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Study the effect of heavy metals and Nano TiO_2 on stevioside and Rebadioside A production in *Stevia rebaudiana* Bertoni hairy roots

Nesa Moradpour[1], Ghasemali Garoosi[1*], Raheem Haddad[1]

[1]Biotechnology Department, Faculty of Agriculture and Natural Sciences, Imam Khomeini International University, P. O. Box: 34149-16818, Qazvin, Iran.
[*]Corresponding author, Email: garoosi@eng.ikiu.ac.ir. Tel: +98-28-33901239.

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Abstract

Stevia rebaudiana Bert. is a medicinal plant with anti-oxidant, antimicrobial, antifungal and antiviral drug properties which contains two valuable secondary metabolites, stevioside and Rebadioside A, with very high sweetness capacity. In this research hairy roots were produced from *S. rebaudiana* leaf disks using two strains of 15834 and A4 *A. rhizogenes*. Then, the best hairy root line was selected and propagated. The effect titanium dioxide nanoparticles at 20, 40 and 60 mg/l after 0, 24, 48 and 72 h of elicitation and cobalt chloride and cadmium chloride each at 100 and 200 µmol after 2 and 8 h of elicitation were studied on the production and accumulation of stevioside and Rebadioside A. The results demonstrated that most treatments of elicitors at different times had significant effects on the production and accumulation of target metabolites, but titanium dioxide nanoparticles caused the highest production and accumulation of stevioside (96.88 mg/l and 24.28 mg/g DW), respectively at 40 mg/l after 24 h. However, at 60 mg/l and 48 h it had the highest effect on production and accumulation of Rebadioside A after 48 h (89.39 mg/l and 21.28 mg/g DW), respectively. Also the results demonstrated that treatment of cobalt chloride and cadmium chloride both at 100 µmol after 8 h had a strong effect on the production (114.19 and 183.98 mg/l) and accumulation (29.54 and 42.87 mg/g DW) of stevioside, respectively. Cadmium chloride at 200 µmol had the highest effect on the production and accumulation of Rebadioside A after 2 h (105.51 mg/l and 25.42 mg/g DW), respectively. Meanwhile, no production of Rebadioside A was observed at 100 and 200 µmol in 8 h treatment.

Key words: Elicitation, Hairy root, Rebadioside A, *Stevia rebaudiana Bert*, Stevioside.

INTRODUCTION

Medicinal plants are one of the most important sources of drugs used for thousands of years. The world Health Organization estimates that more than 80 percent of people have used traditional or modern herbs. Many chemical drugs are also made by modeling herbal substances (Tripath *et al*., 2003). *Stevia rebaudiana* belongs to the family Asteraceae and is native to Paraguay and the leaves contain about thirty steviol glycosides (Ceunen *et al*., 2013). *Agrobacterium rhizogenes* contains Ri plasmid which has different parts. T-DNA is known as the T region when present on the Ti or Ri plasmid. At the time of *Agrobacterium* infection, a piece of DNA is transferred from the bacterium to the plant cell coding T-DNA. The hairy root is produced by the transfer of a piece of DNA from the T-DNA to the plant cells DNA (Chilton *et al*., 1982). The hairy root system is a stable system that has relatively high production capacity in a hormone-free environment. High growth rates, reduced cropping time, easy maintenance, and the ability to synthesize chemical compounds make hairy roots a suitable source for producing high-quality secondary metabolites. (Nikravesh *et al*., 2012). Hairy root culture using *Agrobactrium rhizogenes* is one of the methods used

