

Effect of the Plant Probiotic Bacteria on TIA Biosynthesis Pathway Gene Expression Profiling, Vinblastine and Vincristine Content in the Root of *Catharanthus Roseus*

Maryam Ahmadzadeh

Bu Ali Sina University Faculty of Agriculture

Amir Hossein Keshtkar (✉ akesht@gmail.com)

Bu Ali Sina University Faculty of Agriculture <https://orcid.org/0000-0002-5358-448X>

Kobra Moslemkhany

Seed and Plant Certification and Registration Institute

Masoud Ahmadzadeh

University of Tehran University College of Agriculture and Natural Resources

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Abstract

Catharanthus roseus is the sole resource of vinblastine and vincristine, which are two of the biggest concerns of TIAs because of their powerful anticancer activities. Increasing the concentration of these alkaloids in various organs of the plant is one of the important goals in *C. roseus* breeding programs. Plant probiotic bacteria (PBB) act as biotic elicitors and can induce the synthesis of secondary products in plants. The purpose of this research is to study the individual and combined effects of *P. fluorescens* and *A. brasilense* on expression of the TIA biosynthetic pathway genes (G10H, DAT, T16H and CrPRX) using qRT-PCR and the content of vinblastine and vincristine alkaloids using HPLC method in roots of *C. roseus*. *P. fluorescens* drastically increased the content of vinblastine and vincristine alkaloids, compared to the control in the roots, up to 174 and 589 ($\mu\text{g/g}$), respectively. According to the molecular analysis, bacterium significantly increased the expression of more genes in the TIA biosynthetic pathway compared to the control. *P. fluorescens* increased the expression of the final gene of the biosynthetic pathway (CrPRX) 47.9 times compared to the control. Therefore, the findings indicate the coordination of transcriptional and metabolic outcomes. The same result was also observed for *A. brasilense*. According to the results, it can be concluded that, the seed priming and root of the seedling treatments of probiotic bacteria can be used as a good tool in the enhancement of alkaloid contents in medicinal plants, as it provides an eco-friendly approach.

Introduction

Medicinal plants are recognized as an important therapeutic tool to alleviate the ailments of human's kind (Sain and Sharma, 2013). *Catharanthus roseus* is one of the medicinal plant belonging to the family Apocynaceae (Almagro *et al.*, 2015). *C. roseus* produces more than 130 types of terpenoid indole alkaloids (TIAs) (Gupta *et al.*, 2007). This plant is the only source of vinblastine and vincristine, which are among the largest types of TIA due to their powerful anti-cancer activities (Zhu *et al.*, 2014 and Sun *et al.*, 2016). However, the production of these beneficial drugs has been limited due to their small amount in the plant (Pan, *et al.*, 2010 and Almagro *et al.*, 2015) and the major challenge in the pharmaceutical industry is the low production rate of these alkaloids (Soltani *et al.*, 2020). Due to the pharmaceutical importance and the low content of vinblastine and the related alkaloid vincristine in plants. *C. roseus*, became one of the best-studied medicinal plants (Van der Heijden *et al.*, 2004), and increasing the concentration of important medicinal alkaloids in its various organs is one of the most important goals in *C. roseus* breeding programs (Gupta *et al.*, 2007).

All parts of the plant have alkaloid, but, maximum concentrations are found in the root organ, particularly during the flowering stage (Jaleel, *et al.*, 2008). Therefore, this plant is considered as a very important medicinal plant in most pharmacopoeias due to the presence of valuable alkaloids in the shoots and roots.

The multi-step TIA biosynthetic pathway is quite complex and is under strict molecular regulation (Dutta *et al.*, 2005). Many of the genes involved in this pathway of *C. roseus* have been cloned and sequenced for the analysis of their expression in various plant organs (Gupta *et al.*, 2007). Critical early steps in the biosynthesis of TIAs include the reactions catalyzed by tryptophan decarboxylase (TDC), geraniol 10-hydroxylase (G10H), and strictosidine synthase (STR). TDC and G10H catalyze the first committed steps towards TIA biosynthesis in the indole and terpenoid precursor branches, respectively (Goklany *et al.*, 2009). Strictosidine is the central intermediate in the biosynthesis of different TIAs, which is formed by the condensation of secologanin and tryptamine. Secologanin is derived from terpenoid (isoprenoid) biosynthetic pathway, while tryptamine is derived

from indole biosynthetic pathway. Then various specific end products are produced by different routes during downstream process (Zhu *et al.*, 2014). The downstream TIA pathway genes include deacetylindoline-4-O-acetyltransferase (DAT), Tabersonine 16-hydroxylase (T16H), and *Catharanthus roseus* peroxidase (CrPRX) (Wang *et al.*, 2016) (Fig. 1).

