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Full Length Research Paper

Effect of plant growth regulator combinations on the biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus*

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The effects of plant growth regulator combinations on the contents of vindoline, catharanthine and vinblastine were investigated by short-term spray on an ornamental and medicinal plant *Catharanthus roseus* (L.) during the blooming period for commercial use. The combination groups such as ethylene (0.1 mM) + chlormequat chloride (0.1 mM), salicylic acid (0.1 mM) + chlormequat chloride (0.1 mM) and salicylic acid (0.1 mM) + ethylene (0.1 mM) + chlormequat chloride (0.1 mM) resulted in a significant increase of the three alkaloids contents. The combination groups salicylic acid (0.1 mM) + ethylene (0.1 mM) + chlormequat chloride (0.01 mM) and salicylic acid (0.1 mM) + ethylene (0.1mM) + chlormequat chloride (1 mM), affected vindoline and catharanthine contents but had no effect on vinblastine. Among the combination treatments, salicylic acid (0.1 mM) + ethylene (0.1 mM), ethylene (0.1 mM) + chlormequat chloride (0.1 mM) and salicylic acid (0.1 mM) + ethylene (0.1 mM) + chlormequat chloride (0.1 mM) could significantly increase the vinblastine content by 209% at 48 h, 246% at 48 h and 213% at 24 h respectively. Thus compared to the single PGRs treatments, the combination treatments increase alkaloids accumulation more effectively. The result may provide a method for increasing TIAs in *C. roseus* for industrialized production.

Key words: *Catharanthus roseus*, plant growth regulator combination, vindoline, catharanthine, vinblastine, blooming period.

INTRODUCTION

Catharanthus roseus (L.) G. Don. (Madagascar periwinkle, Apocynaceae), which produces a very large number of pharmaceutically valuable terpenoid indole alkaloids (TIAs) is widely studied as a model medicinal plant. The main alkaloids present in *C. roseus* are vincristine, vinblastine and ajmalicine (Abdul et al., 2006). Among them bisindole alkaloids, vinblastine and vincristine, were the powerful antitumor drugs (El-Sayed and Verpoorte, 2007; Zhao and Verpoorte, 2007).These two important bisindole alkaloids are synthesized by monomeric alkaloids catharanthine and vindoline present in the vacuole of *C. roseus* leaves and stem cells (Roytrakul and Verpoorte, 2007). These anticancer products had a high price ranging from $ 1 million to $ 3.5 million per kilogram many years ago and a huge demand in the world market, so it became an interested area of widespread research (Verpoorte et al., 1991).

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Abbreviations: **PGRs,** Plant growth regulators; **TIAs,** terpenoid indole alkaloids; **MeJA,** methyljasmonate; **SA,** salicylic acid; **CCC,** chlormequat chloride; **AVLBS,** 3', 4'-anhydrovinblastine synthase (EC 4.1.3.27); **ANOVA,** analysis of variance.

In *C. roseus* leaves cells, vindoline which synthesized in the cytosol was transferred to the vacuole where a basic peroxidase-like enzyme 3', 4'-anhydrovinblastine synthase (AVLBS) couples vindoline to catharanthine (Sottomayor et al., 1998), and this coupling process results into a 3', 4'-anhydrovinblastine, the direct biosynthetic precursor of anticancer products is then converted into vinblastine, then further derived into vincristine (Verpoorte et al., 1997). As these studies clearly demonstrated the importance of the two monomeric alkaloids, vindoline and catharanthine, these compounds became prime targets in recent studies. Vindoline is synthesized from tabersonine with five steps while the information about catharanthine biosynthesis from stemmadenine is obscure (El-Sayed and Verpoorte, 2007; Zhou et al., 2009). Vindoline is restricted to the aerial part of the plant because the underground part lacked part of biosynthesis pathway of vindoline, for this reason there was not vinblastine biosynthesized underground though catharanthine is distributed equally throughout the plant (Balsevich et al., 1989; Deus-Neumann et al., 1987; Westekemper et al., 1980).

