Trichoderma and sodium nitroprusside elicitation improves vinblastine and vincristine yield by increasing TIA pathway genes expression in cell suspension of Catharanthus roseus

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Research Article

Keywords: Biotic and abiotic elicitors, Cell suspension, Enzymes activities, Gene expression, Secondary metabolites

Posted Date: June 1st, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2884766/v1

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Abstract

Biotic and abiotic elicitors induce the production of plant secondary metabolites. In this study the effects of *Trichoderma harzianum* and sodium nitroprusside (SNP) as a biotic and abiotic elicitor on biochemical and molecular features of *Catharanthus roseus* cell suspension were investigated. Leaf explant and medium amended with concentration of 8 µM 2,4-D and 2 µM BAP were used for obtain cell suspension. Cell suspension exposed to *T. harzianum* (%1 v/v) and SNP (150 µM) and after 12, 24, 48 and 72 hours harvested. The highest activity of catalase, ascorbate peroxidase, β (1–3) glucanase and chitinase were found 48 hours after application of treatments. Also, the highest level of G10H (2.5-fold), T16H (1.5-fold), D4H (1-fold), DAT (1.9-fold), STR (5-fold) and CrPRX (2-fold) were observed 48-hours after elicitor application. A positive correlation was observed between enzymes activities, T1As biosynthesis pathway genes and vinblastine and vincristine accumulation. These results confirmed that fungus and SNP elicitors in *C. roseus* led to increase secondary metabolites production and gene expression related to biosynthesis of vinblastine and vincristine pathway 1.84 and 1.93-fold, 48-hours after elicitor application, respectively. All these results suggest that fungus and SNP elicitors after 48-hours exposure, effectively improve the pharmaceutical value of *C. roseus*.

Introduction

Alkaloids are various compounds of low molecular weight nitrogen containing which are found in plant species and about 12,000 alkaloids have been known for their chemical structures (Facchini 2001). According to the alkaloids biological activities lots of them have been extremely applied to cure diseases (Zhao et al. 2010). *Catharanthus roseus* is one of the medicinal and ornamental members of Apocynaceae family and it is a sole source of the vincristine and vinblastine as an anticancer drug (Alhaithloul et al. 2019; Birat et al. 2022). In cell biology, vinblastine and vincristine bind to tubulin and cause polymerization and results in inhibiting microtubule assembly that leads to the disorder in the mitotic spindle, finally metaphase stopped (Zandi 2021). Moreover, the accumulation of these beneficial alkaloids is very limited, which rarely meet the global demand (Zhang et al. 2018). Lately studies for finding T1As biosynthesis genes, regulation on T1As biosynthesis pathway and illumination of pathway intermediates transport were successful (Liu et al. 2014). Though, a great progress was obtained, but there are lots of pathway genes, regulators and T1A transporters that need to further studies for a better understanding of the production of anticancer drug components by *C. roseus* (Pan et al. 2018). Secondary metabolites accumulated in plants when they are faced with various stresses, elicitors or signal molecules (Thakur et al. 2019; Siddiqui et al. 2023).

Depending on the source, elicitors can be classified in biological or chemical compounds that lead to increase secondary metabolates biosynthesis and accumulation by induction of defensive responses and physiological changes in plants (Baldi et al. 2009; Ramezani et al. 2018). On the other hand, one of the most efficient ways to enhancing secondary metabolite production is application of biotic or abiotic elicitors in plants *in vitro* cells and organs culture (Sahu et al. 2013). The use of biotic or abiotic elicitor techniques also reduces processing time in obtaining active compounds in volume (Coste et al. 2011). Between various biotic elicitors, fungal elicitors have been widely used for stimulating of secondary metabolite production in *in vitro* cultures (Singh et al. 2018). In order to realize the role of elicitor on plant defense responses varied antioxidant enzymes activity were considered as the addition of elicitors induced cellular stress on tissues (Tonk et al. 2016). Previous studies declared that the expression of T1As biosynthesis pathway genes is regulated by tissue and cell specific controls in response to biotic and abiotic stimuli in *C. roseus* (Kellner et al. 2015). Although the enhancement of alkaloids is depended on treatment and cell line specific, but the utilization of biotic and abiotic elicitors in medium is an affordable approach to enhancing valuable alkaloids of medicinal aims (Tonk et al. 2016). As well as, biotic elicitors have been used to increase the production of secondary metabolites in cell culture of many medicinal plants (Ramezani et al. 2018). Several investigations were showed fungus elicitation effects on secondary metabolites enrichment such as hypericin (Gadzovska Simic et al. 2015), ginsenoside (Tonk et al. 2016) and vincristine and vinblastine (Tonk et al. 2016). The accumulation of ajmalicine in the cell suspension of *C. roseus* by amended medium with *Aspergillus niger*, *Fusarium moniliforme* and *T. viride* as biotic elicitors was considered. The result demonstrated 5% v/v concentrations of fungal elicitors had a great effect on accumulation and content of ajmalicine (Namdeo et al. 2002). Prasad et al. (2013) reported that fungal elicitation by *T. harzianum*, *Colletotrichum lindemuthianum* and *Fusarium oxysporum* application increased biomass and asiaticoside accumulation in *Centella asiatica*. Moreover, induction *T. atroviride* and polysaccharide fragments on the hairy roots of *Salvia officinalis* led to enhancing tanshinone (Ming et al. 2013). Ramezani et al. (2018) reported that after 48 hours of treatment, the highest D4H and DAT gene expression increased 1.2 times 0.7-fold, respectively and increase the vinblastine and vincristine production obtained from *C. roseus* cell suspension amended with *T. tomentosum* elicitor and it was more effective in comparison with *Piriformospora indica*.