to study the pathway of biosynthesis of secondary metabolites. Two strains of ATCC15834 and A4 caused the formation of hairy roots in *Allium sativum* (Moradi *et al*., 2019) In the *Althea officinalis* plants, the highest production rate of the secondary metabolite is due to sample inoculation with *Agrobacterium rhizogenes* A13 strain (Tavassoli *et al*., 2018). Strain A4M70GUS has resulted in the production of hairy roots in *Gentiana utriculosa* L. plants (Vinterhalter *et al*., 2019). One of the most common applications for enhancing the yield of secondary metabolites is the use of elicitors (Sivanandhan *et al*., 2013). Elisitors, by inducing defense responses, cause biosynthesis of secondary metabolites (Zhao *et al*., 2005). Nanoparticles have a large surface area and therefore have different physical and chemical properties and are highly reactive. Nanoparticles have both positive and negative effects on biological systems (Kołodzi *et al*., 2014). In studies with similar elisitor, effect of TiO_2 nanoparticles on plant activities has recently been studied. For example in *Hyoscyamus niger* L. Titanium dioxide caused activation of antioxidant enzymes, hyoscyamine and scopolamine biosynthesis (Ghorbanpour *et al*., 2015). In another example, TiO_2 nanoparticles affected the expression of a key gene in the pathway of biosynthesis of tioguinone in *Nigella sativa* (Kahila *et al*., 2018). TiO_2 nanoparticles can be used as an exogenous stimulus for the improvement of shoot growth and essential oil content in plants (Mohammadi *et al*., 2016). Nano Silver had a positive effect on *Dasha* callus culture (Golkar *et al*., 2018). Javed and Mohamed, (2017) investigated the effect of copper nanoparticles on the production of Steviol glycosides and antioxidant properties of *S. rebaudiana*, which showed that nanoparticles had a positive effect on the production of Stevols and had the greatest effect on the amount of 10 mg/l of CuO (Javed *et al*., 2017). Zahir *et al.* (2019) investigated the effect of silver nanoparticles on cell suspension cultures of *Linum usitatissimum* L. and observed that Zinc oxide nanoparticles (NPs) caused an increase in the amount of lignans and neolignans. Chung *et al*. (2018) investigated the effect of silver ion (Ag) and biologically synthesized silver nanoparticles (AgNPs) to enhance biomass accumulation and phenolic compound production as well as biological activity in hairy root cultures of *Cucumis anguria*. They observed that the biomass of hairy root cultures was significantly increased by AgNPs while it showed a decrease by Ag at 1 and 2 mg/l. Also, AgNPs, produced significantly higher amounts of individual phenolic compounds and flavonoid contents than Ag-elicited hairy roots (Chung *et al*., 2018).

In this study, we aimed to produce *S. rebaudiana* hairy roots using *A. rhizogenesis* strains and enhance the production of secondary metabolites of Stevioside and Rebadioside A by non-biological elicitation.

MATERIALS AND METHODS

Plant materials, bacterial culture and hairy root establishment

The *Stevia rebaudiana* Bert. plants were obtained from the Faculty of Agriculture and Natural Resources of Tehran University in Karaj. Then, for sterilization, young and 10-15 cm long seedlings were selected and rinsed for 30 minutes. Then they were placed in 70% ethanol for 1 minute, and washed with enough distilled water and placed in sodium hypochlorite 1.5% (v/v) for 15 min. The seedlings were then washed 5 times with sterile distilled water for 6 minutes. The stems of the plants containing lateral buds were placed on the basal MS medium containing 3% (w/v) sucrose. The pH of the culture medium was set at 5.7. The cultures were placed in a growth room with a photoperiod of 16/8 light/darkness and 25±2 °C. *A. rhizogenes* strain 15834 and A4 were cultured on Luria Bertoni (Kolb *et al*., 2001) medium. Both strains were grown separately in a solid medium containing 25 ml of LB medium. The samples were incubated at 28 °C. After 48 h of culture, the bacteria were kept in a growth room and cultured in the liquid LB medium. They were then kept in a shaker at a speed of 110 rpm at 28 °C. Absorbance of *A. rhizogenes* cultures were measured using a spectrophotometer at 600 nm. The leaves of the plants were cut into 1-2 cm pieces and were wounded by Insulin syringe and were immersed into the suspension of *A. rhizogenes* strains with OD=600 nm for 10-15 minutes. Inoculated leaf samples were cultured on a complete solid MS medium containing 3% (w/v) sucrose and free of antibiotics for 72 and 48 h at 25 °C and kept in dark. After washing the samples were transferred onto a MS solid culture medium without hormone containing 300 mg/l of cefotaxime and kept in a growth chamber with a photoperiod of 16/8 light/darkness. The samples were sub-cultured every four days to the new MS medium with a gradual reduction of antibiotic concentration. After several subcultures (3-4) they were transferred onto the antibiotic-free MS medium.