Several decades ago, many researchers aimed to produce the two important bisindole alkaloids vincristine and vinblastine by cell suspension culture and induction of hairy roots on account of their trace amounts in *C. roseus* plants. It was found that *C. roseus* cell cultures also lacked part of biosynthesis pathway of vindoline and failed to form these valuable and complex compounds for commercial use (Zhao and Verpoorte, 2007) because there was spatial isolation of vindoline biosynthetic steps in *Catharanthus roseus* (Guirimand, 2011). Thus the recent research hotspots shifted from cell engineering to the metabolic regulation of these alkaloids biosynthetic pathway by genetic engineering method (Ayora-Talavera et al., 2002; Peebles et al., 2005; Peebles et al., 2009; Wang et al., 2010; Zhou et al., 2010). But there were also some difficulties to enhance TIAs in *C. roseus* by genetic engineering pathway, firstly the transgenic hairy root and cell also lacked vindoline to produce vinblastine, secondly the method of regeneration plants by transgenic was not mature at present and the time was very long by this method. So it is urgently to find a rapid and simple method to increase TIAs content in *C. roseus* for commercial use.

The effects of plant growth regulators (PGRs) on the contents of *C. roseus* terpenoid indole alkaloids had been extensively studied (El-Sayed and Verpoorte, 2007; Decendit et al., 1992; Pan et al., 2010; Pasquali et al., 1992; Roytrakul and Verpoorte, 2007; Zhao and Verpoorte, 2007). PGRs such as methyl jasmonate (MeJA) and jasmonate (Aerts et al., 1994; El-Sayed and Verpoorte R 2005; Lee-Parsons et al., 2004; Peebles et al., 2009; Ruiz-May et al., 2008), abscisic acid (ABA), salicylic acid (SA) (Godoy-Hernandez and Loyola-Vargas, 1997; Bulgakov et al., 2002; Mustafa et al., 2009) and gibberellic acid (GA_3) (Amini et al., 2009; Masoud et al., 1968; Srivastava and Srivastava, 2007; Verpoorte et al., 1997), have significant influence on terpenoid indole alkaloids production and enzymes activities of the biosynthesis pathways in *C. roseus* cell suspensions cultures, hairy roots and seedlings (Aerts et al., 1994; Bulgakov et al., 2002; El-Sayed and Verpoorte, 2004; Lee-Parsons et al., 2004; Ruiz-May et al., 2008). For commercial use, *C. roseus* plants will be harvested in three months after germinating when they reach the height of 45 to 55 cm and begin to flower (Pan et al., 2010). To our knowledge, there is only our previous report about effects of PGRs on alkaloids production in *C. roseus* plants during this period (Pan et al., 2010). Previous research about the effects of PGRs on TIAs in *C. roseus* was mostly focused on the production of ajmalicine, serpentine, tabersonine, ajmaline, vindoline and catharanthine (Decendit et al., 1992; Pasquali et al., 1992; Aerts et al., 1994; Godoy-Hernandez and Loyola-Vargas, 1997; Verpoorte et al., 1997; Bulgakov et al., 2002; Lee-Parsons et al., 2004; El-Sayed and Verpoorte, 2005; Peebles et al., 2005; Roytrakul and Verpoorte, 2007; Srivastava and Srivastava, 2007; Zhao and Verpoorte, 2007; Ruiz-May et al., 2008; Amini et al., 2009; Mustafa et al., 2009; Peebles et al., 2009). There are few reports about the effect of PGRs on production of vinblastine (Masoud et al., 1968; Pan et al., 2010).

A short-term pre-harvest PGRs application to mature plants of *C. roseus* during the blooming time was carried out to collect plant biomass with high yields of alkaloids for commercial use in our previous research (Pan et al., 2010). The results showed that SA and ethephon treatments resulted in a significant increase of vinblastine, vindoline and catharanthine. While ABA and GA_3 inhabited the accumulation of these three important alkaloids, MeJA showed no great effect on the production of these valuable alkaloids. CCC highly enhanced the accumulation of vinblastine but greatly decreased the contents of vindoline and catharanthine (Pan et al., 2010). Since this research was only executed with single PGRs to find their respective effect on alkaloids accumulation, while perhaps there are interactions among their mixture used in the field.

To obtain plant materials with the highest yields of alkaloids for commercial use and find the interactions among them, a short-term applying of PGRs combination groups of SA, ethephon and CCC to mature plants of *C. roseus* during the blooming time was performed. This research mainly studied the roles of PGRs combinations in regulating the contents of vinbastine, vidoline and catharanthine. Considering different concentrations of CCC (0.01, 0.1 and 1 mM) had different effects on the three alkaloids accumulation (Pan et al., 2010) in this study, we investigated the effects of the three concentrations of CCC (0.01, 0.1 and 1 mM), together with SA (0.1 mM) and Ethephon (0.1 mM) respectively on the accumulation of TIAs in *C. roseus*. The TIAs included vinblastine, vindoline and catharanthine. This reach may

provide a simple and effective way in commercial use to increase TIAs content in *C. roseus*.

