* benzylaminopurine

concentrations of Ag NPs (30, 45 and 60 mg/l) and salicylic acid (Sigma-Aldrich) (0.25, 0.5 and 0.75 mg/l) on callus growth and SGs (ST and RebA) production were then studied. Before being added to the cultures, elicitors were filter-sterilized (0.45 µm). The fresh calli (20 days) were transferred to the selected media having 16/8 light/-dark cycle, light intensity of 30 µmol m^{-2} s^{-1} and relative humidity of 55%, and containing 1 mg/l, 2,4 D+1 mg/l Kin, at the temperature of 24±2 °C. Different traits were then measured at different time intervals after the elicitation (2, 4 and 6 days).

Trait Measurement

Callus-Related Traits

The callus induction (CI) (%) was calculated as: [(*n*/*N*) × 100], where n equaled the total number of calli; *N* was equal to the total number of cultured explants (Compton 1994). The callus growth rate (CGR) was measured by the mean of callus growth rate (cm/day) on different days (7, 15, 23 and 31 days) after callus induction (2, 4 and 6 days) and elicitation. To do so, callus diameter (CD) (di) (root square of callus length × callus width) was divided by the number of each period (Compton 1994). After determining the callus fresh weight (CFW), calli were dried overnight in an oven at 60 °C for 24 h (Javed et al. 2017) for further analysis.

Quantitative Estimation of SGs by HPLC Analysis

Twenty milligrams of the dried callus samples was mixed with ethanol (70% v/v) in water bath at 70 °C for 5 h (Javed et al. 2017). The solution was centrifuged for 10 (min) at 14,000 rpm and then filtered through 0.45-µm filters. The samples were injected to high-performance liquid chromatography (HPLC) by RI detector, having a flow rate of 0.6 ml/min and an injection volume of 20 µl (JASCO, Tokyo, Japan) at 40 °C. The separation was done with fluid-phase methanol and water in the ratio of 70:30 and the wavelength of 210 nm by a UV detector, C_{18} column (TSK gel-ODSC-18.5 µm, 4.6 × 25 mm). The content of ST and RebA were determined based on the calibration curve of standard compounds, including stevioside (Sigma-Aldrich) and rebaudioside A (Sigma-Aldrich) (Gupta et al. 2014).

Statistical Analysis

All experiments were repeated with three replicates for the investigation of callus-related traits. Eight samples in each replicate were studied. Analysis of variance (ANOVA) was carried out using SAS software (SAS 9.1). The means were compared using LSD (least significant difference) at 5% level of significance as the mean±standard deviation. For HPLC analysis, all data were reported as the mean±standard deviation for three independent samples.

Results and Discussion

Effects of Different Plant Growth Regulators on Callus-Related Traits in Stevia

Callogenesis and callus-related traits were studied in response to various plant growth regulators in *Stevia.* Among different plant growth regulators, two combinations containing MS+1 (mg/l) 2,4 D and MS+0.5 (mg/l) 2,4 D+1 (mg/l) Kin were not capable of inducing callus formation (Table 2). The MS media supplemented with 2.5 (mg/l) 2,4 D and 0.5 (mg/l) BAP were recognized as the best combination for callus induction in *Stevia* (Mahmud et al. 2014). The responsive media (MS_1, MS_2, MS_3, MS_6) significantly increased both the callus fresh weight and callus growth rate (Table 2). The highest (0.096 g) and the lowest (0.035 g) callus fresh weight were observed in culture media containing 1 (mg/l) NAA+0.5 (mg/l) BAP and 0.5 (mg/l) 2,4 D+1 (mg/l) Kin combinations, respectively (Table 2). These findings are consistent with those reported by Janarthanam et al. (2010), who have observed that MS culture medium supplemented with 1 (mg/l) NAA +0.5 (mg/l) BAP resulted in the highest callus fresh weight in *Stevia*. These inconsistencies between the findings of the present study and the previous reports can be due to the effects of many factors on callus induction in Stevia,

Table 2 The effects of different plant growth regulators on the callus-related traits of *Stevia*

Traits	Plant growth regulators (mg/l)			
	0.5 (2,4 D)+1 (Kin)	1 (2,4 D)+1 (Kin)	0.5 (NAA)+1 (BAP)	1 (NAA)+0.5 (BAP)
CI (%)	65.93[a]	64.37[a]	54.68[a]	73.43[a]
CFW (g)	0.035[b]	0.079[a]	0.038[b]	0.096[a]
CGR(cm/day)	0.02[b]	0.038[a]	0.033[a]	0.04[a]

