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Concentration and gene expression analyses of dragon's blood flavonoids in different tissues of *Dracaena cochinchinensis*

Yanqian Wang [1,2✤iD], Shuang Li [1,2iD], Chunyong Yang [1,2iD], Yanfang Wang [1,2iD], Jianming Peng [1,2iD], Ge Li [1,2iD], Zhen Yan [1,2iD], Yan Mou [1,2iD], Er Li[1,2iD], Jianhe Wei [3iD], Jianjun Qi [3iD], Lixia Zhang [1,2iD]

[1] Yunnan Branch, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, China
[2] Yunnan Key Laboratory of Southern Medicine Utilization, China
[3] Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China

ECOLOGY

ABSTRACT

Background: Dragon's blood is a well-known traditional Chinese medicine, isolated mainly from the trunks of *Dracaena* trees. Overexploitation of the *Dracaena* resource has resulted in this plant becoming endangered. This work aimed to assess the potential to use various tissues of the *Dracaena cochinchinensis* tree, such as leaves and roots, to produce dragon's blood.

Results: We found that many dragon's blood components accumulated in the bark of trunk and roots of *D. cochinchinensis* under natural conditions. In addition, small amounts of loureirin A were detected in leaves, suggesting that the leaves could be a potential source of dragon's blood. Real-time quantitative PCR analysis showed that most of the genes tested in this study, which encoded enzymes involved in the biosynthesis of dragon's blood flavonoids, were highly expressed in the bark of roots and trunk.

Conclusion: We confirmed that the bark of roots and trunk of *D. cochinchinensis* tree were the main tissues for the synthesis and storage of dragon's blood under natural conditions. This study demonstrated the potential to extract dragon's blood from the roots that have been abandoned due to mining difficulties without destroying the tree, a process which would be beneficial to the protection of the endangered wild *D. cochinchinensis* tree populations.

Keywords: *Dracaena* tree; Dragon's blood accumulation site; Gene expression; Loureirin A; Loureirin B

HIGHLIGHTS

The bark of trunk and roots are the main tissues for the synthesis and storage of dragon's blood in *Dracaena* tree.
The roots of *Dracaena* tree can be used to extract the dragon's blood.
The leaves of the *Dracaena* tree have the potential to produce dragon's blood.
The production of the red substance can be used as a marker of dragon's blood synthesis in *Dracaena* tree.

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✤Corresponding author e-mail: yanqwang@implad.ac.cn Submitted: 01/07/2021 Accepted: 13/04/2022

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INTRODUCTION

Dragon's blood is a deep red-colored resin produced from plants of *Dracaena* spp., *Daemonorops* spp., *Croton* spp. and *Pterocarpus* spp. (Wang et al., 2015; Saxena et al., 2019), which has been used as a well-known traditional medicine and as an artistic material for thousands of years in various civilizations (Lang et al., 2020; Wang et al., 2011). In China, the dragon's blood is a traditional Chinese medicine (TCM) extracted from the trunk of *Dracaena* plants and called 'long xue jie' (Tsai and Xu, 1979). Wild specimens of the tree *Dracaena cochinchinensis* (Lour.) S.C. Chen have been used as the main source of dragon's blood since the 1970s, with the trees being found mostly in Yunnan Province, China (Tsai and Xu, 1979).

Composition analysis shows that flavonoids are the main chemical constituents in dragon's blood, which making up approximately 50–80% of the total (Helal et al., 2021; Sun et al., 2019). The remainder of dragon's blood is made up of small amounts of other classes of compounds, such as phenolic acids, terpenes, steroids and saponins (Zheng et al., 2006, 2012).