TIA pathway is affected by biotic and abiotic factors (Favali, *et al.*, 2004). According to some researchers, rhizosphere microorganisms act as biotic elicitors and can induce the synthesis of secondary products in plants (Sekar and Kandavel, 2010). Among these microorganisms, some have positive effects on plant growth promotion constituting the plant growth promoting rhizobacteria (PGPR) such as *Azospirillum*, *Azotobacter*, *Pseudomonas fluorescens* and several gram positive *Bacillus* sp (Jaleel *et al.*, 2007b). These rhizobacterias induce the jasmonic acid and ethylene responses in plants (Pieterse *et al.*, 2001 and Beneduzi *et al.* 2012). Tissue culture studies have also shown that the external application of these compounds has induced the production of secondary metabolites in some plant species (Sekar and Kandavel, 2010). Generally, the phytohormone jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are major elicitors of TIA biosynthesis in *C. roseus* (Shen *et al.*, 2017). The Naeem *et al.*, (2017) review provides information regarding the role of potent PGRs such as gibberellic acid (GA3), in boosting the growth, metabolism, and other plant processes, particularly the production of anticancer alkaloids (vinblastine and vincristine) in *C. roseus* plants. Therefore, the mentioned bacteria can induce the synthesis of secondary metabolites in plants, through the production of phytohormones (Sekar and Kandavel, 2010). Singh *et al.*, (2021) identifies the best combination of endophytes consisting of plant growth-promoting and alkaloid enhancing endophytes that can maximize the plant growth and TIAs yield in various *C. roseus* plant cultivars under field conditions.

It should be noted that the fluorescent strains of *Pseudomonas* and *A. brasilense* are both beneficial bacteria and have been mentioned in various sources as the most important and largest group of probiotic bacteria (Ahmadzadeh and Sharifi Tehrani 2021). A review of the resources shows that the only effect of these bacteria on growth parameters and metabolite levels has been studied in *C. roseus* (Jaleel *et al.*, 2007b and Karthikeyan *et al.*, 2009), while, in the present study, the effect of rhizobacteria on the expression of TIA biosynthetic pathway genes in *C. roseus* was evaluated for the first time. The purpose of the research is investigation of the individual and combined effect of two species of rhizobacteria as a biotic treatment on expression some of the upstream and downstream TIA biosynthetic pathway genes, vinblastine and vincristine alkaloids content in roots of *C. roseus*.

Materials And Methods

Bacterial strains and growth conditions

The bacterial strains used in this study were *Pseudomonas fluorescens* and *Azospirillum brasilense*. The isolates provided from the Soil and Water Research Institute (Soil Biology Research Department), Karaj, Iran. The both of the bacteria were grown on nutrient agar (NA) for routine use. A single colony was transferred to 500 mL flasks containing NB grown aerobically in flasks on a rotating shaker (150 rpm) for 48 h (Karthikeyan *et al.*, 2010). The bacterial suspensions were then diluted in distilled water to a final concentration of 10^8 colony forming units (CFU) per milliliter, and the resulting suspensions were treated with *C. roseus* plants. Bacterial inoculation treatment was applied to the plant in three stages including seed, root inoculation and inoculation of soil around the roots.

Plant test

The periwinkle seeds were provided by Pan American Seed Company (www.panamseed.com) and were surface sterilized in 0.1% NaClO for 15 min with frequent shaking and rinsed thoroughly in distilled water to remove NaClO. Surface-sterilized seeds were then soaked in a bacterial inoculum for 30 minutes, with shaking, and sown in culture trays, superficially. After irrigation, the culture trays were kept under plastic cover for complete absorption of moisture and natural light at a temperature of 20 to 25°C. Seedlings after emergence and in the six-leaf stage were removed from the culture trays and immersed in a bacterial inoculum and then planted in separate pots (15 cm diameter × 15 cm height), filled with a 2:1:1 mixture of farm soil, sand and peat, with the same weight (500 g).

Inoculation of root and soil around the roots were performed at the time of seedling transfer to the pot. For root inoculation, the root of the seedlings were washed with distilled water and then immersed in each of the inoculants for 20 minutes, in the six-leaf stage. Distilled water was used in equal volume with bacterial inoculation treatment as a control (no bacterial inoculation). The plants were uprooted at flowering stage the day 30th after transferring from culture tray to pots for analyzing gene expression and estimation of vinblastine and vincristine content in the root.

Influence of bacterial treatments on expression of G10H, T16H, DAT and CrPRX genes

RNA extraction, cDNA synthesis, and primer designing

Root samples were flash-frozen in liquid nitrogen upon harvesting at the flowering stage and ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted from 100 mg of roots using the RibospinTM Seed/Fruit according to the manufacturer's instructions (GeneAll, South Korea). RNA concentration was quantified using the NanoDrop 2000c Spectrophotometer (Thermo Scientific NanoDrop 2000, USA) and were qualified by 1% agarose gel electrophoresis. The first strand cDNA was synthesized from 1 µg of total RNA using the Hyperscript RT-PCR master mix[®] according to the manufacturer's instructions in the final volume of 10 µl (GeneAll, South Korea). The cDNA was diluted to 100 ng/µL as the template for the real-time PCR analysis (Soltani *et al.*, 2020).