CI callus induction; *CFW* callus fresh weight; *CGR* callus growth rate

Treatments with the same letters in each row were not significantly different based on mean comparison by LSD's test at $P<0.05$

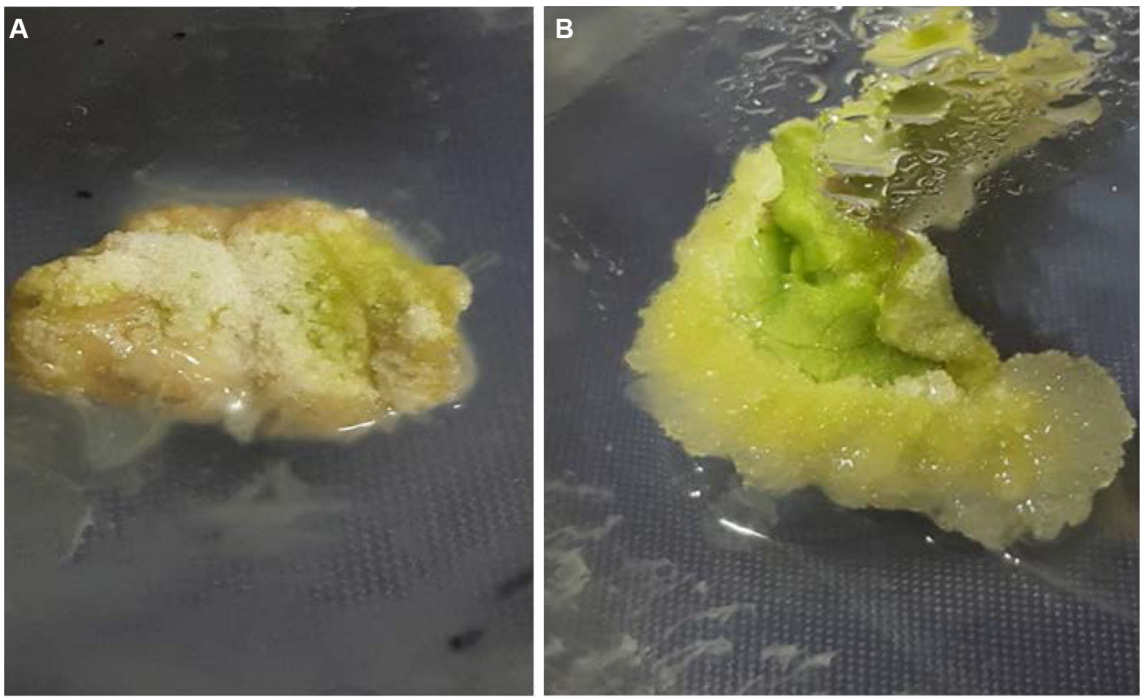

Fig. 1 Callusing from leaf explants of *Stevia rebaudiana* on Murashige and Skoog medium supplemented with 1.0 (mg/l) 2,4 D+1.0 (mg/l) Kin (A), 1 (mg/l) NAA+0. 5 (mg/l) BAP (B)

including explants, genotypes, plant growth regulators and light intensity (Mathur and Shekhawat 2013). The highest (0.038 cm/day) and the lowest (0.02 cm/day) callus growth rates were detected in culture media containing 1 (mg/l) 2,4 D+1 (mg/l) Kin and 0.5 (mg/l) 2,4 D+1 (mg/l) Kin, respectively (Table 2). The most effective initiator of callus from *Stevia* leaf explants was the combination of auxins and cytokinins (Fig. 1). According to the findings of this study, the effect of NAA on callus culture growth was more significant than that of 2,4 D, while BAP exhibited a stronger effect on callus growth compared to kinetin in *Stevia*.

Elicitation Results

The effects of different concentrations of SA and Ag NPs on callus-related traits (callus dry weight, callus relative fresh weight, callus diameter and callus growth rate) and glycosides content (ST and Reb A) were evaluated at three different post-elicitation time points (2, 4 and 6 days) (Table S1). The results of ANOVA test indicated that all the examined traits (CDW, RFW, CD, CGR, ST and RebA) were significantly affected by the elicitors. Elicitation duration, however, only affected the callus diameter, callus growth rate, ST and Reb A, significantly (Table S1). The elicitors × days post of elicitation interaction was significant for all the callus-related traits, except relative fresh weight content (Table S1).