Sodium nitroprusside (SNP) is a nitric oxide (NO) donor. NO has fundamental role in plant growth (Khurana et al. 2011), signaling and regulating plant defense or stress responses (Amooaghaie and Korrani 2018), induce secondary metabolites like alkaloids accumulation (Xu and Dong 2005) and balances the ROS damage by affecting the expression of antioxidant genes (Khan et al. 2017). Mahendran et al. (2021) reported *Gymnema sylvestre* cell suspensions exposed to 20 µM SNP treatment led to increase deacglynnemeric acid, gynemagenin and gynemamic acid XVII 13.43, 13.86 and 17.33-fold higher than the control, respectively. According to Cai et al. (2012) report, elicitors cause cellular stress that could be consider by antioxidant enzymes, which scavange stress levels in tissues. Several enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities are most measured to determine the stress rate in plant tissues (Fatima et al. 2015). Tonk et al. (2016) declared addition of *Aspergillus flavus* as a fungal elicitor on *Catharanthus roseus* callus made extracellular stress and finally led to improves vincristine and vinblastine yield and increase antioxidant enzyme activities. Therefore, this study was performed in two steps. In first step, *in vitro* callus of selected *C. roseus* explants in different hormonal medium induced, cell suspension obtained and elicitors applied. In the second step, the activity of some enzymes and the expression of genes involved in the biosynthesis of vinblastine and vincristine including Geraniol 10-hydroxylase (*GlOH*), Stricotsidine synthase (*STR*) and *Catharanthus roseus* peroxidase (*CrPRX*) by real-time quantitative PCR (qRT-PCR) and evaluating the production of mentioned alkaloids under the influence of *T. fungii* and SNP as elicitor in periwinkle cell suspension were investigated. Finally, the merged results of enzyme activities, qRT-PCR and HPLC data led to get further insights overview into the effect of elicitors on TAs content in *C. roseus* cell suspension.
Materials and methods

Plant material and in vitro culture conditions

*C. roseus* seeds were purchased from Syngenta Company, Basel, Switzerland. Seeds surface sterilized was done with modified (Tonk et al. 2016) protocol by using 70% ethanol for 5 minutes, 1.5% sodium hypochlorite for 12 minutes, and then rinsed with sterile distilled water 6 times for 10, 8, 6, 4, 2, 1 minutes, respectively. Seeds were placed on basal medium (Murashige and Skoog 1962) without plant growth regulators. Different types of explants such as hypocotyl, cotyledon, nod, leaf and petiole were excised and all explants cultured in the MS medium which supplemented with 2, 4-D (2, 4 and 8 µmol/l) and BAP (0.5, 1 and 2 µmol/l) (Table 1). The media were solidified with 7 g/l of agar and the pH of them were adjusted to 5.7 ± 1 before autoclaving at 121°C for 15 min. Cultures were incubated at 25 ± 2°C under 16-h photo period regime with cool white fluorescent (100 µmol m⁻² s⁻¹ PFD).

**Table 1**

<table>
<thead>
<tr>
<th>Medium name</th>
<th>PGRs (µmol L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS1</td>
<td>2 µmol L⁻¹ 2,4-D + 0.5 µmol L⁻¹ BAP</td>
</tr>
<tr>
<td>MS2</td>
<td>2 µmol L⁻¹ 2,4-D + 1 µmol L⁻¹ BAP</td>
</tr>
<tr>
<td>MS3</td>
<td>2 µmol L⁻¹ 2,4-D + 2 µmol L⁻¹ BAP</td>
</tr>
<tr>
<td>MS4</td>
<td>4 µmol L⁻¹ 2,4-D + 0.5 µmol L⁻¹ BAP</td>
</tr>
<tr>
<td>MS5</td>
<td>4 µmol L⁻¹ 2,4-D + 1 µmol L⁻¹ BAP</td>
</tr>
<tr>
<td>MS6</td>
<td>4 µmol L⁻¹ 2,4-D + 2 µmol L⁻¹ BAP</td>
</tr>
<tr>
<td>MS7</td>
<td>8 µmol L⁻¹ 2,4-D + 0.5 µmol L⁻¹ BAP</td>
</tr>
<tr>
<td>MS8</td>
<td>8 µmol L⁻¹ 2,4-D + 1 µmol L⁻¹ BAP</td>
</tr>
<tr>
<td>MS9</td>
<td>8 µmol L⁻¹ 2,4-D + 2 µmol L⁻¹ BAP</td>
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</tbody>
</table>

*MS* = Murashige and Skoog, 2,4-D = 2,4-Dichlorophenoxyacetic acid and BAP = BenzylAminoPurine

Callus induction and cell suspension

A factorial experiment in completely randomized design was done in triplicate for consider the effect of PGRs and explants types on callus formation. The soft callus derived from explant with highest vinblastine and vincristine percentage selected and shifted to the liquid MS medium with the best hormonal treatment for callus growth and then placed in the incubator shaker (120 rpm shaking at 25°C) for callus proliferation. Cell suspensions subculture was performed every 7 days until optimal cell growth rate was obtained and for drawing cell growth curve, used settled cell volume and cell count (Farjaminezhad et al. 2013).