The genes monitored in this study were G10H (gene at the beginning of the pathway biosynthesis of TIAs, in the terpenoid pathway), DAT, T16H (vindoline pathway genes) and CrPRX (Terminal gene of the pathway). Primer sequences for target genes and the 40s ribosomal protein S9 (Rps9) reference gene were obtained from various resources (Table 1) and blasted in the National Center for Biotechnology Information (NCBI) genomic database (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1). Rps9 was validated as an appropriate housekeeping gene for the *C. roseus* by verifying the Ct profile for Rps9 remained nearly constant for all treatments (Goklany *et al.*, 2009). To ensure the specific amplification of designed primers for these genes the PCR reaction was performed using cDNA.

Table 1

Name, accession number, T_m and sequence of primers used for qRT-PCR analysis in *C. roseus*

Gene name	Primer sequence (5'-3')	T _m (°C)	Accession	PCR Product (bp) ¹	Source
G10H	F:TAGCAGGGACGGACACAACATCAA R:TCACGTCCAATTGCCCAAGCATTC	60	KF561461.1	304	Goklany <i>et al.</i> , 2009
T16H	F: GCTTCATCCACCAGTTCCAT R: CCGGACATATCCTTCTTCCA	61	FJ647194.1	242	Wang <i>et al.</i> , 2016
DAT	F: TTCCCTCCGGAAGCCATAGA R: GCTGATTTCCCTGCTACCGT	60	LN809931.1	125	Mokhaberi <i>et al.</i> , 2013
CrPRX	F: GCAACATCTCCAGACCACA R: GTTCTCCCAACTATGAGCACC	64	KT032115.1	117	Wang <i>et al.</i> , 2016
Rps9 ²	F:TCCACCATGCCAGAGTGCTCATTA R:TCCATCACCACCAGATGCCTTCTT	64	A749993.1	203	Goklany <i>et al.</i> , 2009

1. Products were analyzed by running the RT-PCR products against a DNA ladder on a 1% agarose gel.

2. Rps9 was used as the housekeeping gene.

G10H Geraniol-10-hydroxylase; *T16H* Tabersonine 16-hydroxylase; *DAT* Deacetylvinoline-4-Oacetyltransferase; *CrPRX* *Catharanthus roseus* peroxidase.

Gene expression profiling and analysis

The qRT-PCR analysis (Step One Plus Real-time PCR system, Applied Biosystems, USA) was performed using the specific primers to ensure amplification of the target genes. The expression level of the target and reference genes was determined using the RealQ Plus 2x Master Mix Green, High ROXTM. Three biological replications and two technical replications were considered for each gene. Each qRT-PCR reaction consisted of a mixture containing 16 ng/μL of cDNA (2 μl of initial concentration in 100 ng/μL), 0.4 μM of each of the forward and reverse primers, and Master Mix Green. The thermal cycling conditions included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, Temperature specific to each primer °C for 10 s, and 72 °C for 15 s.

Vinblastine and vincristine extraction and quantification

Collected root samples were dried at 58 °C for 48 h and pulverized in a mortar (Pan *et al.*, 2010). Extraction of alkaloids were performed according to the method of Singh *et al.* (2000). Samples were applied in two copies for quantification of vinblastine and vincristine. Isolation and measurement of two alkaloids were performed using

high performance liquid chromatography (HPLC) model Infinity 1260 (Agilent, USA) with RP-C18 column. The alkaloids were quantified by using the regression equation of calibration curve.

To prepare a standard sample, 1 g of vinblastine sulfate and 10 g of vincristine sulfate were dissolved in 10 ml of distilled water. Then, the standard solution of vincristine and vinblastine (1000 µg/ml) was prepared by dissolving a certain amount of vinblastine sulfate and vincristine sulfate solution in methanol. The standard calibration curve was drawn. Finally, the amount of these two alkaloids in the samples was estimated by matching the standard curve.

The yield of alkaloids is calculated by multiplying the amount of alkaloids (µg/g of dry root weight) by the dry weight of the roots (g).

Statistical analysis

In this research, the effect of two bacterial strains at four levels is studied. A control (no bacteria), *P. fluorescens*, *A. brasilense* and combined inoculation are investigated on *C. roseus* plants in a factorial experiment based on a randomized complete block design (RCBD) with three replications. The relative gene transcription was quantified using the comparative threshold cycle (CT) method and the data were analyzed using the REST® software [Pfaffl *et al.*, 2002] according to the $\Delta\Delta CT$ method [Livak and Schmittgen, 2001]. Analysis of metabolite data was performed using SAS Ver. 9.2. LSD (the least significant difference) procedure was used to compare the means.

Results

Monitoring TIA gene expression in roots of *C. roseus*

The transcript level of G10H gene in the terpenoid pathway (Upstream TIA pathway gene) was significantly up-regulated. Figure 2-A shows the effect of different treatments on the relative expression of G10H gene compared to the control in the flowering stage of *C. roseus*. Individual inoculation treatments of two bacteria had a significant effect on G10H gene expression. The highest transcript level was observed in *P. fluorescens*. In combined inoculation, no significant increase was observed in gene expression.

Among the studied treatments, *P. fluorescens* inoculation treatment increased the expression of T16H gene up to 22 times compared to the control. Combined inoculation resulted in increased gene expression, which was significant at the 5% probability level (Fig. 2-B).