The various flavonoids of plants are biosynthesized *via* the phenylpropanoid pathway and the flavonoid pathway, which have been well established in model plants *Arabidopsis*, petunia, maize and wheat (Pourcel et al., 2012; Koes et al., 2005). The principal enzymes involved in the phenylpropanoid pathway are phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumaroyl-CoA ligase (4CL). PAL plays a key role by linkage between primary metabolism and secondary metabolism, by generating a range of important structural skeletons *via* the phenylpropanoid pathway (Winkel-Shirley, 2001; Dixon and Paiva, 1995). The first step of the flavonoid biosynthetic pathway is catalyzed by chalcone synthase (CHS), which uses one molecule of malonyl-CoA and three molecules of 4-coumaroyl-CoA as substrates. The next steps are catalyzed by chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H) and dihydroflavonol 4-reductase (DFR), with a range of flavonoid compounds being produced during this process (Zhu et al., 2018; Zhu et al., 2021).

In general, dragon's blood is produced from the trunk of wild-grown *Dracaena* trees more than 30–50 years of age, and which can be even thousands of years old. Production of dragon's blood in the trunk is induced by stress under natural conditions (Ding et al., 2020; Maděra et al., 2020).

However, several studies have shown that the leaves of *Dracaena* can be induced by challenge by fungi to produce dragon's blood, which was similar in physical and chemical profiles to the natural product (Ou et al., 2013). This finding suggests that other organs of *Dracaena* induced by elicitors are potential sources of dragon's blood, in particular renewable organs such as leaves, flowers, fruits and seeds. This finding may potentially alleviate the problem of the demand for dragon's blood and the concomitant destruction of wild *Dracaena* trees. However, differences in profiles of dragon's blood phytochemicals, as well as in patterns of biosynthetic gene expression in different organs of *Dracaena* trees are still unclear. The present study provides a detailed analysis of the synthesis and accumulation of dragon's blood flavonoids in various organs of the *D. cochinchinensis* tree, and to determine whether organs other than trunks of the *D. cochinchinensis* tree could be exploited to produce dragon's blood, which would reducing the threat to surviving wild *Dracaena* populations.

MATERIAL AND METHODS

Plant materials and collection of *Dracaena* tissues

Three healthy 30-year-old trees of *D. cochinchinensis* (Lour.) S.C. Chen, which had been planted in the Yunnan Branch of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Jinghong, Yunnan, China, were used in this study (Figure 1). The *D. cochinchinensis* trees used in this study are grown in tropical and subtropical monsoon climates, and the soil is latosol. Samples of roots, trunks, leaves, flowers, fruits and seeds were collected using scalpels or branch shears, as appropriate. The fresh tissues for total RNA isolation were promptly snap-frozen in liquid nitrogen, immediately powdered using a mortar and pestle and stored at -80°C.

Extraction of dragon's blood from the test samples

Fresh tissue samples for phytochemical extraction were dried at 60°C for 12 h, and ultrasonically extracted 0.1 g dry tissue samples in 15 mL of methanol three times for 15 min each at 25°C. The methanol in the dragon's blood extracts was removed by evaporation, using a vacuum rotary evaporator at 45°C. The residue from each sample was resuspended 3 times with a small volume of methanol, the solutions were pooled and then adjusted to 5 mL in methanol. The samples were filtered through a 0.22-μm microporous membrane to remove non-dissolved material and the filtrate was stored in sample bottles prior to high-performance liquid chromatography (HPLC) analysis (Cui et al., 2013).

Figure 1. Plant morphology of *D. cochinchinensis* tree.

HPLC analysis of organ extracts

HPLC analysis was performed at 40°C by using a Shimadzu-LC2030C HPLC system (SHIMADZU, Japan) with a C18 column (ZORBX SB-C18, 5 µm particle size, 4.6 mm × 250 mm, Agilent, USA) and a pulsed amperometric detector (PAD, SHIMADZU, Japan). A gradient of 30% acetonitrile–0.3% acetic acid in water (A) and acetonitrile (B) was used as the mobile phase at a solvent flow rate of 1.0 ml.min^{-1}. The mobile phase started with 100% A and introducing a gradient to achieve 80% A at 5 min, 80% A at 30 min, 100% methanol at 31 min to 40 min, and 100% A at 40 min to 55 min. The injection volume was 10 µL and the detection wavelength was 278 nm (Cui et al., 2013). The standard compounds loureirin A and loureirin B were purchased from the National Institute for Food and Drug Control (Beijing, China). The concentration/absorbance linear regression equations for loureirin A and loureirin B were:

