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增强 UV-B 辐射对喜树生理指标及喜树碱含量的影响

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摘　要:喜树(*Camptotheca acuminata* Decne.)隶属于蓝果树科(Nyssaceae)喜树属(*Camptotheca*),为抗癌药物喜树碱的主要资源,提高喜树碱的积累以满足临床需求是喜树碱开发的重要途径。该研究运用 UV-B 辐射对 2 年生喜树进行每天 8 h 辐射处理,对 1 年生喜树分别设置每天 2 h、4 h、6 h 和 8 h 的辐射处理,连续处理 12 d 后分别测定各处理喜树叶的叶绿素、MDA、游离脯氨酸(Fpro)含量和 SOD 活性,以及幼叶、幼枝和根中喜树碱含量,分析 UV-B 辐射对喜树生理指标和次生代谢物的影响,以揭示喜树碱为喜树适应 UV-B 辐射逆境的防御产物。结果显示:(1)2 年生喜树经 UV-B 每天 8 h 辐射处理 12 d 后,叶绿素含量较对照显著降低,而 MDA、Fpro 和喜树碱含量均增加,说明每天 8 h UV-B 辐射对 2 年生喜树产生了较强的胁迫伤害。(2)1 年生喜树经 UV-B 辐射处理 12 d 后,随着每天 UV-B 辐射时间的增加,叶绿素含量不断降低,Fpro 含量显著增加;每天 2～6 h 处理的 MDA 含量与对照无显著差异,但总体随处理时间增加呈上升趋势;每天 8 h UV-B 辐射的 MDA 含量较对照显著增加;SOD 活性随每天处理时间的延长呈先下降、后上升、再下降的变化趋势,说明每天 8 h 的 UV-B 辐射对一年生喜树也产生了胁迫伤害。(3)1 年生喜树幼叶、幼枝和根中喜树碱含量随着每天 UV-B 辐射时间的延长均呈递增趋势,而且每天 8 h 辐射处理的喜树碱含量均最高,其中幼叶和幼枝中喜树碱含量显著高于根中含量。实验结果表明,增强 UV-B 辐射对喜树造成了一定的伤害,而喜树通过改变生理以及次生代谢机制,以进一步产生喜树碱来响应增强 UV-B 的胁迫。

关键词:喜树;UV-B 辐射;喜树碱含量;生理指标

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Effects of Enhanced Ultraviolet-B Radiation on Physiological Indices and Camptothecin Content in *Camptotheca acuminata* Decne

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Abstract: *Camptotheca acuminata* Decne. (Nyssaceae) is a major source of anticancer camptothecin (CPT). It is imperative to induce CPT accumulation in order to develop CPT production strategies to satisfy clinical uses of CPT. In this study, two-year-old *C. acuminata* were dealt 8 h each day with UV-B radiation for 12 days, and one-year-old *C. acuminata* were respectively arranged to radiate 2 h, 4 h, 6 h and 8 h

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with UV-B radiation each day for 12 days. The contents of chlorophyll, MDA and free proline (Fpro), the activity of SOD in leaf and CPT content in young leaves, young shoots and root were separately measured after UV-B treatment. In order to reveal that camptothecin is the defense product of UV-B stress, the effects of UV-B radiation on the physiological indices and secondary metabolites were analyzed. The results showed that: (1) in two-year-old *C. acuminata*, chlorophyll content was significantly decreased, MDA, Fpro and CPT contents were significantly increased after 8 h UV-B treatment daily. It indicated that 8 h UV-B radiation caused great stress influences on two-year-old *C. acuminata*. (2) In one-year-old *C. acuminata*, with the time of UV-B radiation increasing, chlorophyll content was gradually decreased; Fpro content was significantly increased; MDA content had no significantly difference between 2 h and 6 h UV-B radiation, but significantly increased after 8 h radiation compared with the control; SOD activity decreased firstly, then increased, lastly decreased with the time of UV-B radiation prolonging every day. It showed that 8 h UV-B radiation also caused stress influences on one-year-old *C. acuminata*. (3) CPT contents in vegetative organs of one-year-old *C. acuminata* were gradually increased with the time of UV-B radiation prolonging, and the contents were the highest after 8 h UV-B radiation each day. Moreover, CPT content increased more obviously in young leaves and young shoots than those in roots. It confirmed that enhanced UV-B radiation caused certain damage to *C. acuminata*, and *C. acuminata* responded to this stress by not only changing physiological indices, but also changing secondary metabolism to accumulate CPT.