According to Fig. 2-C, treatment with *P. fluorescens* had a significant effect on DAT gene expression compared to the control (34-fold). Combined inoculation showed a positive and significant effect at the level of 5% probability on the expression of the gene.

In the case of CrPRX gene, the final gene of TIA biosynthetic pathway, *P. fluorescens* and *A. brasilense* had a positive and significant effect on the 1% probability level and combined inoculation had a positive and significant effect on the 5% probability level (Fig. 2-D). *P. fluorescens* increased the expression of CrPRX gene 47.9 times compared to the control.

Metabolite analysis in roots of *C. roseus*

Based on different concentrations of standard solutions injected into the device, the retention time (minutes) is specified for the vinblastine and vincristine alkaloids. The graphs of the other samples were interpreted based on the retention time of the alkaloids.

HPLC analyzing the extracts of *C. roseus* root inoculated with bacterial treatments showed a significant difference for both alkaloids and vincristine yield per plant (Table 2). According to mean comparison (Table 3 and Fig. 3), *P. fluorescens* and *A. brasilense*, respectively, were characterized as the best treatments to induce significant production of vincristine and vinblastine alkaloids. In addition, these treatments individually caused increase of vincristine yield per plants root in the same manner as amount per gram of dry root weight.

Comparatively, the vincristine content was estimated at approximately 589 µg/g of dry root weight in plant roots treated with *P. fluorescens* indicating remarkable value, which was significantly more than the control. Also, applying this treatment increased the vinblastine contents by up to 175 µg /g of root dry weight compared to the control. However, combined inoculation elicited the minimum production of vinblastine alkaloid, which is not significant content compared to the control sample.

Table 2

Analysis of variance of the effect of bacterial inoculation treatment on vinblastine and vincristine in the root and alkaloids yield per plant of *C. roseus*

Source of variation	df	Vinblastine	Vincristine	Vinblastine yield per plant	Vincristine yield per plant
Bacterial inoculation	3	38499.49**	216646.22**	1321.22 ^{Ns}	6905.43*
Error	4	611.88	3460.33	285.05	1017.74
Coefficient of variation %	-	6.71	6.83	23.33	18.99

Ns, *, **: Non-significant, significant at the 5% and 1% probability, respectively level.

Table 3

Mean comparisons of vinblastin and vincristine alkaloids in the roots and vincristine yield per plant of *C. roseus* as influenced by different bacteria

No.	Bacterial inoculation	Vinblastine (µg/g)	Vincristine (µg/g)	Vincristine yield per plant
1	Control	339.28 c	649.72 c	142.10 b
2	Combined inoculation	189.85 d	529.07 c	111.73 b
3	<i>P.f</i>	513.22 a	1238.71 a	248.74 a
4	<i>A.b</i>	432.03 b	1026.78 b	169.42 ab

The means with different letters have a significant difference with each other at the 5% probability level.
P.F. *P. fluorescens* and *A.b.* *A. brasilense*

Discussion

Given the molecular results, inoculation of two bacteria treatments individually enhanced the expression of the interested genes in the roots of *C. roseus*. Among them, *P. fluorescens* had a positive and significant effect on the expression of genes involved in first step (G10H) and the last step (T16H, DAT and CrPRX) of the biosynthetic pathway. Previously, up-regulation of TDC genes had been reported in the roots of *C. roseus* treated with this elicitor (Ahmadzadeh *et al.*, in press). Li *et al.* (2013) found the significant changes in the expression level of genes involved in the TIA biosynthesis, such as Tdc and G10H, by overexpressing the transcription factor ORCA2 in the hairy roots of *C. roseus*. In other words, these gene have a common transcription factor. Liu *et al.* (2017) showed that the transcription factors ORCA2 and ORCA3 regulate the expression of G10H and TDC genes. These transcriptional factors are activated in response to methyl jasmonate and jasmonic acid. However, in the present study, they probably were activated under the bacterial treatments, especially *P. fluorescens*, in the roots. Researches revealed that the G10H promoter contains unique binding sites of several transcriptional factors, suggesting that the G10H promoter may be regulated by a different transcriptional cascade (Suttipanta *et al.* 2007).

In the vindoline biosynthesis pathway, the two genes T16H and DAT involved in first and last steps respectively (Shabani *et al.*, 2014) were elected for investigation. Owing to the production of vindoline with organ-dependent manner in green tissues, the expression of genes is not predicted through this branch of TIA biosynthesis pathway in root tissue under normal conditions (Dutta *et al.*, 2005). However, molecular analysis demonstrated the expression of both genes in the roots under all treatments. The expression of some genes in this pathway, including the D4h in root tissue, has already been reported by Dutta *et al.* (2005). In order to prove the correspondence of observed hybridization signals with the real expression of the D4H gene in the root, they sequenced RT-PCR products and finally obtained more than 99% homology by blasting with sequences in the data bank (GenBank Acc. No. 004847).