MATERIALS AND METHODS

Plant materials and cultivation methods

C. roseus seeds (Pacifica cherry red) purchased from Pan American Seed Co. (Illinois, USA; website is http://www.-panamseed.com) were germinated in Petri dishes with MS (Murashige and Shoog, 1962) agar solid medium in tissue culture room. After germination, the seedlings were transferred into 60-cell plug tray containing soil and organic manure mixture in greenhouse for 2 weeks at 25 ± 3°C for acclimation. Then, they were shifted to field. When *C. roseus* plants began to bloom with an average height of about 50 cm, short-term treatments of different PGR combinations were applied. The average temperature was 20 to 26°C and relative humidity was 65.3 to 73.1% during the period of the treatments.

PGRs combinations and their treatments

SA, Ethephon and CCC were purchased from Sigma-Aldrich. Each plant growth regulator was dissolved in ethanol to gain stock with a concentration of 0.1 M. The PGRs combinations were consisted of three groups with 0.1 mM SA, 0.1 mM CCC, and 0.1 mM ethephon pair combined and another three groups with 0.1 mM SA + 0.1 mM ethephon paired 0.01 mM CCC, 0.1 mM CCC or 1 mM CCC. The treatments included six PGRs combination groups and a blank without PGRs spraying. The working solution was prepared by diluting the stocks in water. The prepared PGRs combination treatments were sprayed on each part of the plants and the plan was conducted in randomized block design with three replications.

Leaf sample preparation and alkaloids extraction

The leaf samples were collected in the next five afternoons following spraying at interval and the control leaves were collected before spraying. Each sample was collected from each part of the plant. The samples were dried at 45°C for 48 to 60 h and pulverized in a mortar. Pulverized samples (100 mg) were immersed and shaked in 1.5 ml Eppendorf tubes with 1 ml methanol overnight at 4°C. Then the samples were transferred into Ultrasonic aqueous bath (DL-60D) with the power of 80 W for 30 min and centrifuged at 12000 rpm/min for 10 min at room temperature. The precipitated samples which had been shaken were put into Ultrasonic aqueous bath for 30 min and centrifuged once again. Each supernatant was filtered into a new 1.5 ml Eppendorf tube with 4 µm organic filter membrane for HPLC assay. The processed samples were stored at 4°C for later determination.

Quantification of alkaloids by HPLC

For HPLC analysis individual stock solution of standard samples, catharanthine, vindoline and vinblastine (Sigma-Aldrich, USA), was prepared at a concentration of 1 mg•L-1 in methanol and stored at -20°C. The HPLC analysis was performed using a Sapphire-C18 (4.6 mm×250 mm, 5 µm) column at a column temperature of 35°C and Hitachi L-2000 series HPLC system. This system consists of L-2000 Organizer, L-2130 Pump, L-2200 AutoSampler, L-2300 Column Oven and L-2455 Diode Array Detector. The injection volume was 10 µl. The mobile phase (acetonitrile and diethylamine buffer solution: 1: 1) continued at a constant flow rate of 1 ml per minute. The DAD detection wavelength was 220 nm. A mixture standards and alkaloids were detected (Figure 1A). Alkaloids of vindoline, catharanthine and, vinblastine was identified after UV analysis of absorbance chromatograms (Hisiger and Jolicoeur, 2007) (Figure 1B).

Quantification analysis was repeated in triplicate of each line in parallel of catharanthine, vindoline and vinblastine. The alkaloids were quantified by using regression equation of calibration curve.

Statistical analyses

The experiments were conducted by three independent replications. Data were analyzed with one way analysis of variance (ANOVA) and the significant means were separated by the Student's *t* test. The values are mean ± SD for three samples in each group and difference between treatments was considered as significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of Ethephon+SA on alkaloids accumulation of *C. roseus*

Ethephon (0.1mM) + SA (0.1mM) treatment on *C. roseus* plants during the blooming time significantly enhanced the yields of catharanthine and vinblastine (Figure 2A). There was a fast increase in catharanthine content from 0 h to 24 h and then the content slightly decreased. The maximum increase was observed at 72 h by 207% above the control. Significant increase in vinblastine content was observed at about 48 h after spraying, the content reached 209% above the control and there was no significant difference between the treatment and the control at other tested time point (Figure 2A). However, vindoline accumulation had a significant reduction after treating with the PGRs combination at 48 h, reaching 18.4 % of that in the control. As well as the difference of vindoline between the treatment and the control at other tested time point was not significant (Figure 2A).