PCR analysis of hairy roots

The molecular analysis of roots was performed by polymerase chain reaction (PCR) to confirm the transfer of *rol B* gene. For this purpose, transgenic and control roots were used. Extraction of DNA was carried out according to Japelaghi *et al*. (2011) method. The quality

of DNA extracted from the hairy root was confirmed by electrophoresis. Plasmid extraction from *A. rhizogenesis* of 15834 and A4 strains was carried out using Sambrook *et al*. method, (1989). The primers for the *rol B* and *vir D* gene were designed (Table 1) (Fu *et al*., 2005). Polymerase chain reaction was performed using *rol B* and *vir D* gene using thermocycler (TECHNE) TC-512 in 35 cycles for 5 min at 94 °C 1 min at 60 °C, 3 min 72 °C. Then the PCR products were electrophoresed.

Table 1. Sequence of primers for *rol* B and *vir* D genes.

Gene	Sequence (5'–3')
F *rol B*	5'-TACTGCAGCAGGCTTCATGCA-3'
R *rol B*	5'-GCTTTCCCGACCAGAGACTG-3'
F *vir D*	5'-CCTGCCGTAAGTTTCACCTCACC-3'
R *vir D*	5'-CCTGCCGTAAGTTTCACCTCACC-3'

Treatment of elicitors

For treatment of Nano TiO_2, the stock was prepared and autoclaved, and the treatments were carried out at 50, 100 and 150 mg/l concentrations in three replicates. Sampling was carried out at 24, 48 and 72 h after the elicitation (Putalun *et al*., 2007). Cobalt and cadmium chloride were used at 100 and 200 µmol with three replications and samples were collected 2 and 8 h after elicitation (Wang *et al*., 2017). The initial weight of the inoculum in all samples was 264 mg and the volume of the culture medium was 25 ml.

Extraction, chromatography and analysis of data

Extraction was carried out using Kolb *et al*. (2001) method with minor changes. Chromatography was performed using HPLC and Hézode *et al*. (2013) method.The experiment was conducted as factorial in a completely randomized design with 3 replications. The accumulation and production of metabolites were measured in mg/g DW and in mg/l, respectivily. Statistical analyses were carried out using SPSS 19.0 software. The mean comparison was also performed using Duncan test at 1% probability level. Charts were drawn up by Microsoft Office Exel 2013 software.

RESULTS

Hairy root establishment

The earliest hairy roots appeared about 12 days after infection of seedlings using *A. rhaizogens*, strains 15834 and A4. When the hair roots were approximately 2 cm long, they were separated from the leaf tissue, and each root was transferred separately into a 100 ml eyeliner containing 25 ml of complete liquid MS medium (Figure 1).

Transformation of hairy roots

The extracted DNA (Japelaghi *et al*., 2011) was electrophoresed. Transformation analysis using PCR results on the agarose gel showed that two replicated lines were transgenic (Figure 2).

Nano titanium dioxide treatment

The results of analysis of variance showed that the effect of different concentrations of titanium dioxide, sampling time and their interactions on the accumulation and production of Rebadioside A and Stevioside was significant at 1% level (Table 2). The comparison of the mean interactions of titanium dioxide concentration with sampling time on the accumulation of Stevioside (Figure 3) and accumulation of Rebadioside A (Figure 4) showed that the highest production and accumulation of Stevioside occurred at 40 mg/l and 24 h and the lowest production level occurred at 20 mg/l and 48 h. The highest level of production and accumulation of Rebadioside A was obtained at 60 mg/l and at 48 h.