Key words: *Camptotheca acuminata*; ultraviolet-B (UV-B) radiation; camptothecin (CPT) content; physiological index

Camptotheca acuminata Decne. belongs to the Nyssaceae family and is a perennial deciduous plant that is unique to China, and is mainly distributed along the Yangtze River and Southwest provinces of China[1]. The plant is known to botany and medicine because its various organs contain the alkaloid camptothecin (CPT) and its derivatives which have important biological activities.

CPT, a pentacyclic quinoline alkaloid, is an effective medicine in cancer treatment, which was first isolated from the stem of *C. acuminata*, and the structures of this alkaloid were determined by Wall and his collaborators[2]. CPT is known for its remarkable anti-cancer activity to inhibit the eukaryotic DNA topoisomerase I[3]. It also inhibits retroviruses such as the human immunodeficiency virus (HIV) and the equine infectious anemia virus[4]. CPT is a valuable compound as a chemical precursor of topotecan and irinotecan which were approved by the US Food and Drug Administration in 1996 for the treatment of ovarian and colorectal cancers[5].

Although much is known about many factors which affect the accumulation of CPT in *C. acuminata*, still only little is known about the effect of UV-B radiation on CPT accumulation. Because of the promising clinical uses of CPT, it is important to investigate the factors affecting CPT yield in plant material. In the past study, drought can increased CPT content in leaves of *C. acuminata*[6]. Methyl jasmonic acid and the treatments of yeast extract on leaf discs punched from *C. acuminata* seedlings promoted the mRNA expression of tryptophan decarboxylase (TDC), a key enzyme involved in CPT biosynthesis[7]. Similarly, methyl jasmonic acid and yeast extract treatments on *C. acuminata* cell suspension cultures increased CPT accumulation[8]. It can enhance CPT production by ethanol addition in the suspension culture of the endophyte, *Fusarium solani*[9]. Wang *et al*[10] reported CPT content decreased after 10 days UV-B radiation, and increased after 40 days UV-B radiation. But they did not study the change of CPT content between 10 days UV-B radiation. This study investigated the effects of UV-B radiation between 12 days on the accumulation of CPT in *C. acuminata*, which would provide a basis for maximizing CPT yield and designing an affective CPT production system.

1 Materials and methods

1.1 Plant materials

One-year-old and two-year-old *C. acuminata* plants were grown from seeds. Seeds of *C. acuminata* were selected to gather from Botanical Garden of Xi'an in November of 2012 and 2013, then respectively embedded in wet sand at 25 ℃ in March the next year. After the seeds geminated, the uniform seedlings were selected and transplanted into flowerpots in the botanical garden of Northwest University (Xi'an, China).

1.2 UV-B radiation

Supplemental UV-B radiation was provided by filtered Gucun brand (Gucun Instrument Factory, Shanghai, China) 30 W sunlamps. Lamps were suspended above and perpendicular to the planted rows and filtered with either 0.13 mm thick cellulose diacetate (transmission down to 290 nm) for UV-B irradiance or 0.13 mm polyester plastic films (absorbs all radiation below 320 nm) as a control. The desired irradiation was obtained by changing the distance between the lamps and the plants. The spectral irradiance from the lamps was determined with an Optronics Model 742 (Optronics Laboratories, Orlando, FL, USA) spectroradiometer. The spectral irradiance was weighted with a generalized plant response spectrum and normalized at 300 nm to obtain the desired level of biologically effective UV-B radiation. The lamp height above the plants was adjusted to maintain a distance of 0.15 m between the lamps and the top of the plants and provided supplemental irradiances of 2.1 effective uw · cm^{-2}. Two-year-old plants were irradiated for 12 days and 8 h daily. In order to further illustrate the effects of supplemental UV-B irradiance, one-year-old plants were irradiated for 12 days and respectively arranged 2 h, 4 h, 6 h and 8 h radiation daily. Filtered UV-B radiation was regarded as the control (CK).