In previous study, ethephon was exogenously applied in *C. roseus* cell lines and seedlings, resulting in increase of ajmalicine accumulation and higher production of serpentine, tabersonine, catharanthine and vindoline respectively (Yahia et al., 1998; El-Sayed and Verpoorte, 2004). Our previous research found both of Ethephon and SA treatment increased vindoline, catharathine and vinblastine contents in *C. roseus* (Pan et al., 2010). Compared with Ethephon or SA treatment alone on *C. roseus* during the blooming time, the significant variance was the reduction of vindoline accumulation after treated with their combination. Maybe the interaction of these two PGRs combination enhances the gene transcription whose product catalyzes stemmadenine to form more catharanthine, resulting in little metabolic flow to form vindoline. Our previous research showed maximum vinblastine accumulation occurred at 72 h after treating with ethephon and at 24 h after treating with SA respectively. Present study revealed with a maximum accumulation of vinblastine at 48 h after the treatment,

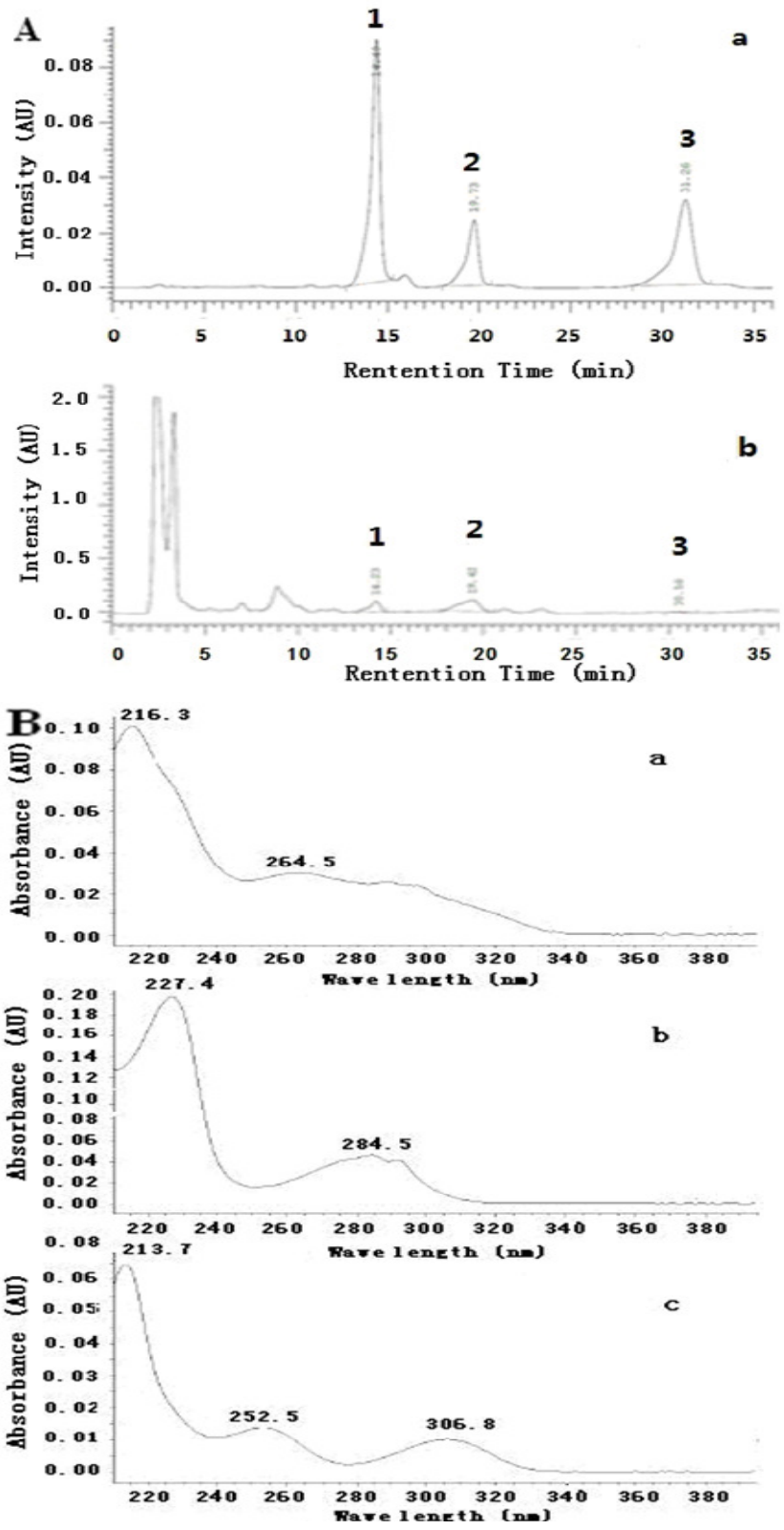

Figure 1. Chromatograms of standards and samples. Fig. 1*A*, Chromatograms of mixed standards (panel a) and *C. roseus* extract (panel b). Peak 1: vindoline; Peak 2: catharanthine; Peak 3: vinblastine; Fig. 1*B*, UV absorb chromatograms of vinblastine (panel a), catharathine (panel b) and vindoline (panel c) standards.