Effects of Elicitation on Callus-Related Traits

The effects of silver nanoparticles and SA on callus growth and secondary metabolites of *Stevia* have not yet been clearly depicted. Ag NP_S are considered as new frontier in abiotic stress elicitation (Vannini et al. 2013). Identifying superior elicitations might be a preferential strategy for the commercial production of SGs applying in vitro callus culture. Moreover, evaluating the effects of NPs on callus growth properties is of great importance in terms of physiological action of NPs on callus cells. Among different elicitors (SA and NPs), the highest amount of CDW (0.06 g) was found in the experimental group treated with 0.75 mg/l SA; however, this amount was not significantly higher than that detected in the control group (0.054 g) (Table 3). The results of the present study revealed that RFW and CGR were increased up to a certain limit (45 mg/l) in Ag NPs-treated group; although their amounts were then significantly decreased (Table 3). A yellowish brown callus was observed in the elicitation process using Ag NPs at the concentration of 60 mg/l. Similarly, Javed et al. (2017) have reported that 0.01 mg/l ZnO NPs increased the callus dry weight in *Stevia*; however, ZnO NPs induced an adverse effect at higher concentrations. This result can be due to the toxic effects of Ag NPs on cell growth at higher concentrations (Barbasz et al. 2016). However, a relatively positive response of the calli-related traits (RFW, CD and CGR) to the low and medium concentrations of Ag NPs

Table 3 Effects of different elicitations (salicylic acid and Ag NP_s) on callus-related traits in calli of *Stevia*

Traits	Chemical elicitors							
		Salicylic acid (mg/l)[I]			Ag NPs (mg/l)			
	Control	0.25[I]	0.5[I]	0.75[I]	15	30	45	60
Callus dry weight (g)	0.054^{ab}	0.045^{bc}	0.041^{c}	0.062^{a}	0.027^{d}	0.041^{c}	0.043^{c}	0.045^{bc}
Relative callus fresh weight	0.018^{c}	0.021^{c}	0.076^{b}	0.085^{a}	0.026^{b}	0.067^{b}	0.064^{b}	0.056^{b}
Callus diameter (cm)	0.54^{d}	0.57^{c}	0.66^{b}	0.79^{a}	0.57^{c}	0.55^{cd}	0.56^{c}	0.52^{e}
Callus growth rate (cm/day)	0.023^{de}	0.037^{c}	0.075^{b}	0.1^{a}	0.031^{cd}	0.038^{c}	0.038^{c}	0.016^{e}

[I] Treatments with the same letters in each row were not significantly different based on mean comparison by LSD's test at $P<0.05$

elicitation was in line with the reports regarding the seedling growth of *Trigonella foenum-graecum* L. using Ag NPs (Jasim et al. 2017), calli growth of barley using TiO_2NPs (Mandeh et al. 2012) and calli growth of *Prunella vulgaris* using silver and gold NPs elicitation (Fazal et al. 2016). On the other hand, an irregular trend was observed in callus-related traits from 30 to 60 mg/l (Table 3). Considering the fact that Ag ions inhibit the action of ethylene (Kumar et al. 2009), the incremental effects of the low-to-medium concentrations of Ag NP_S on the growth rate of undifferentiated cells of callus can be explained. The effects of NPs on different plant growth parameters depend on the plant species, physiological states of the plant cells, types and age of the tissues and the method applied to induce nanoparticle preparation (Vannini et al. 2013). For instance, Javed et al. (2017) have reported that all concentrations of CuO NPs induced inhibitory effects on calli growth rate of *Stevia* at the cellular level.

Among various applied concentrations of SA, treatment with 0.75 mg/l SA resulted in the highest level of RFW (0.085), the maximum callus diameter (0.79 cm) and the greatest rate of CGR (0.10 cm/day) (Table 3). Once the concentration of SA was increased from 0.25 (mg/l) to 0.75 (mg/l), an ascending trend was observed in RFW, CD and CGR traits (Table 3), which was different from that detected in the NPs-treated group (Table 3). The results of the present study indicated that the application of SA induced positive stimulatory effects on callus growth and callus weight. These findings are consistent with those reported in a study performed on *H. perforatum* L. (Gadzovska et al. 2013).