1.3 Determination of physiological parameters

Physiological parameters were determinated in leaves of *C. acuminata*. Chlorophyll was extracted with 96% alcohol, and chlorophyll content was measured according to the method of Zhang[11]. Malonaldehyde (MDA) content was determined according to the method of Heath and Packer[12]. Fpro content was extracted from leaves in 3% aqueous sulphosalicylic acid and estimated using ninhydrin reagent[13]. Superoxide dismutase (SOD) activity was determined according to the method of Giannopolitis and Ries[14].

1.4 CPT content analysis

1.4.1 HPLC analysis The HPLC system consisted of a HPLC pump (LC-10ATvp), a reversed phase column (VP-ODS, 150 mm×4.6 mm, 5μm) and a UV-VIS detector (SPD-10Avp) for the detection of CPT at 254nm[15]. Sample injection volume was 10 μL according to the presumable alkaloid content. The flow rate was 1.0 mL · min^{-1}. The mobile phase used was methanol/water (62/38, V/V). Column temperature was 25 ℃. Under this condition, the HPLC chromatograms of the standard and sample solutions were showed in Fig. 1. CPT standard was kindly supplied by Dr. H. Bischoff of Boehringer Ingelheim Pharma KG. in Germany.

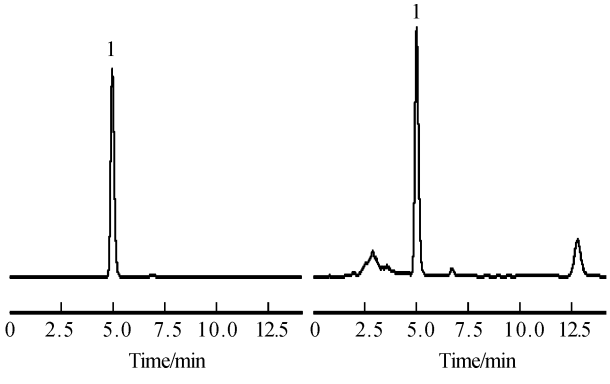


Fig. 1 The HPLC chromatograms of the standard and sample (peak 1: camptothecin)

1.4.2 Standard curve CPT standard 2.5 mg was put in 50 mL volumetric flask, suitable amount of chromatographic methanol was added, and metered volume after the ultrasonic helping dissolve. The solution was shaked and filtered with 0.45 μm microporous membrane, then 0.05 mg · mL^{-1} standard solution was got. 2, 3, 5, 8 and 10 mL of CPT standard solution were precisely measured and put in 10 mL volumetric flask, diluted with chromatographic methanol, then respectively metered vol-

ume. The standard curve for CPT was constructed by separate injection of 10 μL of the above-mentioned standard solution according to the above chromatography conditions. The regression equation between peak area Y and the concentration of camptothecin X (μg · mL^{-1}) was: $Y=16\ 686X-6\ 577.5$, $R^2=0.999\ 3$.