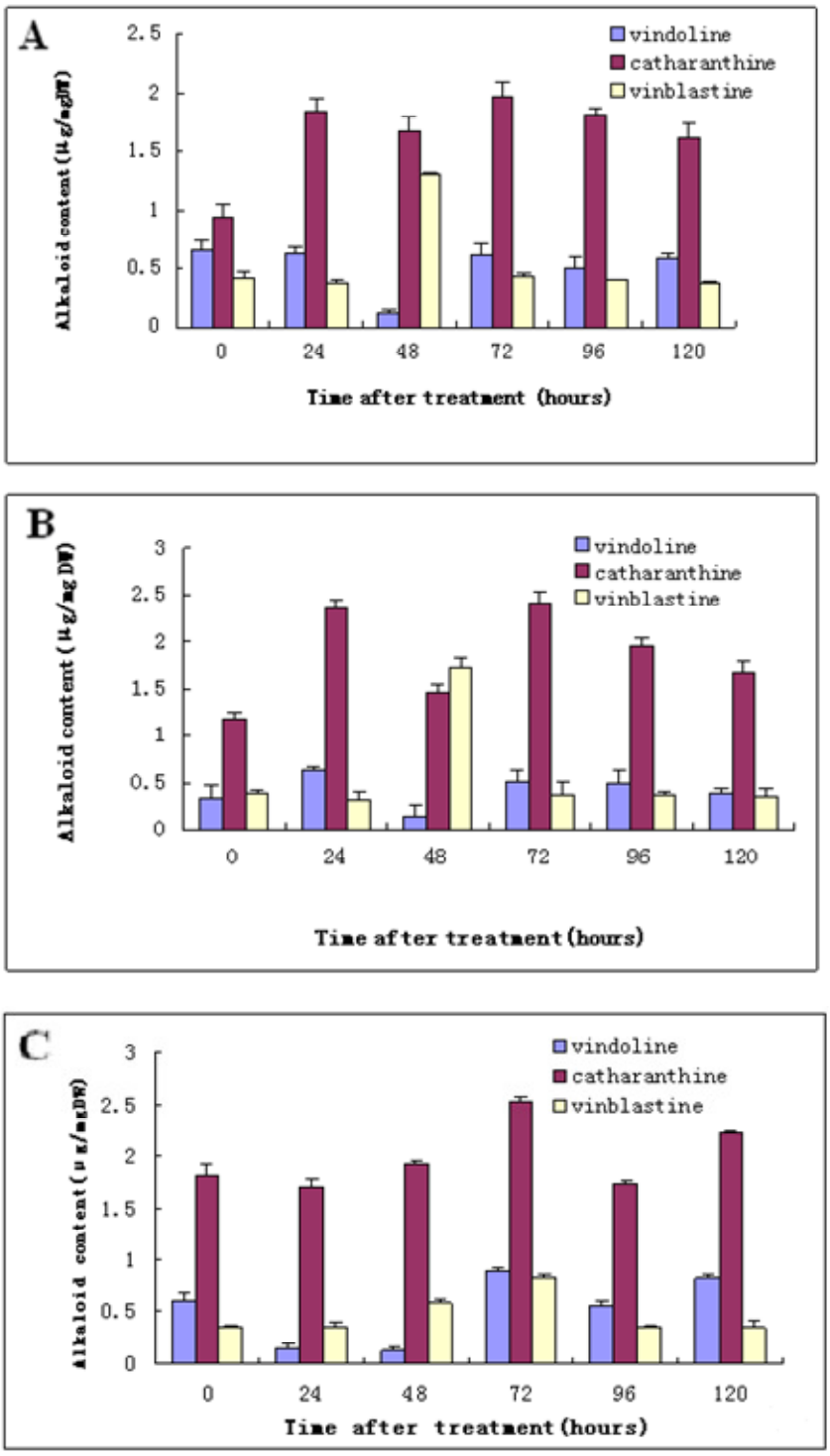

Figure 2. Effect of PGRs combinations on vindoline, catharanthine, vinblastine contents of *C. roseus* at different time after treatment. Fig. 2*A*, 0.1 mM Ethephon and 0.1 mM SA combination; Fig. 2*B*, Ethephon and 0.1 mM CCC combination; Fig. 2*C*, 0.1 mM SA and 0.1 mM CCC combination.

and this combination effect resulted in higher maximum accumulation of vinblastine than treating with ethephon or SA alone. Instead of treating the two PGRs alone, the combination treatment resulted in maximum

Catharanthine production.

