Effects of PEG-induced drought stress on regulation of indole alkaloid biosynthesis in *Catharanthus roseus*

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**ABSTRACT**

*Catharanthus roseus* (C. roseus) plants were used to investigate the terpenoid indole alkaloids (TIAs) accumulation under the condition of PEG-induced drought stress. Multivariate analysis showed that 35% PEG6000 could induce more obvious and stable accumulation on proline (PRO) content and the relative water content (RWC). The results indicated that there were gradual increase and then decrease (p < .05) in the contents of vindoline (VIN) and catharanthine (CAT) under 35% PEG6000 stress, but the content of vinblastine (VBL) increased gradually. In addition, the expression levels of tryptophan decarboxylase (TDC), strictosidine synthase (STR) and deacetylvindoline-4-O-acetyltransferase (DAT) were upregulated in plants under 35% PEG6000 stress. Further correlation analysis indicated that CAT accumulation was significantly correlated with TDC gene expression, and VBL accumulation was significantly correlated with peroxidase (p < .05). Our results suggest that the cultivation of *C. roseus* in drought stress would serve as effective treatment for accumulating TIAs.

**Introduction**

*Catharanthus roseus* (C. roseus) is a highly exploited perennial herbaceous flowering medicinal plant for its many valuable terpenoid indole alkaloids (TIAs) (Ganapathi & Kargi 1990) such as anti-cancer bisindole alkaloid vinblastine (VBL) and vincristine (VCR) (Sreevalli et al. 2004; Verma et al. 2007). Currently, chemical synthesis and metabolic engineering plant cell or tissue culture (Whitmer et al. 1998; Hughes & Shanks 2002) are alternative methods for the synthesis and production of TIAs. However, its low productivity and high cost (Rischer et al. 2006; Mujib et al. 2014) still hamper the development of industrial-scale production. Therefore, it is urgent to study the physiological basis of *C. roseus* to allow more sustainable indole alkaloids’ production.

Drought, one of the greatest abiotic stresses, can be characterized as the absence of adequate moisture for normal plant growth, which has been reported to become an increasingly serious environmental issue that almost influences the growth and production of all crop production (Ludlow & Muchow 1990; Zhu 2002). When plants are exposed to drought condition, the ability of water stress tolerance is shown to be altered (Atkinson & Urwin 2012; Prasch & Sonnewald 2013) and often varies from species to species (Chaitanya et al. 2003). Previous studies have demonstrated that osmotic adjustment and compatible solutes’ accumulation are two typical responses for all plants to contribute stress tolerance (Hare et al. 1998; Reddy et al. 2004). Proline (PRO) accumulation enabled the plant to maintain low water potentials, thus increasing compatible osmolytes, which could help buffer the immediate effect of water shortages within the organism (Sairam et al. 2002; Kumar et al. 2003). In addition to this, antioxidant mechanisms also help plants equipped with antioxidant molecules, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), to survive under stress conditions (Procházková et al. 2001). For the past several years, many efforts to have focused on testing the *C. roseus*’ drought stress tolerance capacity (Qureshi et al. 2005; Cui et al. 2016), but little information is obtained about the physiological basis and molecular mechanisms underlying the PRO and POD metabolism under drought stress in *C. roseus*.

It seems necessary to conduct research related to *C. roseus* in drought stress induction to help provide valuable data to improve total TIAs’ accumulation. In the present study, the effect of drought stress on the dynamic accumulation of indole alkaloids and the induction of TIA biosynthetic pathway genes has been determined. The potential of these pathway genes for use in screening alkaloids accumulation has been investigated.

**Materials and methods**

**Plant materials and reagents**

The seeds of *C. roseus* (L.) G. Don. (family: Apocynaceae) were provided from the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeastern Forest University. Standard samples of vindoline (VIN) and catharanthine (CAT) and vinblastine (VBL) were purchased from Merck (Sigma) company. Chromatography column of diamonsil TMC18 ODS (4.6 mm × 250 mm) was purchased from Dikma Company. RNA Extraction Kit (RNAiso reagent) was obtained from Bao Biotechnology (Dalian) Company. Reverse Transcription Kit and PCR reagents were from Takara (Dalian) Company.