loureirin A: y = 38276x - 8635.7 (r = 0.9999) in the linear range of 1.16–100.17 µg/ml^{-1};

loureirin B: y = 24312x + 131.67 (r = 0.9999) in the linear range of 0.48–10.0 µg/ml^{-1}.

Total RNA isolation and cDNA preparation of test samples

Total RNA was extracted from approximately 0.5 g fresh tissue of *D. cochinchinensis*, using the Tiangen RNAprep Pure Plant Plus Kit (Tiangen, China), with electrophoresis on 1% agarose gel being employed to check RNA quality. Then, any contaminating DNA was removed using DNase I. The first-strand cDNA was synthesized from total RNA, using TaKaRa PrimeScript™ reverse transcription reagent kit (TaKaRa, Japan), following the reverse transcription kit experimental instructions (Wang et al., 2016). Total RNA and cDNA concentrations were determined, using a Nanodrop One^{c} spectrophotometer (Thermo Scientific, USA).

Quantitative real-time PCR analysis (qPCR)

Quantitative real-time PCR was conducted using TB Green Premix Ex Taq II (TaKaRa, Japan) in the CFX96 Touch Real-Time PCR System (BIO-RAD, USA) with the following program: 95 °C for 30 s; 40 cycles of 5 s at 95 °C, and 30 s at 60 °C. The actin gene of *D. cochinchinensis* was used as an internal control in qPCR analysis. The primers used in this study were designed using Primer Premier5 software (Premier Biosoft, Canada), and the primer sequences are listed in Table 1.

RESULTS

Chemical profile and physical properties of dragon's blood extracted from various organs

The concentrations of loureirin A and loureirin B were analyzed by HPLC, and the concentrations varied between extracts of different organs of *D. cochinchinensis* (Figure 2, 3). As shown in Figure 2, high and similar concentrations of loureirin A were detected in BDT (bark of the damaged trunk) and BR (bark of the root). In addition, low concentrations of loureirin A were also found in XR (xylem of the root), BUT (bark of the undamaged trunk) and XDT (xylem of the damaged trunk). Interestingly, YL (young leaves) also contained trace amounts of loureirin A, although there was no loureirin A detected in SL (senescent leaves), FL (flowers), FR (fruits), S (seeds) or XUT (xylem of the undamaged trunk). The concentration of loureirin A in BDT was 10, 50, 65 and 224 times greater than that in XR, BUT, XDT and YL, respectively; in BR, the corresponding concentrations of loureirin A were 9.9, 48, 63 and 217 times higher.

Loureirin B was found only in BDT and BUT, whereas, most surprisingly, its concentration in BDT was 264 times higher than that in BUT (Figure 2). However, loureirin B was not detected in YL, SL, FL, FR, S, XR, BR, XUT or XDT. By comparison, the concentrations of loureirin A in BDT and BUT were approximately 4.3 and 17 times higher, respectively, than those of loureirin B.

The HPLC chromatograms of dragon's blood in extracts of different tissues are shown in Figure 3. Many phytochemicals were detected in XR, BR and BDT, with most of the components being identical in retention time between extracts of these three tissues. However, the concentrations of these compounds varied between extracts of XR, BR and BDT. In addition, fewer compounds were detected, and the concentration of each compound was lower in BUT and XDT than in XR, BR and BDT.

Phytochemicals of dragon's blood were either absent or present at very low concentrations in YL, SL, FL, FR, S and XUT. The physical properties of extracts of different tissues from *D. cochinchinensis* were quite different (Figure 4). The dragon's blood extracted with methanol from XR, BR and BDT appeared red, whereas, for other tissues, it was green, yellow or colorless.