According to the growth curve, elicitor treatments were applied when the cells reached exponential growth.

The biotic elicitor was *T. harzianum* with 1% v/v ratio, the abiotic elicitor was 150 µM SNP and medium without elicitor used as control. After exposure to the elicitors (12, 24, 48 and 72 hours), the cell suspension was passed through a filter paper and then excess water was removed and the obtained mixture were stored at -80°C for stabilize the samples to investigate the enzyme activities and RNA extraction.

Procurement, culture of fungi and preparation of elicitor

The *T. harzianum* strain obtained from the Department of Plant Pathology, of Bu-Ali Sina University, Hamedan, Iran. The fungi culture was done by Nakano and Asada (1981) method and the 1% (v/v) concentration of fungi extract applied to cell suspension (Tashackori et al. 2016). The abiotic elicitor was 150 µM SNP and medium without elicitor used as control. After exposure to the elicitors (12, 24, 48 and 72 hours), the cell suspension was passed through a filter paper and then excess water was removed and was stored at -80°C in order to stabilize the samples to investigate the enzyme activities and RNA extraction.

Enzyme activities assessment

The activities of catalase (Aebi 1984), ascorbate peroxidase (Aebi 1984), β (1–3) glucanase (Abeles and Forrence 1970) and chitinase (Fan et al. 2008) were assayed.

Extraction and assay of vinblastine and vincristine

According to Pan et al. (2010) protocol, samples harvested and dried at 25°C for a week and pulverized. 0.1 g of sample's powder dissolved in 1 ml of methanol 85% (HPLC grade), shacked for 10 minutes ultra-sonicated at 30°C for 1 hour, centrifuged at 14000 rpm for 15 min and then supernatant collected for assay. The final volume was made up of the residue of each sample reconstituted in ratio 1:10 for high-performance liquid chromatography analysis as
described by (Junaid et al. 2006). Indole alkaloids were evaluated by RP-HPLC (Knauer Scientific Instruments, Berlin, Germany). Detection and quantification were carried out with a Smartline Pump 1050, a Smartline UV Detector 2600 and DGU-14 A degasser. All solutions were filtered through a 0.45 nm filter. Aliquots (80 µl) were injected into a C18 reversed-phase column (250 mm × 4.6 mm length, 5 µm particle size). For stock solution, vinblastine and vincristine sulfate of over 97% purity purchased from Sigma-Aldrich (St. Louis, USA) and 0.5 mg solved in 0.5 ml of methanol and 0.5 ml of acetonitrile. In addition, vinblastine and vincristine compounds were recognized by comparing their retention times with those of the standards. Also, content of compounds was determined using their standards calibration curve.

Total RNA extraction and gene expression analyses

By using the RNX-Plus Kit (Sinaclon, Iran) and according to the manufacturer's instructions, total RNA from control and samples which treated with elicitors extracted. Agarose gel electrophoresis and Nanodrop (Thermo Scientific, Germany) spectrophotometer analyses were used to test quality and quantity of RNA, respectively. The first-strand cDNA manufactured of RNA by the instructions of the Sinaclon First-Strand cDNA synthesize kit. Primers for G10H, STR, T16H, D4H, DAT and CrPrx genes were designed by OligoArchitect online software and synthesized by Pishgam Company (Table 2). Real time PCR reaction performed with Siber Green dye through protocol (Sinaclon, Iran). The RPS9 gene was selected as a housekeeping gene for data normalization. The results were calculated as relative changes (Fold changes) compared to the control sample. The relative expression of each gene was calculated using the \(2^{-\Delta\Delta Ct}\) method (Livak and Schmittgen 2001).

<table>
<thead>
<tr>
<th>Gene and primer name</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>G10H (forward)</td>
<td>TAGCAGGGAGGACACACATCAA</td>
</tr>
<tr>
<td>G10H (reverse)</td>
<td>TCACGTCAATTGCCCAAGCATTC</td>
</tr>
<tr>
<td>T16H (forward)</td>
<td>AGGACCTTGTAGTGCTG</td>
</tr>
<tr>
<td>T16H (reverse)</td>
<td>CATTGCCCAATCGACTGTTG</td>
</tr>
<tr>
<td>D4H (forward)</td>
<td>TACCCTGACATGCCCTCACC</td>
</tr>
<tr>
<td>D4H (reverse)</td>
<td>TTGAAGGCGGCAATTG</td>
</tr>
<tr>
<td>DAT (forward)</td>
<td>TTCCTCGGAAGCCATAGA</td>
</tr>
<tr>
<td>DAT (reverse)</td>
<td>GTCTGATTCTCGTACCG</td>
</tr>
<tr>
<td>CrPRX (forward)</td>
<td>GCAACATCTCCGAGCACCACA</td>
</tr>
<tr>
<td>CrPRX (reverse)</td>
<td>GTTCTCCGAAACATGAGACAC</td>
</tr>
<tr>
<td>RPS9 (forward)</td>
<td>TCCACCATGCCAGTGCTCATTA</td>
</tr>
<tr>
<td>RPS9 (reverse)</td>
<td>TCCATACCACCAGGCTCTTT</td>
</tr>
</tbody>
</table>

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA)s using SAS software (version 9.4 Cary, NC, USA). The presented mean values were separated using Duncan's multiple range Test (DMRT) at \(p \leq 0.05\).