1.4.3 Determination of CPT content The samples were dried in the shade and grounded in a mortar. 100 mg of the samples was transferred to a centrifuge tube and 4 mL methanol was added. After extracted with sonication for 10 min at room temperature, 30 mL water and 40 mL dichloromethane were added and this was mixed vigorously for 5 min on a magnetical stirrer[16], centrifugation for 10 min at 2 000 r · min^{-1} yielded two phases. The dichloromethane phase which was proved to contain CPT, was recovered and evaporated to dryness in vacuum using a rotavapor. The remaining residue was re-dissolved in HPLC-grade methanol (1 mL), filtered with 0.45 μm microporous membrane then got sample solution which was used for the determination of CPT content. Sample peaks with the same retention time to standards were verified by spectral scan analysis.

2 Results

2.1 Effects of UV-B radiation on two-year-old *C. acuminata*

The contents of chlorophyll, MDA and Fpro as well as SOD activity were measured after two-year-old plants were irradiated with UV-B radiation. The results showed in Table 1, chlorophyll a and b contents were reduced, chlorophyll a/b ratio had a lesser extent increase under enhanced UV-B. MDA and Fpro contents increased. The activity of SOD was reduced. CPT contents in young leaves, young shoots and root of *C. acuminata* had obviously increased after UV-B treatment compared with the control, CPT content in root has no significant difference compared with the control.

Table 1 Effects of UV-B radiation on physiological indices and CPT content in two year-old *C. acuminata*

Index		CK	UV-B (8h)
Chla/(mg · g^{-1})		1.507±0.125	1.193±0.266*
Chlb/(mg · g^{-1})		0.646±0.102	0.505±0.142*
Chla+Chlb/(mg · g^{-1})		2.152±0.165	1.698±0.075*
Chla/Chlb		2.333±0.212	2.364±0.179*
MDA/(nmol · g^{-1})		1.104±0.083	1.170±0.133*
Fpro/(μg · g^{-1})		1.987±0.137	3.046±0.153*
SOD/(U · g^{-1})		231.9±10.11	222.4±12.18*
CPT/%	Young leaves	0.219±0.017	0.313±0.019*
	Young shoots	0.163±0.007	0.274±0.004*
	Root	0.045±0.008	0.067±0.013*

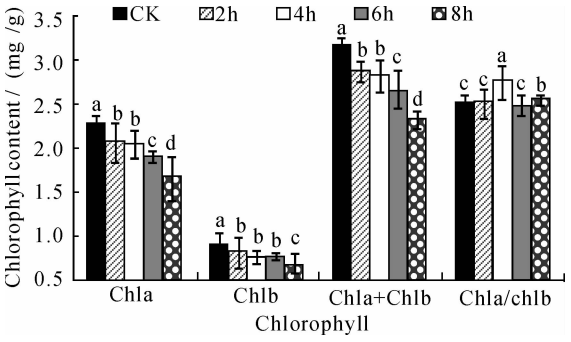
Note: Each assay was repeated three times from three independent experiments. The data are the means ± SEM of three replicates. Asterisk (*) indicates statistically significant difference ($P<0.05$; ANOVA, Tukey test).

2.2 UV-B radiation effects on chlorophyll contents in one-year-old *C. acuminata*

The effects of enhanced UV-B on chlorophyll contents in one-year-old *C. acuminata* were shown in Fig. 2. The same to two-year-old plant, chlorophyll contents decreased after UV-B radiation, and with the treatment time prolonging, the chlorophyll a and chlorophyll b contents gradually decreased. The total chlorophyll content of 2 h, 4 h, 6 h and 8 h UV-B treatment daily was respectively reduced by 9.4%, 10.8%, 15.7%, 26.8% than the control. While 8 h UV-B treatment daily, chlorophyll a and b contents decreased 26.4% and 27.8%, respectively. Chlorophyll a/b ratio had a little change compared with the control, it is suggested that the difference of destroyed extent about chlorophyll a and b is not obvious.