Primer name	Primer sequence (5'-3')
qPAL-F/R	cgagctcgtcaacgatttcta/ctgctcccttgaatccatagtc
qC4H-F/R	ccattgtggccaaacctaga/gggaaacagacccaggtaataaa
q4CL-F/R	caagtataaaggctaccaggttgca/ttgttgggaaacgggatcaca
qCHS-F/R	ttcaagcgcatgtgcgagaaat/ggctgccccccattccttgat
qCHI-F/R	gctatcgtgtcagctcctgt/agtataagaactcgcaggga
qF3'H-F/R	cggttcgtgcccaaggatat/cggctatcaggtcctgagtgaa
qDFR-F/R	cacaagccaagggaagatac/ccttcgaactgagtagggata
qACTIN-F/R	accgagagagggtactcatt/ccagctcctgctcgtaatc

Tab.1 The primers for quantitative real-time PCR used in this study.

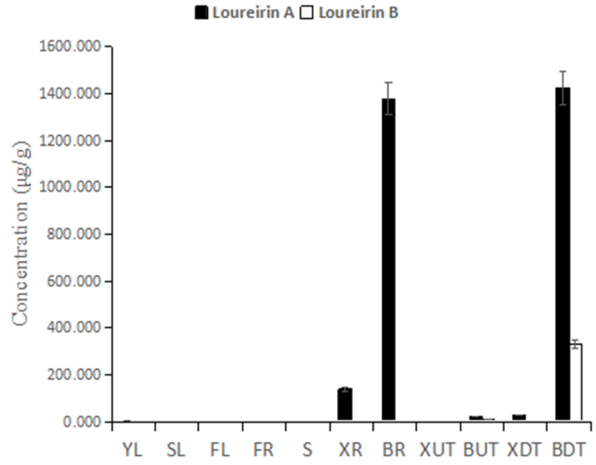

Figure 2. Loureirin A and loureirin B concentrations in different tissues of *D. cochinchinensis* tree. The different organ samples are young leaves (YL), senescent leaves (SL), flowers (FL), fruits (FR) and seeds (S); and the different tissue samples are xylem of the root (XR), bark of the root (BR), xylem of the undamaged trunk (XUT), bark of the undamaged trunk (BUT), xylem of the damaged trunk (XDT) and bark of the damaged trunk (BDT).

The color was identical between the methanol extract, the tissue powder and the original tissue sample from different tissues (Figure 4). Interestingly, the phytochemicals in dragon's blood were detected in extracts of red tissues, but no or few in tissues that were not red. In addition, a phenomenon was observed whereby the red coloration penetrated further into the XR than in XUT and XDT; this result was consistent with the concentrations of loureirins in the extracts of the corresponding tissues in the HPLC chromatograms.

The above results indicated that the bark of trunk and roots are the storage site for dragon's blood under natural conditions, and whether the tissue appears red can be used as a criterion by which to identify the presence or absence of dragon's blood.

Tissue-specific expression analysis of genes related to biosynthesis of flavonoids

The flavonoids are the main chemical constituents in dragon's blood and account for about 50–80% of the total phytochemical content (Helal et al., 2021; Sun et al., 2019). To confirm the reasons for differences in accumulation of dragon's blood components between different tissues of the *D. cochinchinensis* tree, seven genes associated with flavonoid biosynthesis were chosen