Results

Callus induction and cell suspension

In this study after four weeks, callus formation percentage and callus fresh weight were measured. The effect of explants, hormonal treatment and their interaction on callus induction percentage (Fig. 1) and callus fresh weight (Fig. 2) of C. roseus were significant (\(P \leq 0.01\)). Comparison between explants and hormonal treatments on callus formation also showed that the callus formation percentage of different explants was strongly influenced by hormonal treatment in culture medium and in some explants including hypocotyl, nodule and leaf in most hormonal treatments such as 4 µM 2,4-D, 2 µM BAP. Application of 8 µM 2,4-D and 2 µM BAP had 100 percentage callus formation. On the other hand, the lowest percentage of callus formation was seen in the medium with combination hormonal treatment of 2 µM 2,4-D and 2 µM BAP and leaf explants. Interaction effect of explant and hormonal treatment showed that callus fresh weight of various explants in culture media with different hormonal treatments was variable, so that the highest callus fresh weight was observed in 2 µM 2,4-D and 0.5 µM BAP - treated samples and hypocotyl explants and combination of 2 µM 2,4-D and 1 µM BAP and hypocotyl explants, respectively. The lowest callus fresh weight was observed in the medium supplemented with 2 µM 2,4-D and 2 µM BAP and hypocotyl explants (Fig. 2). The HPLC results showed that the simple effect of explants on the vinblastine and vincristine in C. roseus were significant (Fig. 3). The highest amount of vinblastine was obtained from leaf explants (1.348 µg/g dry weight) and hypocotyl (0.317 µg/g dry weight), respectively. The maximum amount of vincristine was obtained from leaf explants (0.657 µg/g dry weight) and hypocotyl (0.183 µg/g dry weight), respectively. There was no significant difference between
petiole, node and cotyledon explants in aforesaid alkaloids (Fig. 3). Leaf explant showed the higher amount of vinblastine and variance therefore used for next stage. Application of 8 µM 2,4-D and 2 µM BAP was the most desirable medium for callus growth, so this medium was used for liquid culture and preparation of cell suspension. The increase in callus formation and percentage by plant growth regulators can related to the provision of necessary precursors for growth. Also, the increase in callus weight in hormonal treatments can go back to the increase in cell number and cell size, which due to photosynthesis have the ability to produce the required carbon source and in the later stages will increase significantly (Tahir et al. 2011). Farhadi et al. (2021) investigated the effects of plant growth regulators BAP, 2,4-D and NAA on the percentage and weight of calluses from the roots and hypocotyl of C. roseus. Their results showed that in comparison with the explants, the highest percentage of calluses was obtained from hypocotyl explant and the highest hormonal treatment on the percentage and weight of calluses was 1 mgL⁻¹ BAP and 1 mgL⁻¹ 2,4-D, reported that C. roseus hypocotyl explant in MS medium supplemented with 4.5 µM 2,4-D with 2 µM BAP had the highest callus formation and fragile and light-colored, fast-growing calluses were produced that could produce somatic embryos. In the present study, hormonal treatments are developed, in particular, the combination treatment of 4 µM 2,4-D and 1 µM BAP and the combined treatment of 4 µM 2,4-D and 2 µM BAP cause callus formation in most of the explants. The negative effect regulators especially 2,4-D and BAP on callus formation percentage and callus weight in many medicinal plants such as Stevia rebaudiana (Keshvari et al. 2018), Taxus baccata, Calotropis procera (Amirkavei Najafabadi et al. 2020), Calotropis procera (Abasi 2017), Mentha piperita (Ahmad et al. 2021) and Salacia macrosperma (Bouguemra et al. 2022) have been reported to be consistent with the results of this study. The balance between organic and inorganic nutrients, carbon source, plant growth regulators, stresses and plant growth stage can affect the biosynthesis pathway of alkaloids. Aslam et al. (2010) investigated the alkaloid content of vincristine in non-embryonic callus and embryogenic callus from leaf, root and node explants. Their results showed, the highest amount of vincristine alkaloid was in the embryonic germination stage (10.04 micrograms per gram of dry weight). Also, plants regenerated from somatic embryos had 2.2 µg per gram of dry weight of vinblastine more than plants grown in the field. Their results showed vinblastine alkaloid were not detected in the roots and the highest vinblastine alkaloid was observed in leaf explant. In the growth conditions comparison vinblastine alkaloid in the in vitro condition was 12.3 µg g⁻¹ of dry weight and in field condition it was 9.4 µg g⁻¹ of dry weight. In our study, the amount of vincristine and vinblastine in leaf-derived callus were 3.6 and 4.2-fold higher than hypocotyl explants, respectively (Fig. 3).