**Growth condition and drought stress induction**

The seeds of *C. roseus* were surface sterilized with 0.2% HgCl₂ solution for 5 min and then washed many times to remove...
HgCl₂ with deionized water. The seeds were cultivated on Petri dishes containing MS (Murashige and Skoog) basal medium to germinate, and then transplanted into soil after 2 weeks. The seedlings were cultivated in the green house in 80% relative humidity at 25–28°C air temperature under the natural light illumination. All the seedlings were watered with ground water up to 45 days after sowing (DAS). On 45 DAS, plants were uprooted randomly, washed carefully and separated into root, stem and leaf for the following analyses.

Plants of similar size were treated with different concentration of PEG6000 (15%, 20%, 25%, 30%, 35%, 40%, V/V) in half strength Hoagland nutrient solution, under the same cultural conditions as used for control at corresponding different time points (0, 3, 6, 12, 24, 48 h), respectively.

### Analysis of proline, relative water content (RWC) and POD activity

The content of proline (PRO) and RWC in the leaves were determined followed the ninhydrin colorimetric method and saturated water method, respectively. The POD activity in the leaves was estimated by the Guaiacol method followed the ninhydrin colorimetric method with a photodiode array detector using Waters 2695 Alliance HPLC system (Waters, Milford, MA, USA) equipped with a reverse phase HPLC and diamonsil TMC18 ODS column (4.6 × 250 mm, Dikon) with a flow rate of 1.5 mL min⁻¹ at 40°C. Gradient elution was employed for qualitative and quantitative analyses using mobile phase of solution A and solution B with the ratio of 64 to 36 in Volume. The mobile phase A was the mixture of deionized water and diethylamine with the ratio of V (H₂O) to V (diethylamine) as 990 to 10 (pH 7.3 with phosphoric acid). The mobile phase B was methanol. Spectra were measured at a wavelength of 220 nm, and peaks were determined by comparing the retention time and UV spectra with those of the standards. For quantification, a calibration curve was constructed using the standard solutions diluted in methanol at a serial of different concentrations.

### Determination of alkaloids content

The analysis of alkaloids including the content of VIN, CAT and VBL in leaves at different time (0, 3, 6, 12, 24, 48 h, respectively) was performed by high-performance liquid chromatography (HPLC) (Jasco, VG, England). The methanol extracts were dried in a rotary evaporator at 50°C. The residue was dissolved in 5 mL of methanol, and then filtered through a 0.45-μm filter (Millipore, Bullerica, MA, USA) for HPLC analysis. Several standard VIN, CAT and VBL samples were used as control. HPLC analysis was performed on a Waters 2695 Alliance HPLC system (Waters, Milford, MA, USA) equipped with a photodiode array detector using reversed phase HPLC and diamonsil TMC18 ODS column.

### Statistical analysis

All experiments were conducted with three replicates. Statistical analysis was performed using analysis of variance with SPSS software, and double factors with repetition followed by T Duncan method. The values are mean ± SD. p Values ≤ 0.05 were considered as significant.

## Table 1. Effect of different concentration of PEG6000 treatments on proline and relative water content.

| Time/h | ck      | 15%     | 20%     | 25%     | 30%     | 35%     | 40%     |
|--------|---------|---------|---------|---------|---------|---------|---------|
| PRO    | 0.83 ± 0.01a | 0.83 ± 0.01a | 0.83 ± 0.01a | 0.83 ± 0.01a | 0.83 ± 0.01a | 0.83 ± 0.01a | 0.83 ± 0.01a |
| RWC    | 2.03 ± 0.02a | 2.03 ± 0.02a | 2.03 ± 0.02a | 2.03 ± 0.02a | 2.03 ± 0.02a | 2.03 ± 0.02a | 2.03 ± 0.02a |