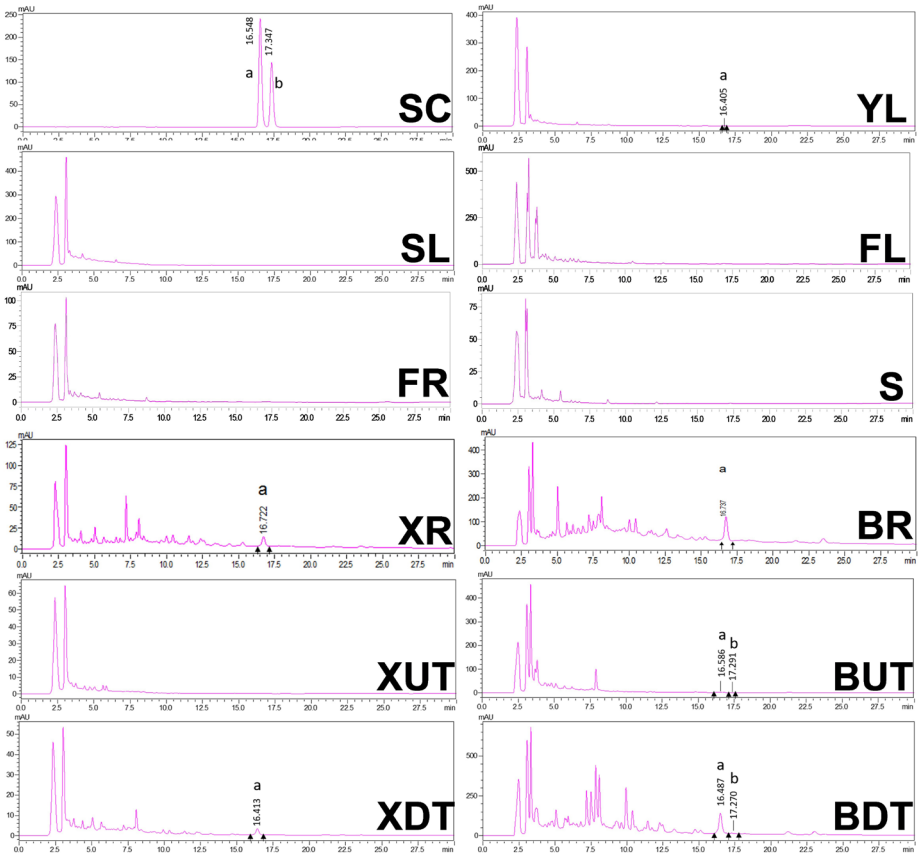

Figure 3. HPLC chromatograms in the test samples (SCa and SCb represent standard compounds loureirin A and loureirin B, respectively). The different organ samples are young leaves (YL), senescent leaves (SL), flowers (FL), fruits (FR) and seeds (S); and the different tissue samples are xylem of the root (XR), bark of the root (BR), xylem of the undamaged trunk (XUT), bark of the undamaged trunk (BUT), xylem of the damaged trunk (XDT) and bark of the damaged trunk (BDT).