Cadmium chloride treatment

The results of the analysis of variance showed that

Figure 1. Induction and propagation of hairy roots in a complete MS medium. **A:** Creating hairy root from leaf tissue after 12 days. **B:** Developed hairy roots, approximately 2 cm in length were removed and transferred onto a complete MS medium. **C:** Propagation of hairy roots in the liquid medium.

the effect of cadmium chloride , the sampling time and their interaction was significant on the accumulation and production of Stevioside, at 1% level (Table 3). The comparison of the interactions of cadmium chloride with sampling time on the accumulation of Stevioside (Figure 5) showed that the highest production and accumulation of Stevioside occurred at 100 µmol and 8 h after treatment (183.98 mg/l and 42.87 mg/g DW, respectivily) and the highest level of production and accumulation of Rebadioside A occurred at 200 µmol after 2 h (105.51 mg/l and 25.42 mg/g DW, respectivily).

Cobalt chloride treatment

The results of analysis of variance showed that the main effect of sampling time and its interaction with cobalt chloride was significant at 1% level on the accumulation and production of Stevioside and Rebadioside A. The interaction of sampling time and concentration of cobalt chloride and also main effect of sampling time was also significant on the accumulation and production of both metabolites at 1% level (Table 4). The comparison of the interaction of cobalt chloride and sampling time on the accumulation of

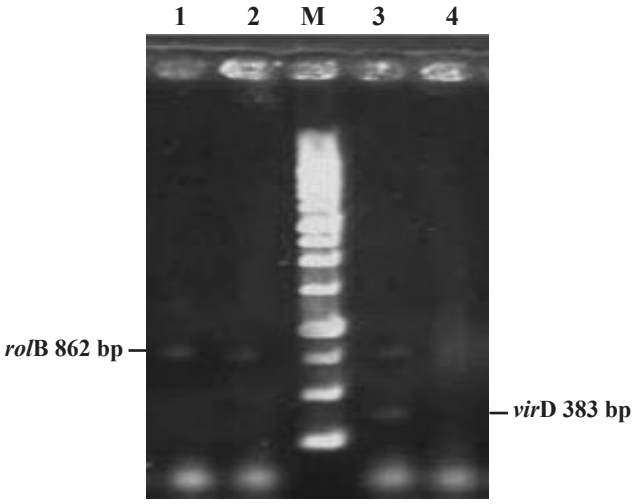

Figure 2. Electrophoresis of PCR product of *rol*B and *vir*D genes. M, One kb molecular marker; lines 1 & 2 hairy roots which were transformed by *A. rhizogenes* strains 15834 and A4 respectively; line 3, Positive control (plasmid DNA) and line 4, negative control.

Table 2. Analysis of variance of application of titanium dioxide and sampling time on the production and accumulation of stevioside and Rebadioside A in the hairy roots of the *S. rebaudiana*.

Source of variation	df	Mean of square			
		Rebadioside production	Rebadioside A accumulation	Stevioside production	Stevioside accumulation
Titanium dioxide (Ti)	3	6089.06**	397.61**	709.67**	40.02**
Time (T)	2	910.95**	206.42**	886.31**	98.10**
Ti×T	6	1393.96**	107.55**	1927.03**	97.34**
Error	24	50.34	2.234	88.48	3.80
Coefficient of variation (%)		26.99	19.64	17.13	17.7