Effects of Elicitation on In Vitro Production of Stevia Glycosides

In vitro elicitation is a useful approach to increase the production of desirable products extracted from in vitro cultures (Gadzovska et al. 2013). The HPLC chromatogram of standard SGs containing ST and Reb A showed sharp picks at the retention times of 1.4 and 2.1, respectively (Figure S1). In the present study, SG was produced in the callus growth culture media with no elicitation in control conditions (Figure S1). These results are contrary to the findings of Yamazaki and Flores (1991) and Swanson et al. (1992), who found no ST and Reb A with no elicitation under control conditions. However, the results of a study conducted by Gupta et al. (2014) have demonstrated the similar findings. The ST (23.79 mg/g DW callus.) and Reb A (2.13 mg/g DW callus) were observed in the control treatment without any elicitation (Fig. 2).

This impressive result could be due to the effective optimization of plant growth regulators and/or the organization of the cells and greening the cultures (Shaoping et al. 1998). With regard to Ag NPs elicitation, two different concentrations of Ag NPs (45 and 60 mg/l) exerted trigging effects on the ST content of calli, i.e., 1.10 and 1.30 times more than the control, respectively (Fig. 2). The highest amount of ST (32.34 mg/g DW callus) was observed in the Ag NPs group treated with 45 mg/l, showing a significant increase compared to that in the control group (23.79 mg/g DW callus) (Fig. 2). It is worth mentioning that Ag NPs mediated an inductive response as a signal transducer in *Stevia* cells and Ag NPs might serve as an important regulator in the GL pathway for ST biosynthesis in *Stevia*. According to Sosan et al. (2016), Ag NPs triggered Ca^{+2} and reactive oxygen species (ROS) signaling through induction of Ca^{+2}-permeable pores and direct oxidation of apoplastic L-ascorbic acid. This result can be supported by the fact that there might be a relationship between ROS and secondary signaling messengers leading to transcriptional regulations of ST biosynthesis in *Stevia*. This, however, requires further investigation. Moreover, a number of studies have indicated that the expression of the genes involved in the biosynthesis of jacalins in *Eruca sativa* is up-regulated in the cells treated with Ag NPs (Vannini et al. 2013). Hence, secondary metabolites are increased in *Prunella vulgaris* L. in response to Ag NPs treatment (Fazal et al. 2016). The stimulating effect of Ag NPs on enhancement of stevioside is also in line with the reports revealed about the effect of

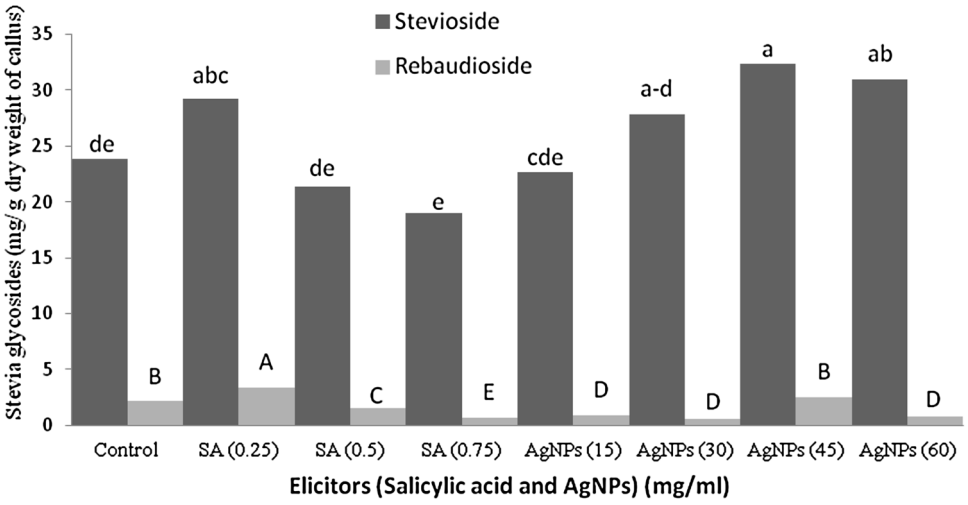

Fig. 2 Stevia glycosides content (mg/g dry weight of callus) treated with different concentrations of salicylic acid and Ag NPs under callus culture in *Stevia*. Treatments with the same lower-case letters (for stevioside) and upper-case letters (for rebaudioside A) were not significantly different based on mean comparison by LSD's test at $P<0.05$

Ag NPs on enhancing the diosgenin content of *foenum-graecum* L. (Jasim et al.2017) and anthocyanin content of *Arabidopsis* (Syu et al. 2014). Furthermore, the related studies have shown that other NP elicitors induced stimulatory effects on increasing the total phenolics content (Fazal et al. 2016; Vecerova et al. 2016), total flavonoids (Fazal et al. 2016) and total antioxidant capacity (Javed et al. 2017) of various plant species.