2.3 UV-B radiation effects on MDA, Fpro contents and SOD activity in one-year-old *C. acuminata*

UV-B radiation had obvious effects on MDA, Fpro content and SOD activity in *C. acuminata*. With the time of UV-B radiation increasing, Fpro content gradually increased; SOD activity decreased firstly, then increased, lastly decreased; MDA content kept increasing with the treatment time prolonging, however, there were no significant difference between 6 h UV-B radiation, MDA content was significantly increased after 8 h UV-B treatment (Fig. 3). UV-B radiation of short time induced the production of reactive oxygen species (ROS), such as $O_2^{\cdot-}$, H_2O_2, and reduced SOD activity. Meanwhile, Fpro content was significantly increased in order to defense UV-B stress. When



Each assay was repeated three times from three independent experiments. The data are the means ± SEM of three replicates. Different letters indicate statistically significant differences ($P< 0.05$; ANOVA, Tukey test). The same as below

Fig. 2　Effect of UV-B radiation on chlorophyll content in one- year-old *C. acuminata*

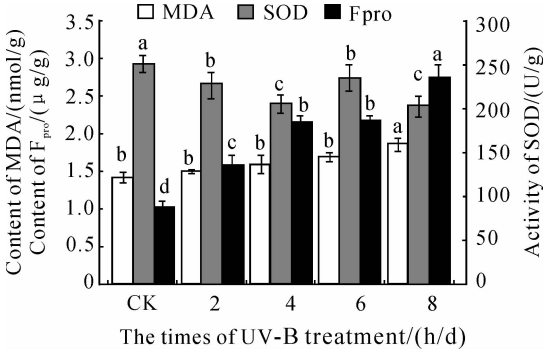


Fig. 3　Effects of UV-B radiation on MDA, Fpro contents and SOD activity in one- year-old *C. acuminata*

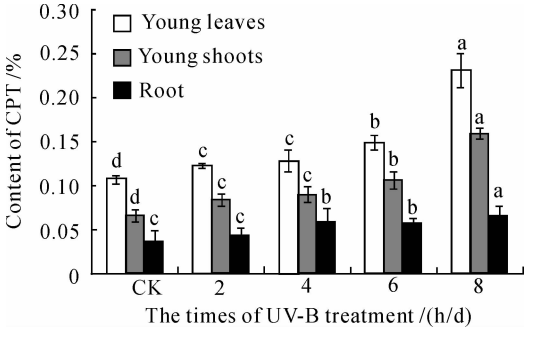


Fig. 4　Effect of UV-B radiation on CPT content in one-year-old *C. acuminata*

the time of UV-B treatment reached to 6 h daily, SOD activity increased in order to eliminate overmuch reactive oxygen species and Fpro content kept in a relative balance at the same time. When the time of UV-B treatment reached to 8 h daily, SOD activity decreased and Fpro content significantly increased.

2.4　UV-B radiation effects on CPT contents in one-year-old *C. acuminata*

UV-B radiation also had obvious effects on CPT contents in young leaves, young shoots and roots of one-year-old *C. acuminata* (Fig. 4). When the time of enhanced UV-B treatment reached to 8 h daily, the CPT contents were significantly increased compared with the control. With the time of UV-B radiation prolonging, CPT contents in young leaves and shoots increased faster than that in the root, and CPT content of young leaves increased to 0.230% from 0.106% of the control group after 8 h radiation daily, young stem increased from 0.064% to 0.158%, and the increase of CPT content in root had a little change, increased from 0.036% to 0.065%.

3　Discussion

UV-B radiation has many effects on plants. Although there are some significant effects about UV-B radiation on plant growth and development in certain species and ecosystems, it turned out that the overall damaging effects of above-ambient UV-B are modest or difficult to detect under natural conditions[17]. An examination of more than 200 plant species reveals that roughly 20% are sensitive, 50% are mildly sensitive or tolerant and 30% are completely insensitive to UV-B radiation[18]. UV-B radiation acts as a kind of environmental stress, and the plant would have to adapt to UV-B radiation to minimum the damage when the stress level reach to a limitation. With a short period of UV-B radiation in *Arabidopsis thaliana* seedlings, roots were elongated and chlorophyll and soluble protein content were increased in leaf, but prolonged UV-B radiation inhibited the elongated root length, caused leaf chlorophyll content, soluble protein content decrease[19]. The effects of enhanced UV-B radiation on plant physiology, morphology, growth and biomass have been investigated extensively. Enhanced UV-B radiation may exert an adverse influence on the physiological and biochemical processes of plants. In this study, we reported the responses of *C. acuminata* plant to