**: Significant at 1% propability.

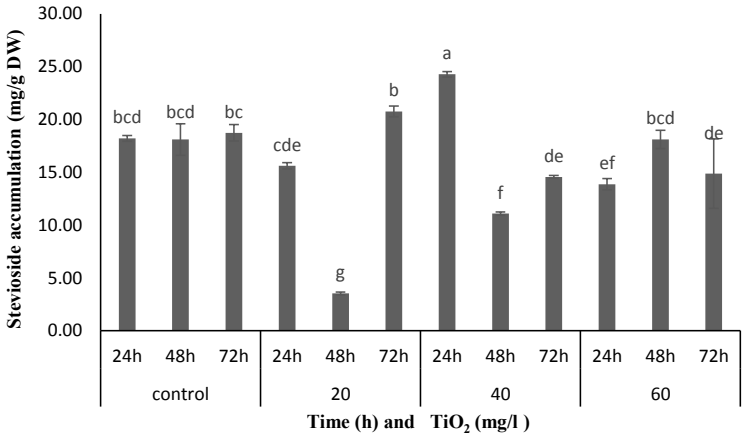

Figure 3. The interaction effect of titanium dioxide and sampling time on stevioside accumulation.

Stevioside and Rebadioside A showed that the highest accumulation of Stevioside and Rebadioside A was at 100 µmol at 8 h (29.54 mg/g DW) (Figure 6) and 100 µmol at 2 h (21.34 mg/g DW) (Figure 7) after treatment, respectively. However, the result indicated that the highest production level of stevioside occurred at 100 µmol and 8 h (114.19 mg/l) (Table 5). On the other hand no production of readioside A was observed at 100 and 200 µmol in 8 h.

DISCUSSION

Various factors such as concentration of elicitor, type of medium and duration of exposure to elicitor affect the production of secondary metabolites (Bota *et al*., 2011). In the study of Javed *et al*. (2017) copper oxide nanoparticles increased the glycoside Steviols and the antioxidant properties of the *S. rebaudiana* plants. Similarly, Raei *et al*., 2014 examined the effect of non-

Table 3. Analysis of variance of application of cadmium chloride and sampling time on the production and accumulation of stevioside and Rebadioside A in the hairy roots of the S. *rebaudiana*.

Source of variation	df	Mean of square			
		Rebadioside production	Rebadioside A accumulation	Stevioside production	Stevioside accumulation
Cadmium chloride (Cd)	2	5994.63**	511.71**	3703.26**	519.43**
Time (T)	1	36.12^{ns}	19.06^{ns}	3385.27**	193.77**
Cd×T	2	51.73^{ns}	5.49^{ns}	2428.76**	131.51**
Error	12	95.34	7.109	49.26	1.81
Coefficient of variation (%)		29.17	23.12	14.18	5.62

**: Significant at 1% probability; ns: None-Significant.

Table 4. Analysis of variance of application of cobalt chloride and sampling time on the production and accumulation of stevioside and Rebadioside A in the hairy roots of the S. *rebaudiana*.

Source of variation	df	Mean of square			
		Rebadioside production	Rebadioside A accumulation	Stevioside production	Stevioside accumulation
Cobalt chloride (Co)	2	1117.15**	68.49**	18457.56**	118.60**
Time (T)	1	14090.33**	805.17**	2019.24**	186.24**
Co×T	2	3813.71**	245.98**	4722.33**	105.79**
Error	12	84.58	7.289	96.98	2.39
Coefficient of variation (%)		28.85	23.99	11.54	9.42

**: Significance at 1% probability.

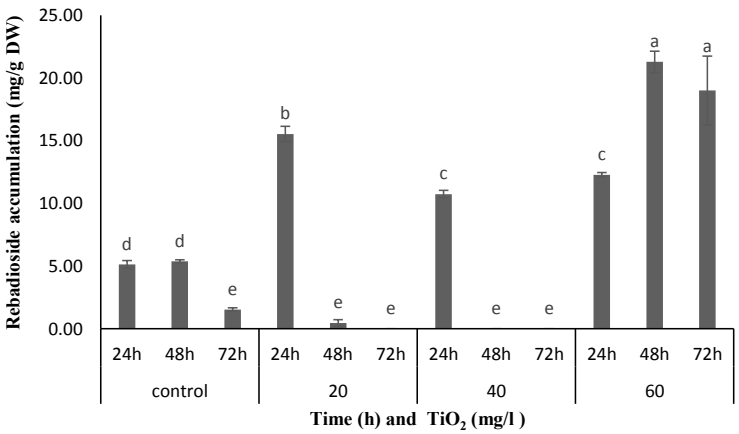

Figure 4. The interaction effect of titanium dioxide and sampling time on Rebadioside A accumulation.