In this study, expression of T16H and DAT genes in the root was affected by *P. fluorescens* and combine inoculation treatments. Also, the response of these two genes to all used treatments was almost the same. Thus, the treatments that increased the expression of T16H gene also increased the expression of DAT gene in the root. This result was not unexpected. This is because these two genes are in a branch of the biosynthetic pathway and it is possible that they have a common promoter to regulate expression or are activated by a common transcription factor. The possibility of co-response of some biosynthetic pathway genes of TIA to elicitors has also been mentioned in other reports. The expression of several genes involved in the biosynthetic pathway of indole alkaloids, including Str and Tdc, is coordinately induced by fungal elicitors such as yeast extract (Pauw *et al.* 2004).

The CrPRX gene is the final gene of the TIA biosynthetic pathway and encodes the enzyme anhydrovinblastine synthase. The product of this gene combines the two substances catharanthine and vindoline and it causes the formation of vinblastine and eventually vincristine (Goklani *et al.*, 2009). CrPRX gene expression in root in response to all treatments was significantly increased compared to the control, but the most effective treatment was *P. fluorescens*. In the study of Wang *et al.*, 2016, the transcript level of CrPRX in *C. roseus* increased in response to different concentrations of ethephon. Maximal amounts of CrPRX transcripts were detected in seedlings treated by 100 μ M ethephon. Since the studied rhizobacteria increase the susceptibility of the plant to

ethylene (Beneduzi *et al.*, 2012), it may be possible to influence the expression of TIA biosynthetic pathway genes in this way.

Overall, *P. fluorescens* was effective than other treatments in root and caused the significant up-regulation of studied genes, particularly last step gene, CrPRX, compared to the control plant. In sequent, *A. brasilense* treatment was able to increase the expression of G10H and CrPRX, significantly.

van der Fits and Memelinc, (2000) showed that methyl jasmonate (MJ) treatment stimulated TIA metabolism in *C. roseus* cell suspension and increased the expression of all genes implicated in the TIA biosynthetic pathway. In addition, the transcription factor ORCA3 was activated in response to MJ, which in turn regulated the expression of some other genes in this pathway, including the STR. Suttipanta (2011) studied the transcription factor WRKY in the *C. roseus* plant, which is expressed predominantly in roots and also in response to phytohormones such as jasmonate, gibberellin, and ethylene. They demonstrated that a high expression of transcription factor CrWRKY2 in response to methyl jasmonate in the hair roots culture of *C. roseus* up-regulates several TIA pathway genes. Besides, it promoted the expression level of the transcription factor activating ORCA3 and the inhibitor of ZCT. Simultaneous induction of activators and inhibitors may be necessary to activate or repress some genes in response to elicitors (Shabani *et al.*, 2014). It is noteworthy that some transcription factors are tissue specific, and some treatments are involved in the simultaneous induction of transcription factors of either activators or inhibitors. According to Pattra *et al.* (2018), inhibitors such as JAZs and RMT1 mediate the interaction of CrMYC2 and BIS regulators, as well as balance the metabolic flux of TIA. Zhang *et al.* (2011) showed that application of methyl jasmonate increases the expression of Tdc, G10H, Str, etc., genes in the biosynthetic pathway of TIA in the *C. roseus*. Another study revealed that ethylene treatment has a positive effect on *C. roseus* alkaloids at transcriptional and metabolic levels (Wang *et al.*, 2016). Also, Shabani *et al.* (2014) found that some genes in the TIA pathway, such as the G10H gene, play critical role in responses to ethylene. Papon *et al.* (2005) reported high expression of this gene in response to cytokine and ethylene. Similarly, in this study, inoculation treatments individually had positive and significant effects on G10H in roots. In agreement with these results and previous research, it can be suggested that bacterial inoculation treatments by producing cytokinin hormone or increasing plant susceptibility to ethylene in the root, have up-regulated the G10H gene in conjunction with other genes studied.

Given the results, it could be inferred that exposure of *C. roseus* to bacterial inoculation treatment probably induced the production of hormones such as gibberellin and cytokinin, as well as activated the jasmonic acid and ethylene responses. These increased the expression level of transcription factor CrWRKT2, subsequently activators ORCA3 and ORCA2 in conjunction with some inhibitors, which in turn influenced the expression level of genes involved in the TIA biosynthetic pathway. Considering the positive correlation between relative expression level of these gene and the accumulation of corresponding alkaloids in *C. roseus* (Dutta *et al.*, 2005; Goklani *et al.*, 2009; Jaggi *et al.*, 2011 and Wang *et al.* 2016), it can be concluded that up-regulation of G10H, DAT, T16H and CrPRX genes under these treatments, especially by *P. fluorescens*, can result in a higher production rate of vinblastine and vincristine alkaloids as final products in the TIA biosynthetic pathway. As an example, it is shown that an increase in the expression level of DAT has been found to result in the accumulation of vinblastine and vincristine alkaloids (Wang *et al.*, 2012 and Khataee *et al.*, 2019). In this case, metabolite studies were performed in the plant.