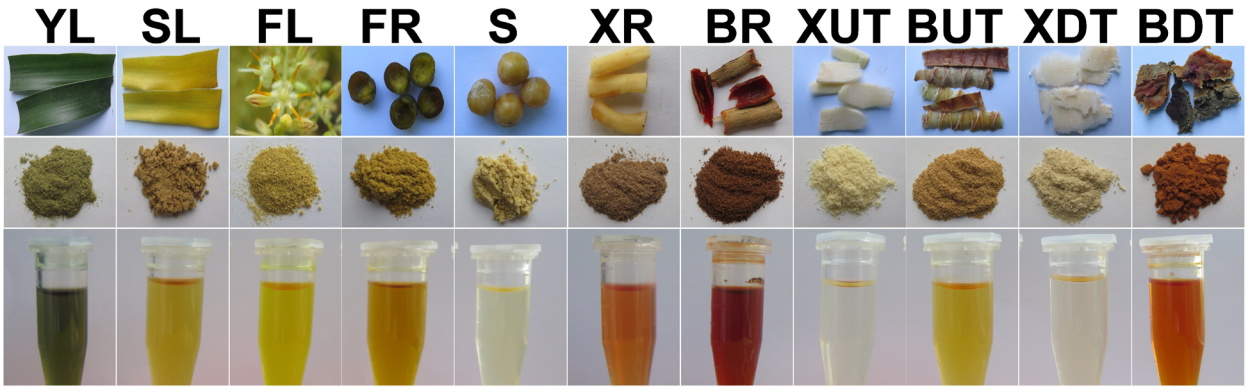

Figure 4. Preparation of different tissue samples of *Dracaena cochinchinensis*, the morphology of different tissues, their powder and their extract. The different organ samples are young leaves (YL), senescent leaves (SL), flowers (FL), fruits (FR) and seeds (S); and the different tissue samples are xylem of the root (XR), bark of the root (BR), xylem of the undamaged trunk (XUT), bark of the undamaged trunk (BUT), xylem of the damaged trunk (XDT) and bark of the damaged trunk (BDT).

for evaluation of gene expression, using quantitative real-time PCR (qPCR) assays. The expression profiles of these genes are shown in Figure 5 and Figure 6. From the phenylpropanoid pathway (Figure 5), the expression levels of the PAL and C4H genes in tissues in which dragon's blood accumulated were higher than those in non-accumulating tissues, such as BR and BDT. The 4CL gene was highly expressed in almost all tested tissues in this study, suggesting that it may be a gene constitutively expressed in most tissues.

From the flavonoid pathway (Figure 6), genes encoding CHS and DFR were highly expressed in tissues that accumulated dragon's blood, such as BDT, BR and XR, but not expressed or expressed at lower levels in tissues which did not accumulate dragon's blood. On the other hand, CHI and F3'H genes were not expressed or were expressed at only low levels in the tissues which accumulated dragon's blood. By comparing the expression patterns of these seven genes in each tissue, we found four genes (encoding PAL, C4H, CHS and DFR) with predominant expression in BDT tissue, one gene (encoding CHI) in flowers and one gene (encoding F3'H) in fruits. Most of the genes mentioned above showed the highest expression in BDT (bark of the damaged trunk) of the *D. cochinchinensis* tree, suggesting that the trunk bark may be not only the main storage site for dragon's blood but also the main site for synthesizing the precursors and components of dragon's blood.

DISCUSSION

The cause of dragon's blood synthesis and storage in the trunk and roots of *Dracaena* tree under natural conditions

The HPLC and qPCR results from this study indicated that the trunk bark of *D. cochinchinensis* tree can accumulate large amounts of dragon's blood and express some of the genes important for the synthesis of flavonoids under natural conditions. Previous studies have reported that dragon's blood is mainly produced from the trunk xylem, and that synthesis is induced by fungal, chemical or mechanical damage (Wang et al., 2011; Zhu et al., 2016; Cui et al., 2013).

Many studies shown that various biotic or abiotic stresses can induce the *Dracaena* tree to produce dragon's blood (Ding et al., 2020; Zhu et al., 2020). The *Dracaena* tree has no special tissue to secrete and store dragon's blood, which is synthesized and accumulated when the plant is stressed (Cui et al., 2013). The strong antimicrobial activities of dragon's blood *in vitro* and *in vivo* have been demonstrated in many studies (Ou et al., 2013). In the *Dracaena* tree, the dragon's blood can degrade the hyphae and prevent the rapid growth of invasive fungi (Cui et al., 2013).

Under natural conditions, the trunk bark of *Dracaena* trees acts as the first line of defense against stresses, such as microbial invasion, so the trunk bark produces considerable amounts of dragon's blood to protect the essential xylem tissue from damage. The root bark has morphological characteristics and profiles of dragon's blood components broadly similar to those of the trunk bark. This indicates that the roots are also under stress in natural conditions, and hence produce dragon's blood. Therefore, we can consider that trunk bark and root bark are the main storage and synthesizing tissues of dragon's blood under natural conditions.