Note: Bold values represent 35% concentration of PEG6000 which could induce more obvious and stable effects on the content of PRO and RWC, thus was selected for subsequent experiments.
Results

Effect of PEG drought stress on the content of PRO and RWC

It has been reported that PRO, a compatible solute, could accumulate under abiotic stress in plants (Reddy et al. 2004). To confirm the C. roseus has been induced drought stress, the PRO content was determined in C. roseus after different concentrations of PEG6000 treatment. As shown in Figure 1 (A), an increase in PRO accumulation in C. roseus under drought stress induced by PEG6000 of different concentrations. In particular, the content of PRO largely increased under PEG6000 treatment at a concentration of 30%, 35% and 40%. Notably, 40% PEG6000 leaded to a significant increase in the content of PRO at 24 h, but slight decrease at 12 h. It has been widely accepted that high levels of PRO make plant maintain low water potentials. Therefore, we further determined the RWC in C. roseus leaves after different concentration of PEG6000 treatment. As expected, RWC in leaves was decreased under the treatment of PEG6000 at different concentrations, especially, when treated with 35% PEG6000, which caused a sharp decrease in the RWC in the C. roseus leaves (Figure 1(B)). In addition, multiplex analysis of variance with double variables revealed that the content of PRO and RWC in C. roseus leaves was more significant with the increase in PEG6000 concentration (Table 1). Compared with 40% PEG6000, 35% concentration of PEG6000 could induce more obvious and stable effects on the content of PRO and RWC, thus was selected for subsequent experiments.

Effect of PEG drought stress on the POD activity

To investigate the effect of PEG6000-induced drought stress on in vivo metabolism of C. roseus, we determined the POD activity under 35% PEG6000 treatment. As shown in Figure 2, the POD activity presented an upward trend after 6 h, 24 h highest, but gradual decline at 48 h, which might be ascribed to physiological metabolism disorders by severe drought stresses.

Effect of PEG-induced drought stress on TIAs accumulation

As shown in Figure 3, the alkaloids contents displayed a dynamic change in the leaves of C. roseus in the control. VIN content displayed a decline-rise trend, and CAT content gradually decreased, while VBL content increased gradually. In the 35% PEG6000 treatment, VIN content gradually increased with the extension of osmotic stress time, and reached the peak as 12.9 mg g⁻¹ FW ± 0.46 at 6 h. CAT

Figure 1. The effect of proline content (A) and relative water content (B) in C. roseus leaves under different concentration of PEG6000 stresses.

Figure 2. Effects of 35% PEG6000 on POD activity of Catharanthus roseus leaves.

Figure 3. Levels of indole alkaloid in Catharanthus roseus leaves after 35% PEG6000 at different time points (0 h, 3 h, 6 h, 12 h and 24 h, respectively) treatment.
content increased gradually and was significantly higher than that of control \((p < .05)\) at 12 h treatment. The content of VIN and CAT decreased after 24 h treatment. VBL content was significantly higher than that of control \((p < .05)\) between 12 and 24 h treatment \((p < .01)\), and VBL content reached the peak as 0.17 mg g\(^{-1}\) FW ± 0.0036, which was 1.4 times higher than that of control. VBL content gradually decreased after 24 h treatment.

Effect of PEG drought stress on the regulatory genes related with indole alkaloid biosynthesis

The accumulation of tryptophan decarboxylase (TDC), strictosidine synthase (STR) and deacetylvindoline-4-O-acetyltransferase (DAT), which are three key enzymes involved in the alkaloid biosynthesis pathway, provides further insights into the induction of the TIAS. As shown in Figure 4, the relative mRNA level of TDC, STR and DAT were significantly higher than that of control in the early 3 h of PEG-induced drought stress, while in the late stage of PEG drought, the expression of STR and DAT remained higher than that of control, while the expression of TDC was comparable to that of control.