Root inoculation with plant probiotic bacteria promoted significant increase in growth and alkaloid content (Karthikeyan *et al.*, 2010). Some studies have shown that different types of probiotic bacteria have a positive effect on alkaloid content in the root of *C. roseus* (Jaleel *et al.*, 2007a; Jaleel *et al.*, 2009; Karthikeyan *et al.*, 2010). Jaleel *et al.* (2007b) studied the effect of *P. fluorescens* along with drought stress on vegetative traits, and ajmalicine content in roots of *C. roseus* plant. They suggested that ajmalicine content increased significantly due to exposure of drought-treated plants with *P. fluorescens* compared to non-treated plants and control under drought stress. Experimentally, Jaleel *et al.* (2009) showed that supplementing plant growth regulators as the same as *P. fluorescens* elicitor significantly changed the constituents of metabolites (ajmalycin, serpentine, catarantine, and vindoline) in the roots of *C. roseus*. Karthikeyan *et al.* (2009) also found, by inoculating *C. roseus* seeds and seedlings with *P. fluorescens* and *A. brasilense* separately or in combination that bacteria can be used as a suitable agent to increase alkaloids in *C. roseus* roots. Also, the effect of these beneficial bacterial strains has been demonstrated on alkaloid contents of *Hyoscyamus niger* L. and increasing the hyoscyamine and scopolamine yield in roots and shoots (Ghorbanpour *et al.*, 2013).

To comparative analysis of molecular and metabolite results, all results were presented in Table 4. As seen in this table, *P. fluorescens* by increasing the expression of all studied genes in the biosynthetic pathway and *A. brasilense* by increasing the expression of gene at the beginning (G10H) and end of the pathway (CrPRX) were able to significantly increase the levels of both vinblastine and vincristine alkaloids compared to the control.

Table 4

Comparative analysis of molecular and metabolite evaluations in the roots of *C. roseus* inoculated with bacteria relative to control

Bacterial inoculation treatments	Genes expression relative to control				Alkaloids amount relative to control	
	Downstream TIA pathway genes			Terminal gene	Vinblastine (µg/g)	Vincristine (µg/g)
Upstream TIA pathway gene	Vindoline pathway			Terminal gene	(µg/g)	(µg/g)
G10H	T16H	DAT	CrPRX			
Combined inoculation	Increase ^{Ns}	Increase [*]	Increase [*]	Increase [*]	Decrease [*]	Unchanged
<i>P. fluorescens</i>	Increase ^{**}	Increase ^{**}	Increase ^{**}	Increase ^{**}	Increase [*]	Increase [*]
<i>A. brasilense</i>	Increase ^{**}	Increase ^{Ns}	Increase ^{Ns}	Increase ^{**}	Increase [*]	Increase [*]
Gene expression was measured relative to the control sample (no bacterial inoculation).						
Ns, *, **: Non-significant, significant at the level of 5% and 1% probability, respectively.						
<i>P.F.</i> : <i>P. fluorescens</i> and <i>A.b.</i> : <i>A. brasilense</i>						
The means with different letters have a significant difference with each other at the level of 5% probability.						

Conclusions

In the present study, *P. fluorescens* drastically increased the content of two alkaloids vinblastine and vincristine, compared to the control and other bacterial treatments in the roots of *C. roseus*. A review of the results of molecular analysis showed that the bacterium significantly increased the expression of more genes in the TIA biosynthetic pathway compared to the control. Therefore, the positive effect of this treatment on the amount of evaluated alkaloids indicates the compatibility of the results of transcription and metabolic. The same result was observed for *A. brasilense* (Table 5). From the results of this investigation, it can be concluded that, the seed priming and seedling treatments of plant probiotic bacteria can be used as a good tool in the enhancement of alkaloid contents in medicinal plants, as it provides an eco-friendly approach.

Considering the positive effect of bacteria on two important alkaloids of *C. roseus*, the results of this research can be considered an important step in the pharmaceutical industry.

Abbreviations

CFU colony forming units

CrPRX *Catharanthus roseus* peroxidase

DAT Deacetylvindoline-4-O-acetyltransferase

D4H Desacetoxyvindoline-4-hydroxylase

G10H Geraniol-10-hydroxylase

HPLC High-performance liquid chromatography

PBB Plant probiotic bacteria

PGPR Plant growth promoting rhizobacteria

qRT-PCR Quantitative reverse transcription PCR

RSP9 40 s ribosomal protein S9

TIAs Terpenoid indole alkaloids

Declarations

Authors' Contributions

Maryam Ahmadzdeh conducted the experiments and drafted the manuscript; Amir Hossein Keshtkar conceived the idea, performed the statistical analysis and edited the manuscript; Kobra Moslemkhany and Masoud Ahmadzadeh helped in the design of some experiments and assisted in interpreting the data; all authors read and approved the final manuscript.

Compliance with ethical standards

Conflicts of interest

The authors report no conflicts of interest.

Ethical approval

All procedures performed in this study did not involve human or animal participants.