The RebA contents in all the Ag NPs-treated groups remained unchanged or even decreased compared to the control group (Fig. 2). In line with these findings, the effects of NPs (both positive and negative effects) on the SM production of plants were related to NPs properties, such as their sizes, concentrations, types and preparation methods (Syu et al. 2014; Goswami et al. 2017; Marslin et al. 2017). It can be concluded that the moderate stresses caused by Ag NPs at 45 mg/l led to an increase in the callus growth rate and ST production, which is contrary to the findings of Javed et al. (2017).

The addition of 0.25 mg/l SA to *Stevia* callus was found to effectively increase both SGs (ST and Reb A) compared to the control group (Fig. 2). Higher concentrations of SA (0.5 and 0.75 mg/l) did not result in any significant changes in ST content compared to the control group (Fig. 2); however, the mentioned concentrations induced inhibitory effects on Reb A content. The inhibitory effect of SA on SG accumulation in this study was similar to the decreasing trend observed in syringin in callus cultures of *Saussurea medusa* (Yu et al. 2006) and in hypericin in *H. perforatum* L. (Gadzovska et al. 2013), at higher concentrations. This result could be due to the effective role of SA as an important activator of the pathways involved in the secondary metabolite synthesis and the enzymes (copalyl diphosphate synthase and ent-kaurene synthase) involved in the production of Kauronic acid (Brandle and Telmer 2007), the precursor of Reb A synthesis. Nonetheless, the effects of elicitors on SG accumulation depend on the type and concentration of elicitors, their physiological action on the pathway involved in the synthesis of glycosides and the duration of post-elicitation. For instance, SGs content was significantly reduced upon the application of saline (Gupta et al. 2014) and drought stresses (Hajihashemi and Geuns 2016). However, it was mainly found that the effect of Ag NPs elicitation on the amounts of ST was greater than that of the SA elicitation in *Stevia* callus (Fig. 1). Conversely, SA elicitation could affect the amounts of Reb A more than Ag NPs elicitation did (Fig. 1). However, the quantities of Reb A did not largely change upon application of different elicitations, except 0.25 mg/l SA. Taking these facts into consideration, it can be concluded that, SA elicitation, at its highest concentration (0.75 mg/l), increased callus-related traits, while SG production was significantly decreased (Table 3 and Fig. 2).

Effects of Elicitation Period on Callus-Related Traits and SGs Content

In this study, CD, CGR and Reb A contents were significantly increased 2, 4 and 6 days post-elicitation (Table 4). The highest levels of CD (0.65 cm) and CGR (0.07 cm/day.) were detected in the third (6 days post-elicitation) and second (4 days post-elicitation) periods, respectively (Table 4). Comparing the mean values of the interaction effects of elicitors × time revealed that 45 mg/l Ag NPs resulted in the production of the highest amount of ST (41.04 mg/g DW callus) 2 days post-elicitation (Figure S1b); the lowest amount (9.25 mg/g DW callus), however, was achieved in the control treatment, 2 days post-elicitation (Fig. 3). This could be due to the longer elicitation time required for the genes involved in ST synthesis to be expressed. Regarding the changes in Reb A during post-elicitation days, the highest amount (5.73 mg/g

Table 4 Effects of elicitation time on callus-related traits and glycosides of *Stevia*

Traits	Days post-elicitation		
	2 (days)	4 (days)	6 (days)
Callus dry weight (g)	0.049[aǂ]	0.048[a]	0.044[a]
Relative callus fresh weight	0.12[a]	0.15[a]	0.38[a]
Callus diameter (cm)	0.53[c]	0.61[b]	0.65[a]
Callus growth rate (cm/day)	0.018[c]	0.078[a]	0.038[b]
Stevioside (mg/g DW of callus)	22.87[b]	28.66[a]	21.61[b]
Rebaudioside A (mg/g dry DW of callus)	0.96[c]	1.43[b]	2.24[a]

ǂ Treatments with the same letters in each column were not significantly different based on mean comparison by LSD's test at $P<0.05$