biotic elicitors of TiO_2 and NH_4NO_3 nanoparticles on the tissue culture of *Aloe vera* (Raei *et al*., 2014). Their results indicated that nanoparticles increased aloin production 48 h after the elicitation. TiO_2 nanoparticles were used in this study may have caused the activation of genes associated with defense mechanisms and biosynthesis of Stevioside and Rebadioside A. Although, nanoparticles easily penetrate the plant tissue

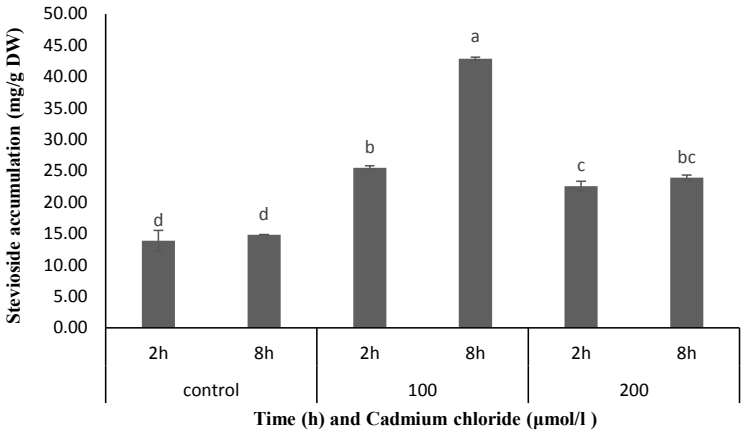

Figure 5. The interaction effect of cadmium chloride and sampling time on stevioside accumulation.

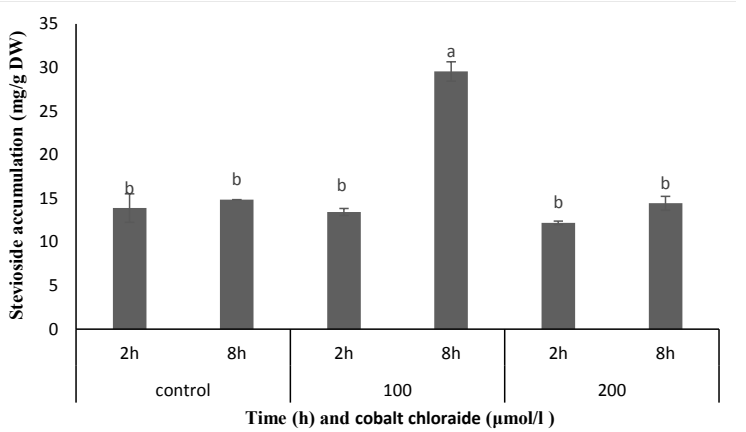

Figure 6. The interaction effect of cobalt chloride and sampling time on stevioside accumulation.

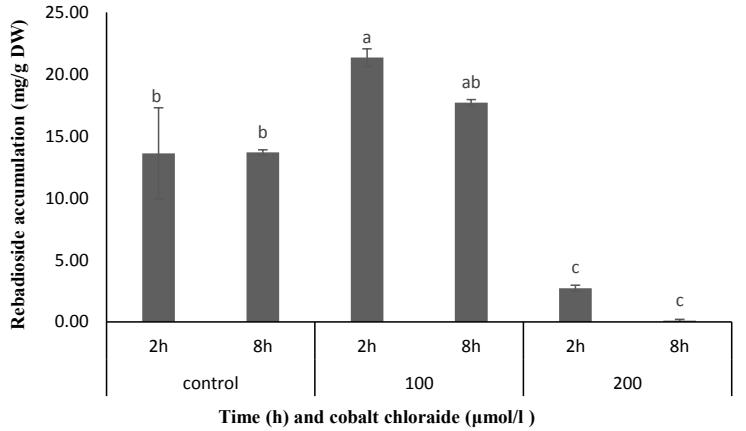

Figure 7. Interaction between cobalt chloride and sampling time on Rebadioside A accumulation.