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Figures

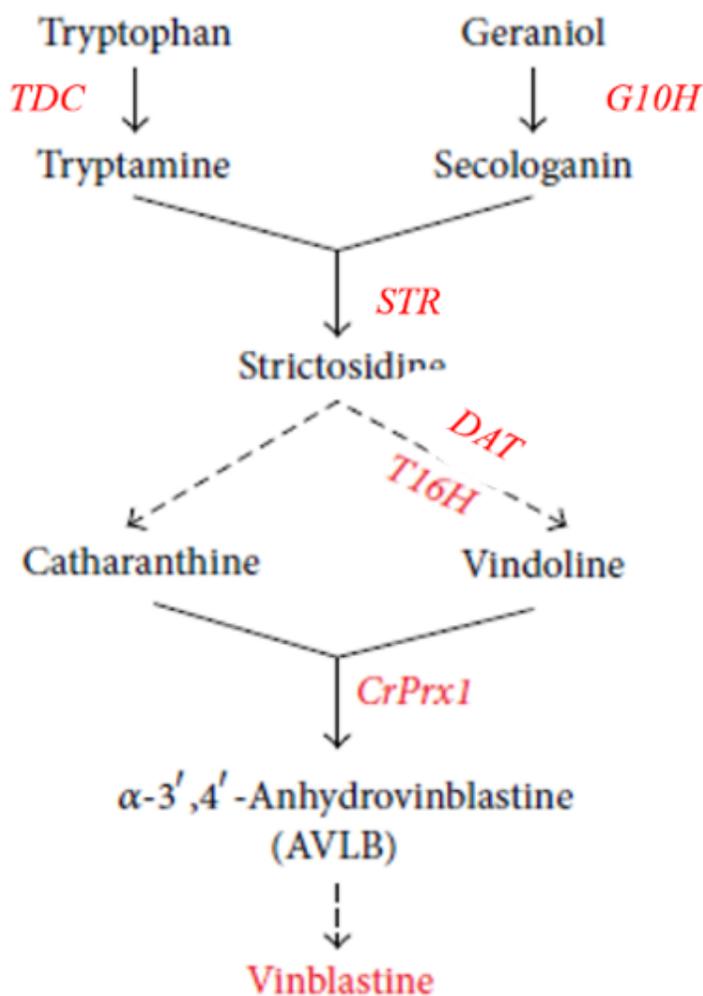


Figure 1

The pathway of terpenoid indole alkaloids biosynthesis in *Catharanthus roseus*. (Taken from Wang et al., 2016)