enhanced UV-B radiation. This is supported by the earlier findings of intraspecific responses in flavonoid metabolism in *Cucumis sativus*[20], soybean[21] and *A. thaliana*[22] and in flavonoid content and chlorophyll content decreased in rice[23].

UV-B radiation had obvious effects on chlorophyll contents of *C. acuminata*. The UV-B radiation mechanism of decreasing chlorophyll contents was still not clear. Changes in chlorophyll contents have often been used as an index to assess the degrees of UV-B radiation sensitivity. In this study, chlorophyll contents in *C. acuminata* leaves were also sensitive to enhanced UV-B radiation. UV-B radiation significantly decreased chlorophyll contents, primarily because UV-B radiation destroyed the structure of the chloroplast, inhibited the synthesis of new chlorophyll and destroyed the structure of chloroplast envelope membrane and increased the degradation of chlorophyll. Yang *et al.*[24] reported that UV-B radiation reduced the photosynthetic pigment in leaves (including the contents of chlorophyll and carotenoid, especially the content of chlorophyll a). Zhang indicated that UV-B radiation of different intensity can make the growth, chlorophyll contents decrease in *Vicia faba* seeding[25]. These results showed that enhanced UV-B radiation made the plant chlorophyll damage, changed the proportion of chlorophyll a and chlorophyll b, affected the formation of photosynthetic protein complexes, and inhibited the formation of organelles.

Little was known of the responses of SOD activity and MDA contents to enhanced UV-B radiation in the past. The primary explanation of cell membrane's damage induced by UV-B was the free radical theory. UV-B radiation may induce the production of reactive oxygen species (ROS), such as $O_2^{\cdot-}$, H_2O_2, and change the SOD activity, result in membrane lipid peroxidation, which further lead to the changes of membrane structure and finally alter membrane permeability. As a result, the cells of plant are injured and MDA content increased. SOD is one of the protective enzymes to defense the injury of ROS. In normal circumstances, the generation and elimination of ROS in plants are in a state of dynamic balance. In our study, SOD activity firstly decreased, then increased, lastly decreased, which is consistent with the change of SOD activity under low temperature stress condition[26]. The stress of UV-B induced the production of a certain amount of ROS under the treatment of 4 h daily, in order to eliminate ROS timely, the activity of SOD that is intrinsical in *C. acuminata* decreased. Meanwhile, Fpro content significantly increased between 4 h radiation daily compared with the control, which indicated the increased Fpro improved the resistance to the UV-B stress. A certain amount of UV-B radiation may increase the content of ROS such as $O_2^{\cdot-}$ in plant, and SOD can catalyze $O_2^{\cdot-}$ disproportionation to generate H_2O_2 and O_2, enhance the activity of antioxidant enzyme system[27]. Feng *et al.*[28] also reported that enhanced UV-B radiation in low dose or short-term treatment can briefly stimulate the activity of SOD and POD to increase. In *C. acuminata*, when the time of UV-B treatment was 4—6 h, a large amount of ROS was generated, SOD activity significantly increased to eliminate superabundant ROS, meanwhile Fpro and MDA contents had no significantly differences. This suggested that higher SOD activity can inhibit membrane lipid oxidation, and keep MDA content in a balance. When the time of UV-B treatment reached to 8 h, as UV-B stress deepened, SOD activity decreased, ROS was accumulated and the lipid peroxidation of membrane system was enhanced, accordingly MDA and Fpro content significantly increased. We came to a conclusion that UV-B stress of 8 h daily decreased the activity of protective enzyme, caused a certain damage of biomembrane. It can be further testified that the leaves of *C. acuminata* became withering after we had studied 10 h UV-B radiation daily.