Effect of Ethephon + CCC on alkaloids accumulation of *C. roseus*

The influence pattern of Ethephon (0.1 mM) + CCC (0.1 mM) on alkaloid biosynthesis in *C. roseus* during the blooming time showed similar to Ethephon + SA (Figure 2B).

This treatment resulted in significant increase of catharanthine and vinblastine, while the increase of vindoline content with a sudden decrease during the early stage. Catharanthine and vinblastine accumulation responded to this treatment similarly as the above treatment.

The maximum increase of catharanthine and vinblastine was 104.7% at 72 h and 246% at 48 h, it is higher than the control values. Accumulation of vindoline was different from catharanthine and vinblastine after the treatment, the vindoline content increased from 0 to 24 h reaching the maximum about 85.5% above the control, its content suddenly decreased 44.1% than the control during 24 to 48 h. After 48 h the content increased again and then slowly returned to the normal level. The next peak was 50% at 72 h above the control.

In this treatment the increases in vindoline and catharanthine was at 24 h, and decreased during 24 to 48 h, simultaneously the maximum accumulation of vinblastine appeared at 48 h. This phenomenon was consistent with the two parent compounds, vindoline and catharanthine accumulation improving the biosynthesis of vinblastine. It could also explain the sharp decrease of vindoline at 48 h as much vindoline flowed to form its product. There was a fast and lasting influence of Ethephon + CCC on vindoline and catharanthine contents while a transient effect on vinblastine accumulation. The increase of vinblastine was direct effect of PGRs combination and increase of its precursor, vindoline and catharanthine. Previous research proved that vinblatine and its precursors were synthesized in different compartments of *C. roseus* cells (Roytrakul and Verpoorte, 2007) which could also explain why the diterpenoid vinblastine sharply accumulated behind the accumulation of the two monoterpenoid.

Different from the effect of CCC resulting in vindoline and catharanthine decreased in our previous research (Pan et al., 2010) Ethephon + CCC treatment increased their contents because Ethephon has positive effect on their accumulation. The much more maximum increase of all the alkaloids treating with Ethephon + CCC than they used alone during the blooming period may be due to additive effect from the interaction between them.

Effect of SA + CCC on alkaloids accumulation of *C. roseus*

The pattern of vinblastine accumulation after treating with SA (0.1 mM) + CCC (0.1 mM) on *C. roseus* during the blooming time was similar to above patterns (Figure 2C). Significant increase in diterpenoid alkaloid was observed after the treatment and it showed a slow response to the treatment induction and began to increase from 48 to 72 h after spraying. The maximum increase of vinblastine was 138.6% at 72 h above control, and then reduced to the level of untreated sample at 96 h.

The effect on vindoline and catharanthine accumulation after being treated with SA + CCC showed a unique pattern. It was different from the treatment using PGRs alone (Pan et al., 2010) or combinations of SA (0.1 mM) + Ethephon (0.1 mM) or Ethephon (0.1 mM) + CCC (0.1 mM). Treatment of SA (0.1 mM) +CCC (0.1 mM) resulted in a slight decrease on vindoline and catharanthine accumulation at first, then these two monomeric alkaloids slowly increased, with the maximum increase of 47.6 and 38.5% both at 72 h higher than the control. Among all of the other treatments, these two alkaloids increased to the highest contents and then they slowly decreased to the untreated level at last. In this treatment after their maximum accumulation at 72 h, there was a next peak at 120 h. This strange phenomenon may be explained as CCC inhibiting the monomeric alkaloids accumulation initially (Pan et al., 2010) from 96 to 120 h and its inhibitory effect decreased owing to lose most of its efficacy. CCC decreased the two monomeric as in our previous study and this reason can also explain why there was a slight decrease in vindoline and catharanthine accumulation at first.

Compared with the two PGRs when used alone, the range of vinblastine accumulation increase was wider in their combination treatment and the maximum content appeared a little later. The two monomeric alkaloids, vindoline and catharanthine were also shown the trend of enhancement.