**Enzymes Activities**

In order to realize the effect of Trichoderma and sodium nitroproside elicitor on plant defense responses and secondary metabolism, several antioxidant enzymes activity evaluated as the addition of elicitor induced cellular stress on cell suspension. The results showed that Trichoderma, SNP and elicitors application time had significant effects on the catalase, ascorbate peroxidase, β (1–3) glucanase and chitinase activities in treated C. roseus cell suspension. There was a significant difference in elicitors and elicitor application time on content of catalasase activity in C. roseus cell suspension. The results revealed that the T. viride, SNP and T. harzianum, SNP treatment after 48 hours elicitors application time led to increase the 2 and 1.9-fold catalase activity in comparison with controls, respectively (Fig. 4). The interaction effect of elicitors and elicitors application time on ascorbate peroxidase activity have not significant difference but the most mentioned enzyme activity was observed in T. harzianum, SNP (2.59 mg⁻¹ protein min⁻¹) and SNP (2.55 mg⁻¹ protein min⁻¹) treatment, respectively (Fig. 5). Also, a significant increase in ascorbate peroxidase activity was seen after 48 hours elicitors application time (Fig. 6). According to the results (Table 4), β (1–3) glucanase was affected by elicitors and elicitor application time. The results showed that β (1–3) glucanase activity increase in T. harzianum and T. harzianum, SNP treatment after 48 hours elicitor application time about 2.4-fold increase rather than control (Fig. 7). Due to the Table 4, elicitors and elicitor application time led to change in chitinase activity so that, T. harzianum and T. harzianum, SNP treatments after 48 hours elicitor application time increase 3.7-fold compared with control (Fig. 8).

In general, the results of this study showed that the effect of catalase and ascorbate peroxidase was higher than in most samples treated with T. harzianum. On the other hand, the activity of these enzymes in comparison with biotic and abiotic treatments was higher in SNP treatment than fungal treatments. The highest activity of these antioxidant enzymes was observed 48 hours after treatments. The most desirable elicitor and application treatment time for increasing the activity of catalase and ascorbate peroxidase enzymes was the treatment of T. harzianum and SNP after 48 hours. After 72 hours, due to the death of most cells in the suspension culture, the amount of activity of enzymes decreased and the amount of activity reached less than the amount of activity in the treated samples after 12 hours. Also, the activity of chitinase and β-1 and 3 glucanase enzymes in samples treated with T. harzianum was higher than that of T. viride, although no significant difference was observed between them. Comparing the effect of biological treatments and SNP elicitors on the activity of chitinase and β-1 and 3-glucanase enzymes, fungal treatments increased the activity of these enzymes compared to SNP treatment. After 24 hours of treatment, the highest activity of these enzymes was observed. In general, the highest activity of chitinase and β-1 and 3-glucanase enzymes was obtained from the treatment of T. harzianum and SNP after 24 hours. After 48 hours of treatment, the activity of these enzymes decreased and after 72 hours, it decreased to less than 12 hours of treatment with biotic elicitors and SNP elicitor. Tonk et al. (2016) in a study investigated the effect of Aspergillus flavus fungi elicitor on vinblastine and vincristine content in periwinkle invitro culture. Their results showed that adding 0.15% fungal elicitor to the culture medium in comparison with other concentrations and calluses derived from leaf explants, led to the highest activity of the antioxidant enzymes superoxide dismutase, catalase and ascorbate peroxidase. Khaskan and Husain (2015) by treating fungi, yeast and bacteria as elicitor in the culture medium containing periwinkle leaf explants, reported the highest activity of catalase enzyme in the T. harzianum treatment (61.49 units) and then in bacteria and yeast treated samples. The highest content of vinblastine and vincristine from fungal elicitor treatment were 31.30 and 17.78-fold compared with control.

SNP as chemical elicitor in plants stimulates enzymes and genes involved in the scavenging of free radicals. On the other hand, it causes stress and responds to the mechanism of stress by stimulating enzymes and genes related to the defense response. Reduction of fusarium wilt by treating biotic and SNP elicitors was investigated in tomatoes (Chakraborty et al. 2021). The results showed that after 48 hours of treatment with biotic elicitor and SNP activity of β-1,3-glucanase, chitinase, polyphenol oxidase, peroxidase and phenylalanine ammonia-lyase were increased and recorded as 3.34, 2.11, 3.34, 2.17 and 2-fold higher than controls. In this study, according to the results of enzymatic activity and non-significant differences between T. harzianum and T. viride elicitors, the experiment was continued with 1% v/v T. harzianum with 150 µM of SNP.
Expression analysis of TAs biosynthetic genes