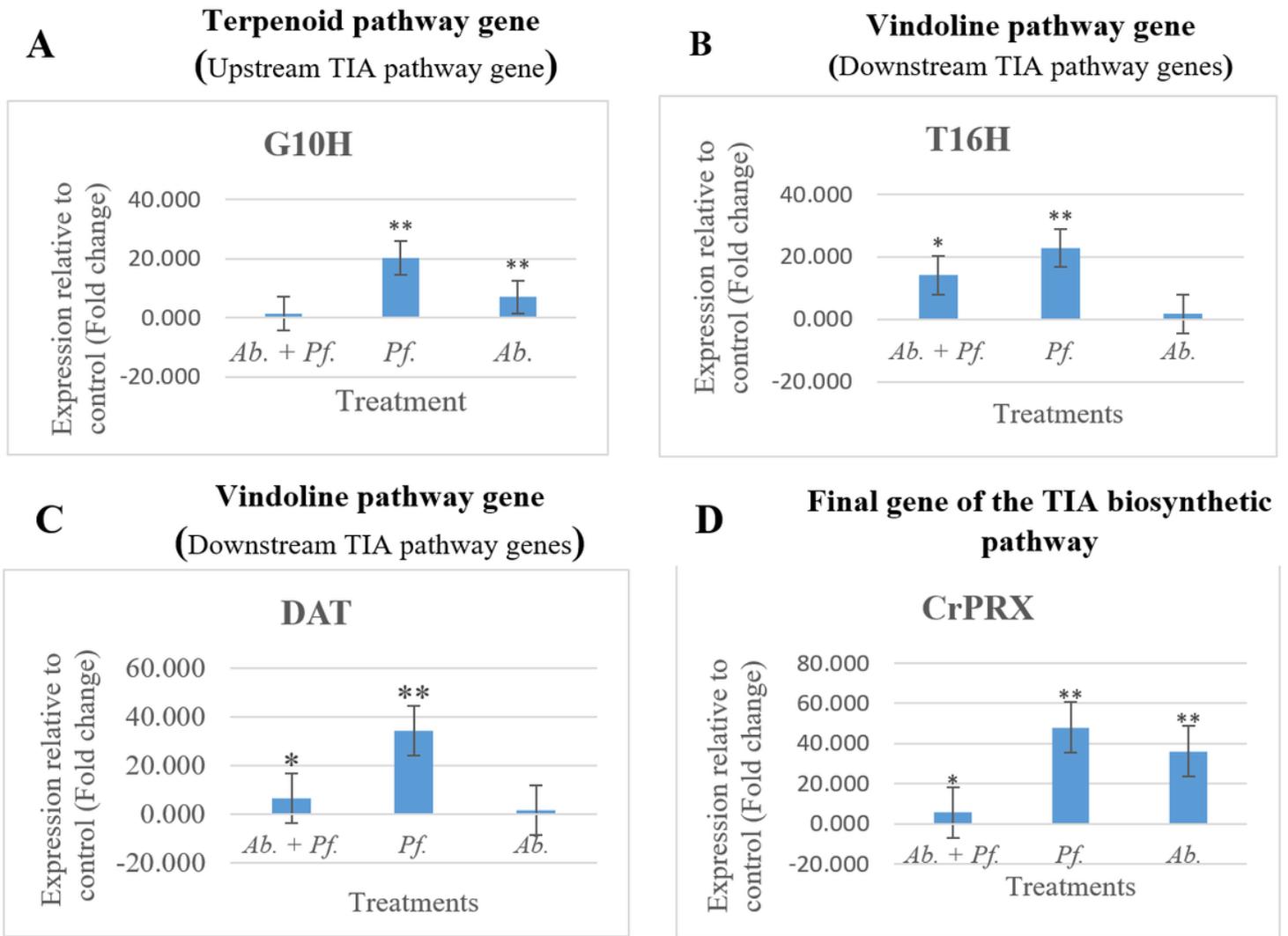


Figure 2

qRT-PCR expression analysis of G10H, T16H, DAT and CrPRX genes following treatment of *C. roseus* root with beneficial bacteria at three different concentrations. (PF: *P. fluorescens* and A.b: *A. brasilense*)

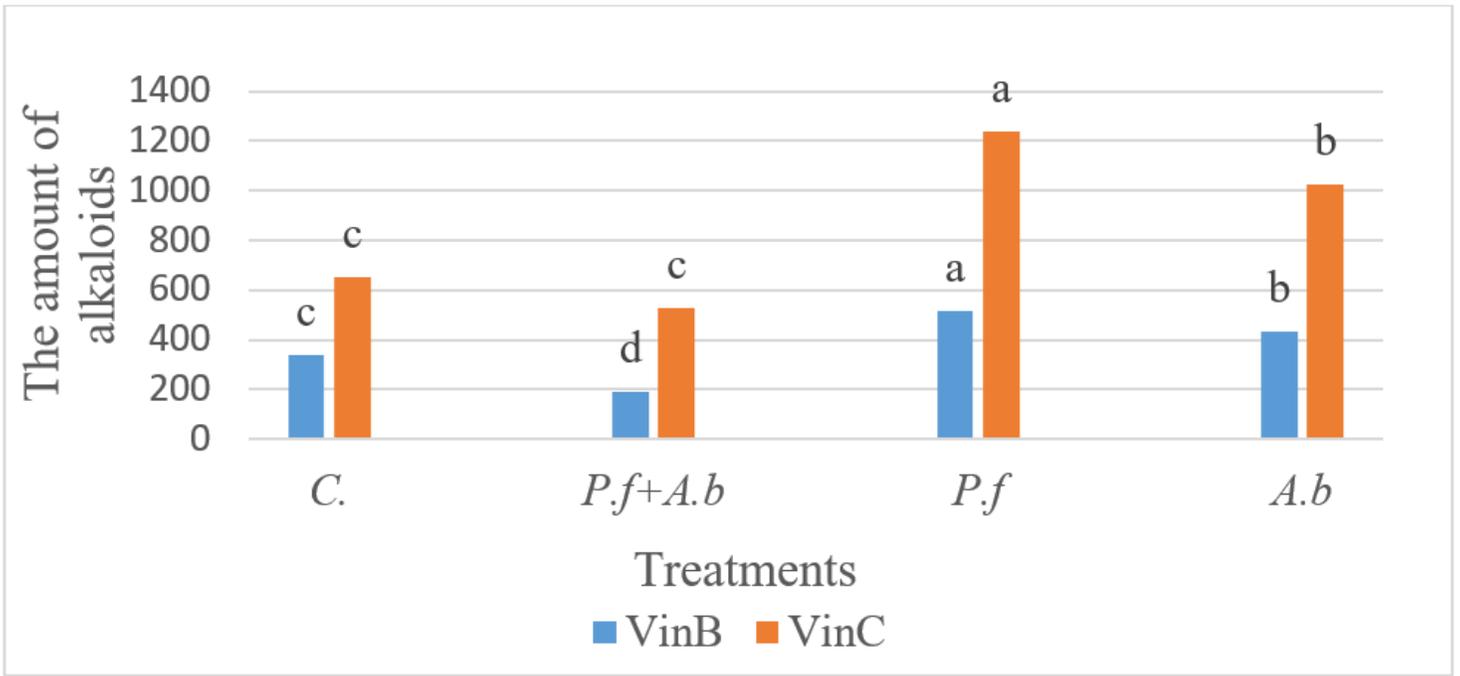


Figure 3

Comparison of the mean effects of bacterial inoculation treatments on the amount of two alkaloids vinblastine and vincristine in the roots of *C. roseus*. The same letters in each column indicate a significant difference in the 5% probability level. P.F: *P. fluorescens* and A.b: *A. brasilense*