Effects of CCC + Ethephon + SA combinations on alkaloids accumulation of *C. roseus*

The treatment of different concentrations of CCC (0.01 mM, 0.1 mM and 1 mM) combined with SA (0.1 mM) and Ethephone (0.1 mM) on *C. roseus* during the blooming time had significant effect on vindoline accumulation (Figure 3A). After spraying SA + Ethephone + CCC solutions with different CCC concentrations (0.01 mM, 0.1 mM and 1 mM) vindoline accumulation decreased at first, and the maximum decrease was 75.8% at 24 h, 77.8% at 48 h and 46.6% at 24 h. Then its content increased with the maximum increase up to 149.2% at 72 h, 166.7% at 120 h and 113.5% at 72 h. After reaching to the maximum content, vindoline directly began to decline to normal level gradually under the treatments of SA + Ethephone + CCC (0.01 mM and 1 mM), while this alkaloid declined initially and then increased to a higher content than previous increase under the treatment of SA + Ethephone + CCC (0.1 mM).

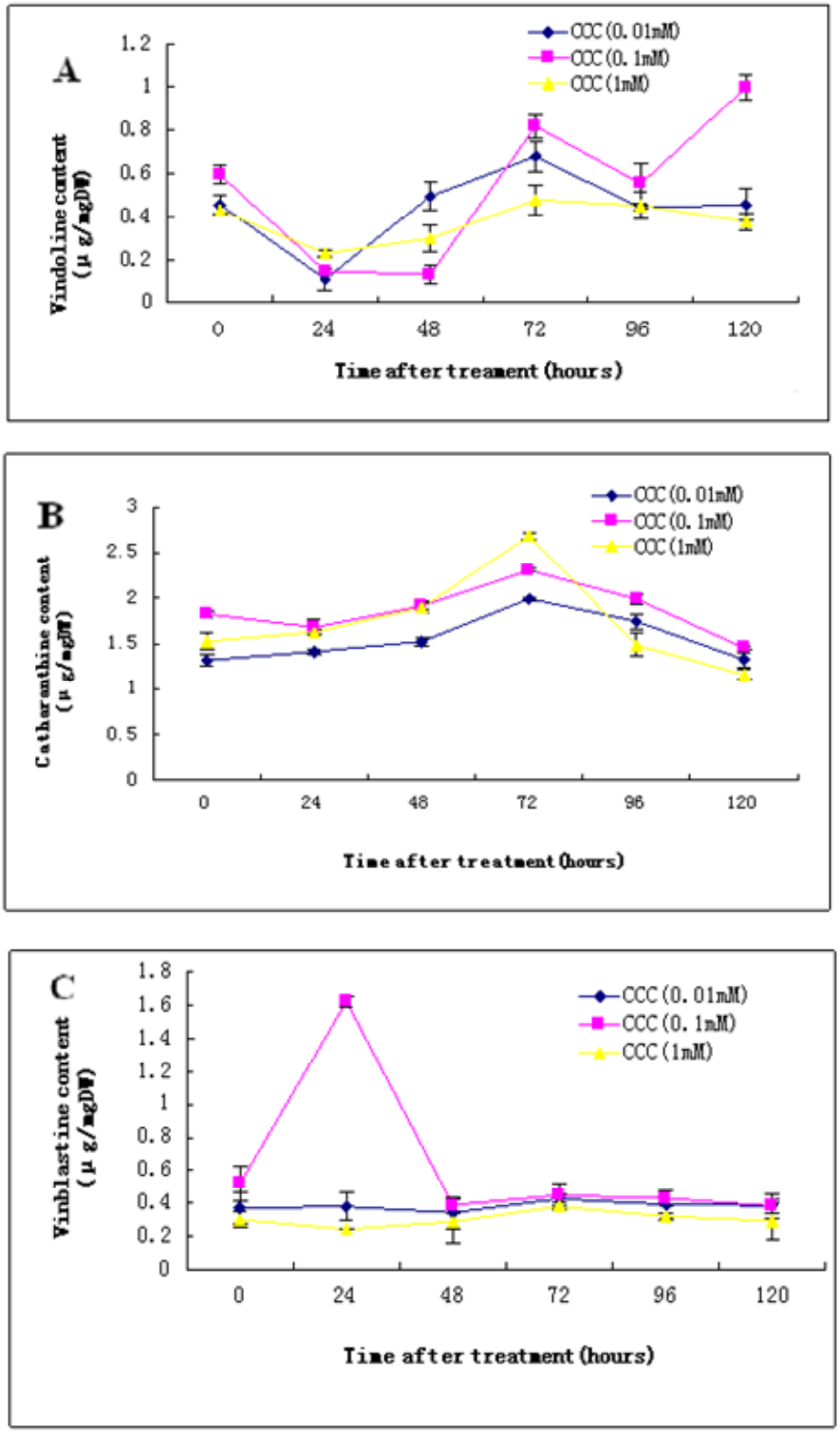

Fig. 3. Effect of 0.1 mM SA + 0.1 mM Ethephon and gradient concentration of CCC (0.01 mM, 0.1 mM and 1 mM) combination on alkaloids contents of *C. roseus* at different time after treatment. Fig. 3*A*, vindoline content variation; Fig. 3*B*, catharanthine content variation; Fig. 3*C*, vinblastine content variation.

SA (0.1 mM) treatment and Ethepone (0.1 mM) treatment both increased vindoline content after spraying on *C. roseus* during the blooming time, while low concentration of CCC treatment decreased vindoline accumulation in

our previous research (Pan et al., 2010). The treatments of SA + Ethephone + CCC (0.01 mM, 0.1 mM and 1 mM) led to vindoline decrease firstly may be because vindoline accumulation fast responded to CCC inhibition and slowly to SA and Ethepone stimulation. Among the three treatments, CCC (0.1 mM) + SA + Ethepone combination treatment had larger range of vindoline content change than CCC (0.01 mM and 1 mM) + SA + Ethepone combination treatments. These two phenomena consisted with our previous research that CCC (0.1 mM) treatment resulted in much more vindoline accumulation decrease than 0.01 mM and 1 mM concentration of CCC treatments (Pan et al., 2010). Another explanation of the decrease was much of vindoline may be as precursor to form vinblastine since the treatments activated the later biosynthesis pathway. CCC (0.1 mM) + SA + Ethepone combination treatment led to a next peak at 120 h of vindoline may be because CCC slowly lost most of its efficacy.