The relative expressions of the $G10H$, $T16H$, $D4H$, $DAT$, $STR$ and $CrPRX$ gene of TAs biosynthetic pathway in $C. roseus$ cell suspension were significantly up-regulated with Trichoderma, SNP elicitor and the effect of elicitor application time. The gene expression began to increase after 12 hours and reached its maximum level in 48 hours and after 72 hours it was decreased to the less than primary level. $T. harzianum$, SNP treatment after 48 hours elicitor application time led to maximum expression of all considered genes. The results of elicitors and application time on the $G10H$ gene expression in $C. roseus$ cell suspension showed the maximum relative expression seen in $T. harzianum$, SNP treatment after 48 hours of elicitor application (2.53-fold compared with control) and $T. harzianum$, SNP treatment after 24 hours elicitor application (2.32-fold compared with control) (Fig. 8). $T. harzianum$, SNP and $T. harzianum$ treatment after 48 hours elicitor application led to increase 1.5-fold and 1.37-fold $T16H$ gene compared with control, respectively (Fig. 9b). The relative expression of the $D4H$ gene was higher in $C. roseus$ cell suspension treated with $T. harzianum$, SNP after 48 hours elicitor application (1.18-fold compared with control) and $T. harzianum$, SNP treatment after 24 hours elicitor application (1.15-fold compared with control) (Fig. 9C). The 1.98-fold and 1.68-fold increase in $DAT$ gene relative expression observed in $T. harzianum$, SNP after 48- and 24-hours elicitor application, respectively (Fig. 9D). The maximum $STR$ gene relative expression obtained from $T. harzianum$, SNP treatment after 48 hours elicitor application (5.08-fold compared with control) and $T. harzianum$, SNP treatment after 24 hours elicitor application (4.46-fold compared with control) (Fig. 9E). $T. harzianum$, SNP treatment after 48 and 24-hours elicitor application caused increase 2.07 and 1.79-fold $CrPRX$ gene compared with controls, respectively (Fig. 9F). Among all, the lowest expression value observed in control. Totally, utilized elicitors stimulate the expression of the six key genes on vinblastine and vincristine biosynthesis in the TAs biosynthetic pathway and leading to the accumulation of TIA.

Vinblastine and vincristine alkaloid yield

The effect of Trichoderma fungi and SNP, the application elicitor time, the interaction effect of Trichoderma fungi and SNP and application elicitor time on vinblastine and vincristine alkaloid amount in $C. roseus$ cell suspension were significant at 1% probability level (Figs. 10 and 11). Based on comparison of interaction effect of Trichoderma fungi and SNP and application elicitor time, the maximum of vinblastine (Fig. 10) and vincristine (Fig. 11) alkaloids observed in $T. harzianum$, SNP 48 hours after elicitor application (1.84 and 1.93-fold compared with control respectively) and combination of $T. harzianum$, SNP after 24 hours application elicitor (1.69 and 1.87-fold compared with controls, respectively). The lowest amount of vinblastine and vincristine alkaloids was obtained from controls with different application elicitor times that had no significant different with each other. It is very evident from this paper that the biotic elicitor stimulates enriched level of alkaloids in $C. roseus$ cell suspension.