Table 5. Mean Data±Standard error interaction effect of different concentrations of cobalt chloride treatment on sampling indices in hairy roots.

Cobalt chloride (µmol)	Sampling time (hs)	Accumulation of stevioside (mg/g dry weight)	Production of stevioside (mg/l)
Control	2	89.13±1.64^{b}	31.62±2.71^{c}
	8	14.85±0.01^{b}	29.30±2.25^{c}
100	2	13.43±0.41^{b}	40.98±5.26^{bc}
	8	29.54±1.11^{a}	114.19±6.22^{a}
200	2	12.19±0.20^{b}	34.70±2.82^{bc}
	8	14.43±0.80^{b}	46.08±3.41^{b}

* Interaction effect of cobalt chloride concentration with sampling time on fresh and dry weight of hairy roots, accumulation of ribadioside and production of ribadioside was insignificant.

system and affect the rate of production of secondary metabolites, but their high concentrations and long-term presence in the environment may cause cytolytic depletion by mitotic index release and the release of toxic ions (Abdelhamid *et al*., 2015). However, its reduction over time may be due to its toxicity on cells, which is associated with the increase in the production of ROS or active oxygen (Lok *et al*., 2007)

The effect of heavy metals treatment such as cadmium chloride and cobalt chloride showed that the highest level of Stevioside production and accumulation in both treatments was obtained at 100 µmol at 8 h, and the lowest level of Stevioside production and accumulation was observed in the control samples. The levels of Rebadioside A accumulation as well as the production and accumulation of Stevioside were highest at 100 µmol after 8 h. Production of Rebadioside A occurred in cadmium chloride treatment at 200 µmol and 2 h. Production of plant secondary metabolites is often created in response to environmental changes (Wu *et al*., 2005). In general, elicitors stimulate the production of secondary metabolites by stimulating the defense pathways of the plants. In many studies non-biological elicitors have increased the production of secondary metabolites in plants (Zheng *et al*., 2004; Georgiev *et al*., 2006; Gangopadhyay *et al*., 2011), and also heavy metals of cobalt chloride and cadmium chloride stimulate secondary metabolites production. In a study by Zhao *et al*. (2010) the effect of biotic and abiotic elicitors was investigated on the production of tanshinon in the cell culture of *Salvia miltiorrhiza*. They showed that non-biological elicitors such as cobalt, cadmium and silver increased the production of tanshinon. Although cadmium chloride is a heavy metal toxic and detrimental to plant growth and development which has been used as an elicitor to stimulate the production of secondary metabolites in hairy root and plant cell cultures such as ajmalicine, podophyllotoxin and tanshinones (J. W. Wang *et al*., 2013). Similarly, Ch, Rao, Gandi, & Giri, 2012 studied the effect of abiotic elicitors such as cadmium chloride, mercuric chloride, silver nitrate, cupric chloride, cobaltous chloride and calcium chloride on the production of Gymnemic acid in suspension cultures of *Gymnema sylvestre*. Their study showed that among different abiotic elicitors used, $CdCl_2$ was proven to be the best in enhancing gymnemic acid accumulation. Their study indicated that application of heavy metal elicitors can enhance the capacity of gymnemic acid production in the suspension cultures of *G. sylvestre*. Also the amount of Taxol was significantly increased in the cell cultures of *Taxus baccata* by the addition of sufficient amounts of silver nitrate and cobalt chloride (Vermeersch *et al*., 2009).

CONCLUSIONS

The results showed that titanium dioxide nanoparticles affected the accumulation of Rebadioside A and the lower concentrations of cobalt and cadmium chloride had greater effects on metabolite production. We propose to investigate other concentrations of cobalt and cadmium chloride and other nanoparticles and elicitors for the production of these metabolites.

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