Free proline accumulation has been observed in response to a wide range of abiotic and biotic stresses in plants. Proline is considered to be one of the first metabolic responses to stress, and is perhaps a second messenger[29]. Environmental factors including water deprivation, salinization,

high and low temperature, heavy metal toxicity, pathogen infection, nutrient deficiency, atmospheric pollution and UV-radiation induce the elevation of the proline level in plants. In *C. acuminata* plant, enhanced UV-B radiation made Fpro content increase, it may be an adaptive response to resist UV-B radiation stress. A negative correlation between SOD activity and Fpro content was found under UV-B stress condition in *C. acuminata*.

MDA is the product of membrane lipid peroxidation when the plant tissue suffered oxidative stress, which reflects the degree of cell membrane lipid peroxidation and the plant to respond to stress. In this paper, MDA contents have no significant difference between 6 h UV-B radiation compared with the control, however, MDA content was significantly increased after 8 h UV-B treatment. A low dose of UV-B stress induced the production of a certain amount of ROS, while inherent SOD in *C. acuminata* rapidly eliminated these ROS, and resulted in the decrease of SOD activity. When the time of UV-B treatment reached to 8 h daily, a large number of ROS were generated and caused membrane lipid peroxidation, so MDA content was significantly increased. In conclusion, there is a certain correlation among the change of SOD activity, Fpro content and MDA content under UV-B stress, the decrease of SOD activity is accompanied by the increase of MDA or/and Fpro content.

Under the condition of adversity, in addition to the plant physiological indices can be induced some changes, the secondary metabolism of plant will also be changed. Plants interact with their environment by producing a diverse array of secondary metabolites, one of which is alkaloid. Biotic and abiotic environment have important roles in the secondary metabolizing of plant. As a secondary metabolite, CPT may play a crucial role during biotic and abiotic stresses, which pose a great impact on alkaloid biosynthesis and accumulation[30]. The increase of alkaloid accumulation during seedlings development was observed and this showed that CPT may play a defensive function for the plants during this vulnerable stage of their life cycle. UV light responsive regions in the promoter of the tryptophan decarboxylase (*tdc*) gene in *Cathatranthus roseus* had been identified[31]. In our study, UV-B radiation can obviously enhance CPT content in *C. acuminata*, we hypothesize that UV-B might stimulate the expression of *tdc* gene and increase CPT accumulation in *C. acuminata*. CPT accumulation induced by UV-B radiation demonstrated that CPT was involved in plant defense against UV-B radiation. When *C. acuminata* suffered UV-B radiation in a low dose (2 h, 4 h), CPT content was increased slowly; with the time of UV-B radiation increasing (6 h, 8 h), CPT content was increased rapidly, which illustrated that the long time of UV-B radiation caused certain damage to *C. acuminata*, and *C. acuminata* defensed primarily by increasing the secondary metabolites-CPT. Moreover, CPT contents in young leaves and shoots were increased more obviously than those in root, it indicated that enhanced UV-B radiation could priority improve CPT accumulation of aerial organ in *C. acuminata*, because aerial organ mainly suffered UV-B stress.

Enhanced UV-B radiation caused certain damage to *C. acuminata*, not only affects the morphology of *C. acuminata*[10], but also affects the physiological and biochemical metabolism. Under the stress of UV-B, *C. acuminata* itself defensed this stress by changing physiological indices (i. e., SOD activity, MDA and Fpro content) and secondary metabolism to accumulate CPT. Therefore, the changes of physiological indices and CPT accumulation are also the adaptive mechanism to response to the stress of enhanced UV-B radiation in *C. acuminata*. Furthermore, in the industry planting of *C. acuminata*, the method of supplementing 8 h UV-B radiation daily to promote the increase of CPT content is feasible.

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