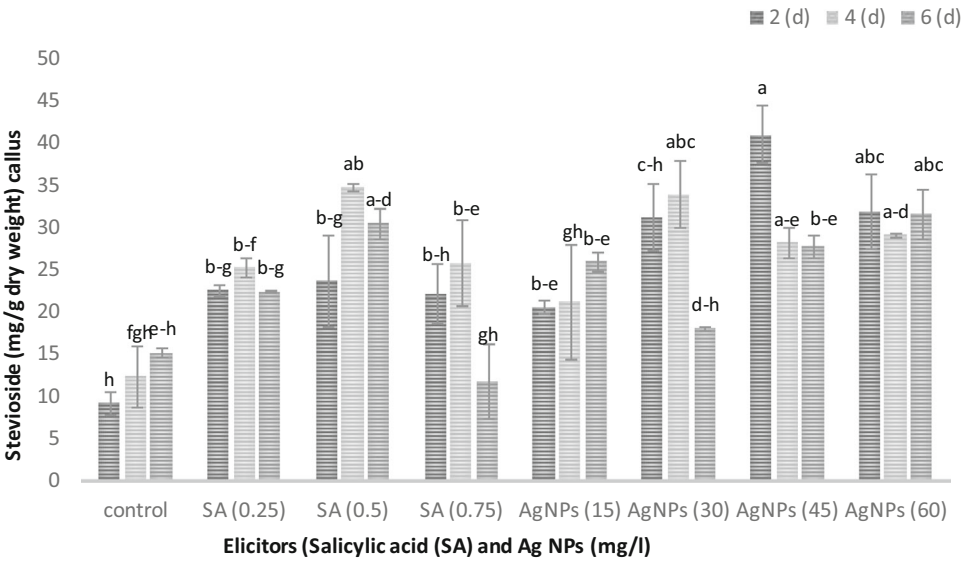

Fig. 3 Interaction effects of elicitors × days post-elicitation on the stevioside (mg/g dry weight) content under callus culture in *Stevia*

DW callus) was observed upon the application of 0.25 mg/l SA (Figure S1a). The lowest value (0.002 mg/g DW callus), however, was detected in the group treated with 60 mg/l Ag NPs, both 6 days post-elicitation (Fig. 4). In all the elicitation treatment groups, the contents of glycosides showed no significant changes with increasing the elicitation time, similar to the findings achieved by Gadzovska et al. (2013). This may be due to the application of elicitors which preserve the high concentrations of secondary metabolites (Rao and Ravishankar 2002).

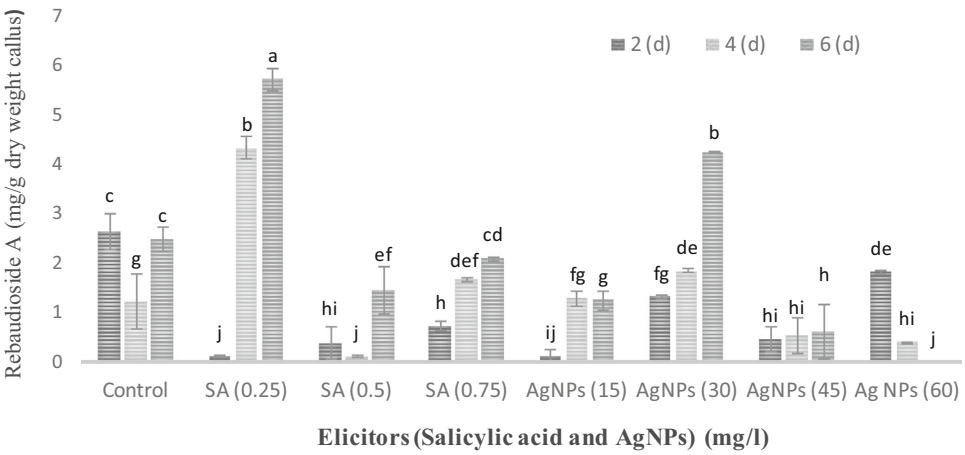

Fig. 4 Interaction effects of elicitors × days post-elicitation on the rebaudioside A (mg/g dry weight) content under callus culture in *Stevia*

Conclusion

Employing plant cells and tissue culture techniques, with varying amounts of culture media supplements, including plant growth regulators and NPs, can be beneficial to increase the yield of SGs, which was the important aim of the present study. Since this new role of nano-elicitors in medicinal and food industries has not yet been largely investigated, the results of this study can significantly contribute to enhance the production of SGs. Moreover, the best found elicitation treatment in this study, including 0.25 mg/l SA and 45 mg/l Ag NPs, can be used to increase the amounts of rebaudioside A and stevioside in callus through the activation of signaling pathways involved in biosynthesis of these SGs. The findings of the present study put forward the view that cell suspension cultures of Stevia with 45 mg/l Ag and 0.25 mg/l SA can be used as a potential approach to produce stevioside and Reb A in plants, 2 and 6 days post-elicitation, respectively. Thus, SGs can possibly be elicited in a shorter period of time, an aim which is of great pharmaceutical importance in the industry.