Correlation between phytochemical, molecular and metabolic evaluations

The results of correlation between enzymatic activity, molecular and metabolic evaluations in $C. roseus$ cell suspension under the influence of Trichoderma fungi and SNP elicitors showed a positive and significant correlation between catalase, ascorbate peroxidase, β-1 and 3-glucanase and chitinase enzyme activity with the relative $G10H$, $T16H$, $D4H$, $DAT$ and $CrPRX$ $STR$ and $CrPRX$ genes involved in the biosynthesis of vinblastine and vincristine alkaloids with the amount of production of these alkaloids. Due to these results, it is inferred that treatments of $T. harzianum$, SNP elicitor and elicitor application time induced the defense response and increased enzymatic activity and thus stimulated the relative expression of the biosynthetic pathway genes in the $C. roseus$ cell suspension. Enhancement of vinblastine and vincristine alkaloids confirms this because the increased production of vinblastine and vincristine alkaloids can be due to increased activity of the antioxidant enzyme (catalase and ascorbate peroxidase) and defense enzymes (β-1 and 3-glucanase and chitinase) or increase the expression of genes involved in the biosynthetic pathway of these alkaloids (Table 3).
The first part of the experiments was performed to screen the best explants and medium with high callus formation percentage, callus fresh weight and amount of vinblastine and vincristine. In second part, the effect of *T. harzianum*, SNP elicitor and elicitor application time, enzymatic activity and expression of the biosynthetic pathway of TIAs genes on *C. roseus* cell suspension were investigated. Liu et al. (2021) stated that cell suspension and hairy roots are the best methods to study the biosynthesis pathway of indole alkaloids in the periwinkle plant, although more recently seedlings (Mortensen et al. 2019), leaves (Sharma et al. 2018) and petals (Schweizer et al. 2018) have also been used to investigate the expression of genes related to the synthesis of valuable alkaloids in this plant. Therefore, in this study, *C. roseus* cell suspension derived from leaf callus was used. The production of the secondary metabolite is genetically controlled, but its accumulation in the plant occurs under the influence of biotic stresses and SNP elicitor (Verma et al. 2017). Fungal elicitors increase the production of secondary metabolites, especially those involved in plant defense mechanisms. Fungi, like plants, have cell walls, but their cell wall composition is different from plants. Elicitors attack cell wall compounds, and in response to the elicitors message, the plant’s defense system is activated (Orbán et al. 2008). Decreased in growth of cells affected by fungal elicitors over time may be due to decreased primary metabolism and the onset of secondary metabolism. On the other hand, there are reports of increased growth affected by fungal elicitors (Wang et al. 2001). SNP is a source of nitric oxide, which is a free radical and has a wide range of physiological consequences in plant cells (Hayat et al. 2010). The role of signaling of this molecule in regulating important growth processes, development and defense responses has been identified (Hong et al. 2008). Nitric oxide activity in plant tissues and cells usually occurs in response to abiotic stresses, pathogen attacks, and fungal elicitor challenges. The most prominent role of nitric oxide is signaling and regulation of defense responses to stresses, which leads to increased production of secondary metabolites (Senthil 2020) and activation of enzymes and expression of genes related to their biosynthetic pathway (Ma et al. 2021). Considered the positive effects of fungal elicitors and SNP in this study, the effect of these elicitors on the change in the relative expression of genes involved in the biosynthetic pathway of vincristine alkaloid was investigated. It should be noted that the relative expression of G10H, T16H, D4H, DAT, STR and CrPRX genes involved in the biosynthetic pathway of vinblastine and vincristine alkaloid in *C. roseus* cell suspension under the treatment of *T. harzianum* and SNP elicitor have not been studied at different application elicitor times. Current findings were similar to publications that reported the positive effect of biotic elicitor on biosynthesis pathway of terpenoid indole alkaloids. Pandey et al. (2016) investigated the effect of endophytic fungi (*Choanephora infundibulifera* and *Curvularia spp.*) on increasing the expression of vindoline genes in periwinkle. Result illustrated after inoculation with *Curvularia spp.* elicitor led to expression of STR (0.7-fold increase), SGD (0.2-fold decrease), T16H (0.3-fold decrease), 16OMT (1-fold increase), D4H (1.7-fold increase), DAT (5.3-fold increase) and PRX1 (2-fold increase) compared with the control. *Choanephora infundibulifera* elicitor led to changes in relative expression of STR (0.9-fold increase), SGD (0.4-fold decrease), T16H (0.2-fold decrease), 16OMT (2-fold increase), D4H (1-fold increase), DAT (4.8-fold increase) and PRX1 (2.4-fold increase). Ramezani et al. (2018) reported that the treatment of
Trichoderma harzianum, Piriformospora indica and T. tomentosum 1% v/v in the periwinkle cell suspension was more effective than T. tomentosum. Gene expression began after 24 hours of treatment and expression of D4H and DAT genes after 48 hours of treatment had the highest gene expression of 1.2-fold and 0.7-fold, respectively. After 72 hours the expression of mentioned genes were reduced and reached the control level. Zhonghai et al. (2011) studied the effect of Fusarium oxysporum extract on increasing the expression of PAL and TDC genes in the biosynthesis pathway of alkaloids. Increased PAL and TDC activity led to the increase in the alkaloid synthesis of C. roseus suspension cell culture. Liang et al. (2018) investigated the effect of biotic elicitor on the expression of indole alkaloid production genes using Aspergillus flavus as an elicitor in the cell suspension resulting from C. roseus meristem. Treatment of different concentrations of the fungus (0, 5, 15 and 25 mg l⁻¹) was applied at different treatments (12, 24, 36, 48 and 60 hours). qRT-PCR results showed that the combined treatment of 25 mg l⁻¹ elicitor was the most desirable treatment after 48 hours and in this treatment the relative expression of D4H, G10H, GES, IRS, LAMT, SGD, STR, TDC and ORCA3 genes were compared with control increased by 49.4, 1.75, 1.71, 1.42, 3.12, 2.33, 2.87, 2.51 and 5.97-fold. These results showed that increasing the accumulation of vindoline, catharanthine and ajmaline alkaloids in periwinkle cells is correlated with increasing the expression of the above genes. Application of SNP elicitor increases the production of secondary metabolites and stimulates the expression of genes in the biosynthetic pathway of valuable medicinal compounds in plants (Zhou et al. 2010). Mahendran et al. (2021) reported that SNP at different concentrations (5, 10, 20, and 40 µM) in the cell suspension of the antidiabetic drug Gymnema sylvestre increases valuable secondary metabolites of this plant and their biosynthetic genes. Concentrations of 20 µM up to day 40 of the treatment resulted in the highest fresh and dry cell weight. The results of HPLC analysis showed that the maximum accumulation of gymnemic diacyl acid (5.51 mg g⁻¹ callus dry weight), gymnemagnine (2.80 mg g⁻¹ callus dry weight) and gymnemic acid (2.08 mg g⁻¹ callus dry weight) in the treatment of 20 µM SNP was observed after 40 days of treatment, which was 13.43, 13.86 and 17.33-fold higher than the control, respectively. They attributed the increase to a change in the expression pattern of genes involved in the biosynthesis of these compounds by 20 µM SNP treatment after 40 days and they recommended this treatment as a good strategy for large-scale production of these secondary metabolites in industrial level. Liang et al. (2012) reported the application of SNP elicitor (100 µM) in salvia hairy root culture significantly increased tanshinone production. This treatment increased the expression of two key genes HMGR and DXR in the biosynthesis pathway of tanshinone alkaloid 16.7 and 14.1-fold, respectively. Wang et al. (2009) by application of cerebroside (30 µg ml⁻¹) and SNP (10 µmol) in the hairy root of the artemisia reported that SNP alone did not change the expression of genes involved in the biosynthesis pathway of artemisinin sesquiterpene alkaid. The results of qRT-PCR showed the combination of the two above-mentioned elicitors resulted in the highest expression of HMGR (9.3-fold) and DXR (6.6-fold) genes compared to the control. Xu and Dong (2005) in a study evaluated the SNP role as a nitric oxide donor in secondary metabolites production in cell suspension of periwinkle. The results showed that production of ajmalicine and catharanthine in the treatment of 10 mmol l⁻¹ SNP increased by 1.6 and 2.9-fold, respectively, which increased followed by high expression of genes involved in the production of ajmalicine and catharanthine alkaloids. Zhou et al. (2010) investigated the transcriptional response of the catharanthine alkaloid biosynthesis pathway in hairy root culture of periwinkle using methyl jasmonate (50 mg l⁻¹) and SNP (10 mg l⁻¹) elicitors. The results showed SNP elicitor treatment alone and in combination with methyl jasmonate caused overexpression of ORCA3 and subsequently decreased catharanthine levels and also significant enhancement of CPR and ZCT transcription. Methyl jasmonate increased the expression of TDC, STR, MECS, SLS, SGD and G10H genes, which was inhibited by SNP. This response of transcription receptors and pathogen genes may indicate the role of methyl jasmonate and SNP antagonists in the biosynthesis of the catharanthine alkaloid. Li et al. (2011) used different concentrations of SNP (0, 0.1, 1 and 10 mM) for investigating its effects on growth and alkaloid compounds production in hairy root culture of periwinkle. Their results showed that 10 mM of SNP was a complete inhibitor of hairy root growth and 1 and 10 mM SNP were toxic and reduced the production of indole-terpenoid alkaloids. 0.1 mM up to day 9 of the treatment increased the alkaloids of ajmalicine and taberzonin and decreased the alkaloid of catharanthine and serpentine alkaloids reached their maximum on day 14 of treatment but decreased by day 30. Treatment of 0.1 mM concentration up to day 12 reduced the expression of STR, ORCA3 and Crgbf1 genes and 21 days after treatment the relative expression of ZCT1 gene doubled compared to the control and until the 28th day the relative expression of G10H gene upward trend.