Correlation analysis

Correlation study (Table 3) revealed that the expression of TDC gene was positively correlated with the content of CAT \((p < .05)\), but no significant correlation with the content of VIN or VBL. The POD activity positively correlated with the content of VBL \((p < .05)\).

Discussion

Drought often has devastating effects on crop production, characterized as the absence of adequate moisture (Zhu 2002). In most plants, there is an increased accumulation of PRO content in their tissues in response to stress, which will help us to tolerate stress by supplying energy for growth and survival (Yadav et al. 2005; Chandrashekar and Sandhyarani 1996). However, the underlying mechanisms of drought tolerance have remained unclear. Therefore, we aimed to investigate the effects of drought stress on PRO metabolism and indole alkaloid accumulation.

Consistently, the present study discovered that 35% PEG6000 could cause a significant increase in PRO and decrease in RWC in C. roseus. Furthermore, drought stress at early stage induced the increase in POD activity in a short period of time. Numerous studies have shown that plants are able to protect against abiotic stress by increasing the activities of antioxidant enzymes POD (Gill & Tuteja 2010; Li et al. 2011). But with the prolonged stress stimulation, the enzymatic activity of POD decreased, which might be ascribed to physiological metabolism disorders by severe drought stresses. Interestingly, we found the increased levels of POD were closely associated with accumulation of TIAS, including VIN, VBL and CAT under the early drought stress. Previous reports showed that the abiotic stresses can increase the alkaloid content in plants by treatment of salinity, various chemicals and bioregulators (Zhao et al. 2001; Karadge & Gaikwad 2003; Misra & Gupta 2006).

To further uncover the molecular mechanisms underlying the TIAS accumulation in C. roseus, we analyzed the expression of TIA biosynthesis genes. The results indicated that the expression levels of DAT, TDC and STR were significantly upregulated under early drought stress in C. roseus. Among all, TDC is a key enzyme for catalyzing the reaction of decarboxylation of tryptophan to production of tryptamine (Rizvi et al. 2016). Further correlation study revealed that the expression level of TDC gene was positively correlated with the content of CAT, suggesting that TDC plays an important role in TIAS biosynthesis. STR is identified as VIN-biosynthetic gene and one of the bottlenecks involved in the biosynthesis of TIAS (Pandey et al. 2016). The expression of TDC and STR affected the accumulation of CAT content (Ramani & Chelliah 2007). In addition, DAT is the key enzyme catalyzing the last step of the reaction of the biosynthesis of VIN from tabersonine (Magnotta et al. 2007). POD has been proved to involve in the coupling process of VIN and CAT to synthesize VBL (Wang et al. 2008), which further verify our result that the POD activity positively correlated with the content of VBL.

Table 3. Correlation analyses among alkaloids content, gene expression and POD activity under PEG6000-induced drought stress.

| Alkaloids | Str\(_{\text{ck}}\) | Str\(_{\text{tr}}\) | Tdc\(_{\text{ck}}\) | Tdc\(_{\text{tr}}\) | Dat\(_{\text{ck}}\) | Dat\(_{\text{tr}}\) | POD\(_{\text{ck}}\) | POD\(_{\text{tr}}\) |
|-----------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| VIN       | –              | –              | 0.670          | 0.399          | 0.013*         | 0.347          | –              | –              |
| CAT       | 0.271          | 0.380          | 0.196          | 0.013*         | 0.182          | –              | –              | –              |
| VBL       | –              | –              | –              | –              | –              | 0.462          | 0.005**        | –              |

\(^*p < .05\) show significant differences at 0.05 level.
Conclusions

In summary, the increase in VBL content in *C. roseus* leaves under PEG6000 drought stress was found to be a consequence of metabolic pathways alteration. Notably, the above results demonstrate that the high expression of pathway genes accelerates the accumulation of VBL in the leaves under PEG6000 drought stress, which may have practical significance in the production improvement of VBL. Furthermore, we could provide a rapid method for estimation of periwinkle alkaloids by the analysis of the expression of TIA biosynthetic pathway genes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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