Acknowledgements The authors wish to express their gratitude to the Research Institute of Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, Iran, for supporting this work.

Authors' ContributionsGAG conceived the idea, PG managed the experiment, MM did the experimental work. PG and MM analyzed the data. MM wrote the initial draft of the manuscript. PG recompleted the manuscript, critically reviewed the manuscript and added to its technical part. All authors have contributed, seen and approved the manuscript.

Compliance with Ethical Standards

Conflict of interest No potential conflict of interest was reported by the authors.

References

Ahmad, N., A. Rab, and N. Ahmad. 2016. Light-induced biochemical variations in secondary metabolite production and antioxidant activity in callus cultures of *Stevia rebaudiana* (Bert). *Journal of Photochemistry and Photobiology B: Biology* 154: 51–56.

Barbasz, A., B. Kreczmer, and M. Ocwieja. 2016. Effects of exposure of callus cells of two wheat varieties to silver nanoparticles and silver salt ($AgNO_3$). *Acta Physiologiae Plantarum* 38(3): 76.

Bourgaud, F., A. Gravot, S. Milesi, and E. Gontier. 2001. Production of plant secondary metabolites: a historical perspective. *Plant Science* 161(5): 839–851.

Brandle, J.E., and P.G. Telmer. 2007. Steviol glycoside biosynthesis. *Phytochemistry* 68(14): 1855–1863.

Compton, M.E. 1994. Statistical methods suitable for the analysis of plant tissue culture data. *Plant Cell, Tissue and Organ Culture* 37: 217–242.

Fazal, H., B.H. Abbasi, N. Ahmad, and M. Ali. 2016. Elicitation of medicinally important antioxidant secondary metabolites with silver and gold nanoparticles in callus cultures of *Prunella vulgaris* L. *Applied Biochemistry and Biotechnology* 180(6): 1076–1092.

Geuns, J.M. 2003. Stevioside. *Phytochemistry* 64(5): 913–921.

Gadzovska, S., S. Maury, A. Delaunay, M. Spasenoski, D. Hagège, D. Courtois, and C. Joseph. 2013. The influence of salicylic acid elicitation of shoots, callus, and cell suspension cultures on production of naphtodianthrones and phenylpropanoids in *Hypericum perforatum* L. *Plant Cell, Tissue and Organ Culture* 113(1): 25–39.

Goswami, L., K.H. Kim, A. Deep, P. Das, S.S. Bhattacharya, S. Kumar, and A.A. Adelodun. 2017. Engineered nano particles: Nature behavior and effect on the environment. *Journal of Environmental Management* 196: 297–315.

Gupta, P., S. Sharma, and S. Saxena. 2014. Effect of salts (NaCl and Na_2CO_3) on callus and suspension culture of *Stevia rebaudiana* for steviol glycoside production. *Applied Biochemistry and Biotechnology* 172(6): 2894–2906.

Hajihashemi, S., and J. Geuns. 2016. Gene transcription and steviol glycoside accumulation in *Stevia rebaudiana* under polyethylene glycol-induced drought stress in greenhouse cultivation. *FEBS Open Bio* 6(9): 937–944.

Janarthanam, B., M. Gopalakrishnan, and T. Sekar. 2010. Secondary metabolite production in callus cultures of *Stevia rebaudiana* Bertoni. *Bangladesh Journal of Scientific and Industrial Research* 45(3): 243–248.

Jasim, B., R. Thomas, J. Mathew, and E.K. Radhakrishnan. 2017. Plant growth and diosgenin enhancement effect of silver nanoparticles in Fenugreek (*Trigonella foenum-graecum* L.). *Saudi Pharmaceutical Journal* 25(3): 443–447.

Javed, R., B. Yucesan, M. Zia, and E. Gurel. 2017. Elicitation of secondary metabolites in callus cultures of *Stevia rebaudiana* Bertoni grown under ZnO and CuO nanoparticles stress. *Sugar Tech* 20(2): 194–201.

Kang, S.M., H.Y. Jung, Y.M. Kang, D.J. Yun, J.D. Bahk, J.K. Yang, and M.S. Choi. 2004. Effects of methyl jasmonate and salicylic acid on the production of tropane alkaloids and the expression of PMT and H6H in adventitious root cultures of *Scopolia parviflora*. *Plant Science* 166: 745–751.