The possible to use roots and leaves to produce dragon's blood

In commercial production, dragon's blood is extracted from the trunk of *Dracaena* trees over 30–50-years-old, although some trees may be thousands of years old (Wang et al., 2011; Tsai and Xu, 1979). However, the roots of the *Dracaena* trees are discarded because they are difficult to dig out and hence are not used to produce dragon's blood for TCM. Our study demonstrated that roots can produce and accumulate

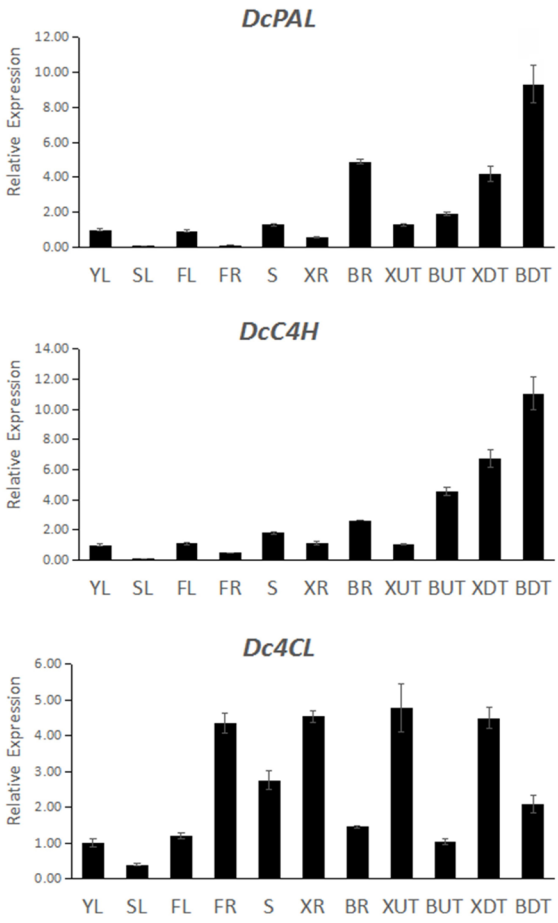

Figure 5. Real-time quantitative PCR (qPCR) analysis of phenylpropanoid pathway genes (*DcPAL*, phenylalanine ammonia-lyase; *DcC4H*, cinnamate-4-hydroxylase; Dc4CL, 4-coumarate-CoA ligase). Gene expression was quantified (as relative expression) from extracts of different tissue samples: young leaves (YL), senescent leaves (SL), flowers (FL), fruits (FR), seeds (S), xylem of the root (XR), bark of the root (BR), xylem of the undamaged trunk (XUT), bark of the undamaged trunk (BUT), xylem of the damaged trunk (XDT) and bark of the damaged trunk (BDT).