Previous studies have shown that elicitors can control large number of transcription factor regulators of key genes in the biosynthesis pathway of terpenoid indole alkaloids and stimulate the expression of these genes at the biochemical and molecular level (Khataee et al. 2019). However, the results of this research showed that simultaneous application of biotic elicitor and SNP have a greater effect on stimulating production of secondary metabolites. These results were consistent with previous studies and showed the combined treatment of Trichoderma elicitor (T. harzianum) with abiotic elicitor (SNP) was more effective on the activity of enzymes, relative expression of mentioned genes and vinblastine and vincristine alkaloids amount in C. roseus cell suspension culture.

Conclusion

In this study we investigate the effect of T. harzianum and SNP on phytochemical, molecular and metabolic change in C. roseus cell suspension. Base on the results, leaf explant and 8 µM 2,4-D and 2 µM BAP were selected for liquid culture and preparation of cell suspension. In addition, the use of T. harzianum elicitor in combination with SNP elicitor 48 hours after application is recommended to increase the enzyme activities, expression of genes involved in biosynthesis pathway of vinblastine and vincristine alkaloids in periwinkle. The results of this paper can be useful to secondary metabolite production with higher quality under in vitro status.

Declarations

Funding

This work was supported by Bu-Ali Sina university (Grant No: 98-936).

Competing interest
The authors have no main and known competing financial interests or important and personal relationships that could have appeared to influence the study reported in this paper.

Author Contributions

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Mohammad Sayyari: Author 2 was supervisor of Ph. D. project of author 1 and cooperated in conducting the experiment, analyzing the data and preparing the manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgements

The support of this research provided by Bu-Ali Sina University (BASU) has been acknowledged.

References


Interaction effect of *C. roseus* explants and hormonal treatments on callus formation percentage. In each column, the averages with the same letters have no significant difference in the 5% probability level based on Duncan's multiple range test.

**Figure 2**

Interaction effect of *C. roseus* explants and hormonal treatments on callus fresh weight.

**Figure 3**

The effect of explants on the vinblastine and vincristine level in *C. roseus*.

**Figure 4**

Elicitor application time
The interaction effect of *T. harzianum*, *T. viride* and sodium nitroprusside elicitor treatment and elicitor application time on catalase enzyme activity in *C. roseus* cell suspension.

![Figure 5](image)

**Figure 5**

The interaction effect of *T. harzianum*, *T. viride* and sodium nitroprusside elicitor treatment on ascorbate peroxidase enzyme activity in *C. roseus* cell suspension.

![Figure 6](image)

**Figure 6**

The interaction effect on elicitor application time on ascorbate peroxidase enzyme activity in *C. roseus* cell suspension.

![Figure 7](image)

**Figure 7**

The interaction effect of *T. harzianum*, *T. viride* and sodium nitroprusside elicitor treatment and elicitor application time on β-1 and 3-glucanase enzyme activity in *C. roseus* cell suspension.
Figure 8
The interaction effect of *T. harzianum*, *T. viride* and sodium nitroprusside elicitor treatment and elicitor application time on chitinase enzyme activity in *C. roseus* cell suspension.

Figure 9
The effect of *T. harzianum*, sodium nitroprusside elicitor and elicitor application time on relative expression of *G10H*, *T16H*, *D4H*, *DAT*, *STR* and *CrPRX* genes in *C. roseus* cell suspension.
Figure 10

The interaction effect of *T. harzianum* elicitor and sodium nitroprusside elicitor treatment and application elicitor time on vinblastine alkaloid amount in *C. roseus* cell suspension.

Figure 11

The interaction effect of *T. harzianum* and sodium nitroprusside elicitors and application time on vincristine alkaloid amount in *C. roseus* cell suspension.