Catharanthine accumulation had a significant change after treating on *C. roseus* plants with SA (0.1 mM) + Ethephone (0.1 mM) + CCC (0.01 mM, 0.1 mM and 1 mM) solutions during the blooming time (Figure 3B). The maximum increase of the content was observed at 72 h, up to 151.6, 126.5 and 176.2% by the control. The decrease of catharanthine content resulted from SA (0.1 mM) + Ethephone (0.1 mM) + CCC (0.1 mM) treatment was more than SA (0.1 mM) + Ethephone (0.1 mM) +CCC (0.01 mM) treatment during 0 h to 24 h, while SA (0.1 mM) + Ethephone (0.1 mM) + CCC (1 mM) treatment had no negative effect on it during this period. This result may explain the maximum catharanthine increase ranked CCC (1 mM) + SA + Ethephon> CCC (0.01 mM) + SA + Ethephon > CCC (0.1 mM) + SA + Ethephon, the increase may result from the effect of other two PGRs alone and their three interations.

Treated with the combinations of SA (0.1 mM) + Ethephone (0.1 mM) + CCC(0.01 mM, 0.1 mM and 1 mM) on *C. roseus* plants during the blooming time, only the treatment of SA (0.1 mM) + Ethephone (0.1 mM) + CCC (0.1 mM) significantly increased vinblastine content (Figure 3C), and the maximum increase was 213.3% at 24 h above the control.

Both SA (0.1 mM) and Ethephone (0.1 mM) could increase vinblastine content after spraying on the plants during the blooming time. CCC alone at 0.01 mM had no effect on vinblastine content, while CCC at 1 mM had slight effect on vinblastine accumulation. However, the combination of SA (0.1 mM) + Ethephone (0.1 mM) + CCC (0.01 mM, 1 mM) had no effect. Perhaps there were some unknown interactions among them which affected vinblastine accumulation.

It can be concluded from the current study that spraying *C. roseus* plants with the combinations of PGRs during the blooming time has significant effects on the accumulations of vinblastine, vindoline and catharanthine. Among the treatments, SA (0.1 mM) + Ethephone (0.1 mM), Ethephone (0.1 mM) + CCC (0.1 mM) and SA (0.1 mM) + Ethephone (0.1 mM) + CCC (0.1 mM) could highly increase the vinblastine content up to 309% at 48 h, 346% at 48 h and 313.3% at 24 h of that in the control. The combinations of PGRs could induce much higher alkaloids accumulation, compared with the single use, with the highest vindoline and catharanthine accumulations occurred at 72 h after treatment.

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