Kumar, V., G. Parvatam, and G.A. Ravishankar. 2009. $AgNO_3$: A potential regulator of ethylene activity and plant growth modulator. *Electronic Journal of Biotechnology* 12: 8–9.

Mahmud, S., S. Akter, I. Jahan, S. Khan, A. Khaleque, and S. Islam. 2014. Comparative analyses of stevioside between fresh leaves and in vitro derived callus tissue from *Stevia rebaudiana* Bert using HPLC. *Bangladesh Journal of Scientific and Industrial Research* 49: 199–204.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15(3): 473–497.

Mandeh, M., M. Omidi, and M. Rahaie. 2012. In vitro influences of TiO_2 nanoparticles on barley (*Hordeum vulgare* L.) tissue culture. *Biological Trace Elements Research* 150: 376–380.

Marslin, G., C.J. Sheeba, and G. Franklin. 2017. Nan particles alter secondary metabolism in plants via ROS burst. *Frontiers in Plant Science* 8: 832.

Mathur, S.H., and G.S. Shekhawat. 2013. Establishment and characterization of *Stevia rebaudiana* (Bertoni) cell suspension culture: an in vitro approach for production of stevioside. *Acta Physiologica Plantarum* 35: 931–939.

Namdeo, A.G. 2007. Plant cell elicitation for production of secondary metabolites: A review. *Pharmacognozy Review* 1: 69–79.

Piasecka, A., N. Jedrzejczak-Rey, and P. Bednarek. 2015. Secondary metabolites in plant innate immunity: Conserved function of divergent chemicals. *New Phytologist* 206(3): 948–964.

Rao, S.R., and G. Ravishankar. 2002. Plant cell cultures: chemical factories of secondary metabolites. *Biotechnology Advances* 20: 101–153.

Shaoping, X., X.Z. Ouyang, L. Hongwei, M.C. Chen, and D. Wang. 1998. Stevia callus growth, differentiation and stevioside accumulation relationship. *Journal of Tropical and Subtropical Botany* 6(1): 8–14.

Sosan, A., D. Svistunenko, D. Straltsova, K. Tsiurkina, I. Smolich, T. Lawson, S. Subramaniam, V. Golovko, D. Anderson, A. Sokolik, and I. Colbeck. 2016. Engineered silver nanoparticles are sensed at the plasma membrane and dramatically modify the physiology of *Arabidopsis thaliana* plants. *Plant Journal* 85(2): 245–257.

Swanson, S.M., G.B. Mahady, and C.W.W. Beecher. 1992. Stevioside biosynthesis by callus, root, shoot and rooted-shoot cultures in vitro. *Plant Cell, Tissue and Organ Culture* 28: 151–157.

Syu, Y.Y., J.H. Hung, J.C. Chen, and H.W. Chuang. 2014. Impacts of size and shape of silver nanoparticles on Arabidopsis plant growth and gene expression. *Plant Physiology and Biochemistry* 83: 57–64.

Tiwari, R., and C.S. Rana. 2015. Plant secondary metabolites: A review. *International Journal of Engineering Research and General Science* 3: 661–670.

Vannini, C., G. Domingo, E. Onelli, B. Prinsi, M. Marsoni, L. Espen, and M. Bracale. 2013. Morphological and proteomic responses of *Eruca sativa* exposed to silver nanoparticles or silver nitrate. *PLoS ONE* 8: 68752.

Vecerova, K., Z. Vecera, B. Docekal, M. Oravec, A. Pompeiano, J. Tríska, and O. Urban. 2016. Changes of primary and secondary metabolites in barley plants exposed to CdO nanoparticles. *Environmental Pollution* 218: 207–218.

Wang, Y.D., J.C. Wu, and Y.J. Yuan. 2007. Salicylic acid-induced taxol production and isopentenyl pyrophosphate biosynthesis in suspension cultures of *Taxus chinensis* var. mairei. *Cell Biology International* 31(10): 1179–1183.

Yamazaki, T., and H.E. Flores. 1991. Examination of steviol glycoside production by hair root and shoot cultures of *Stevia rebaudiana*. *Journal of Natural Products* 54: 986–992.

Yu, Z.Z., C.X. Fu, Y.S. Han, Y.X. Li, and D.X. Zhao. 2006. Salicylic acid enhances jaceosidin and syringin production in cell cultures of *Saussurea medusa*. *Biotechnology Letters* 28(13): 1027–1031.