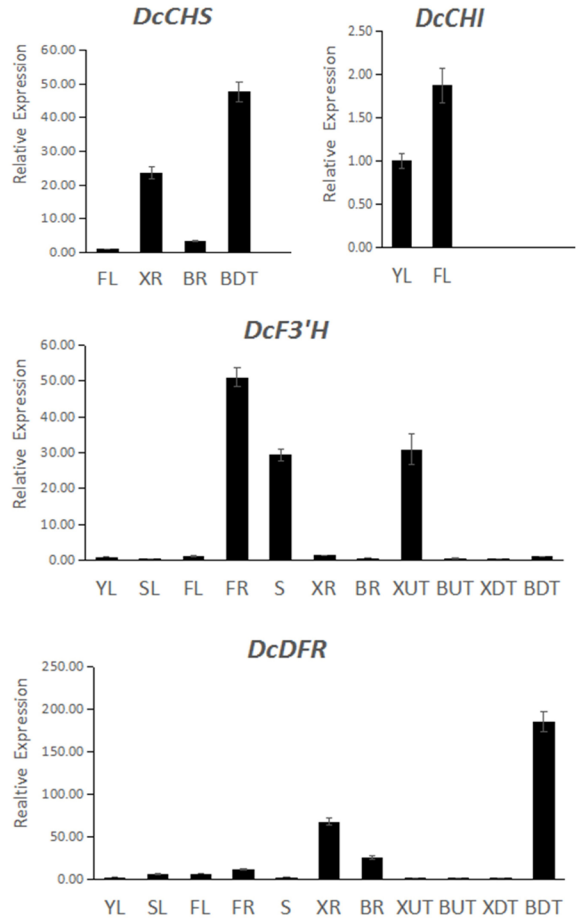

Figure 6. Real-time quantitative PCR (qPCR) analysis of flavonoid pathway genes (*DcCHS*, chalcone synthase; *DcCHI*, chalcone isomerase; *DcF3'H*, flavonoid 3'-hydroxylase; *DcDFR*, *dihydroflavonol* 4-reductase). Gene expression was quantified (as relative expression) from extracts of different tissue samples: young leaves (YL), senescent leaves (SL), flowers (FL), fruits (FR), seeds (S), xylem of the root (XR), bark of the root (BR), xylem of the undamaged trunk (XUT), bark of the undamaged trunk (BUT), xylem of the damaged trunk (XDT) and bark of the damaged trunk (BDT).

dragon's blood, with chemical composition almost identical to that obtained from the trunk.

Some studies have shown that the leaves of *Dracaena* can be induced by fungi to produce dragon's blood, with physical and chemical profiles similar to those of the natural product (Ou et al., 2013; Zhu et al., 2021). We also detected trace amounts of loureirin A, one of the red-colored components of dragon's blood, in the leaves. These results suggested that leaves of the *Dracaena* tree have the potential to produce dragon's blood, with the advantage that leaves are renewable, the harvesting of which will not damage the *Dracaena* tree (Ou et al., 2013).

The red color intensity and loureirin A concentration should be used as a more accurate marker of the quality of dragon's blood

Loureirin B is arbitrarily used as the standard for quality evaluation of dragon's blood preparations in the Pharmacopoeia of the People's Republic of China. However, the concentration of loureirin A was much higher than that of loureirin B in both fungal-induced and commercial dragon's blood (Ou et al., 2013; Cui et al., 2013). Similarly, we detected that the concentration of loureirin A was much higher than that of loureirin B in natural dragon's blood extracted from *D. cochinchinensis*. In addition, the dragon's blood extracted from root bark (BR) contained high concentrations of loureirin A, but no loureirin B was

detected in it. These results indicated that loureirin B is not suitable as the standard marker of the pharmacological activity of dragon's blood preparations. Previous studies have reported that dihydrochalcones are responsible for the red color of dragon's blood (Zheng et al., 2006, 2012). Both loureirin A and loureirin B are dihydrochalcones. Therefore, we propose that the intensity of the red color of dragon's blood, along with the loureirin A concentration, should be used as a more accurate marker of the quality of dragon's blood preparations in quality control.

CONCLUSION

In summary, the components of dragon's blood extracted from various tissues of the *D. cochinchinensis* tree were identified and the expression of genes involved in the biosynthesis of components of dragon's blood was analyzed in this study. We confirmed that the bark of the roots and trunk of the *Dracaena* tree were the main tissues for the synthesis and storage of dragon's blood under natural conditions. The utilization of the roots to produce dragon's blood could allow for a secondary exploitation of *Dracaena* resources, which would allow harvesting without killing the *Dracaena* tree, as harvesting trunk bark does, thus benefiting the wild *Dracaena* tree populations. In addition, it was possible to produce dragon's blood from leaves by artificial induction, which could contribute to the sustainable use of wild *Dracaena* tree resources.

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AUTHORSHIP CONTRIBUTION

Project Idea: YW
Funding: YW, JW, JQ, LZ
Database: YW, SL, CY
Processing: SL, YW, JP
Analysis: YW, SL, GL, ZY, YM
Writing: YW, SL
Review: YW, EL, JW, LZ

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