

Growth, photosynthesis and podophyllotoxin accumulation of *Dysosma versipellis* in response to a light gradient and conservation implications

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Dysosma versipellis (Hance) M. Cheng ex Ying (Berberidaceae) is a rare and vulnerable, perennial herb endemic to China with pharmaceutical significance. Increasing habitat loss and over-exploitation of the plant has severely affected the plant's *in situ* conservation, necessitating *ex situ* conservation and commercial cultivation. The light regime is a critical environmental factor contributing to successful *ex situ* conservation via efficient production of biomass and secondary metabolites. We investigated the responses of 2-year-old *D. versipellis* plants to a light gradient in terms of leaf morphology, growth, biomass production, photosynthesis and podophyllotoxin (PTOX) accumulation. *D. versipellis* responded to the light gradient in terms of plant height, leaf size, leaf photosynthesis and PTOX accumulation. Plants demonstrated optimal vegetative growth, photosynthetic capability, and PTOX production, under a light intensity equal to 30% full sunlight. Half full sunlight led to the lowest production of PTOX despite having no obviously different effects on growth. Full sunlight was able to cause leaf death in mid-summer (July), although these plantlets did not significantly differ in growth or biomass production before July and new leaves emerged in August. *D. versipellis* is therefore proposed to be a shade-tolerant plant, well adapted to variations in irradiance. Irradiance of 10%–50%, particularly ca. 30% full sunlight is strongly recommended for *ex situ* conservation and commercial cultivation.

***Dysosma versipellis*, light response, photosynthesis, podophyllotoxin accumulation, *ex situ* conservation**

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Ex situ conservation has played an increasingly important role in stemming loss of biodiversity [1]. A simulation of a natural habitat can guarantee *ex situ* survival of conserved plants. Light regime is a critical environmental factor affecting plant growth, distribution, and yield [2,3]. To achieve the ultimate goal of biodiversity conservation, commercial plantations of wild economic plants, including medicinal plants, will be essential. Thus, the yield of a desired natural product is the key for industrial scale production. Light variation can be a significant factor affecting

secondary metabolism in plants as well as plant growth [4]. Some surveys have supported the effect of the light regime on plant secondary metabolism [5–7]. An optimal light intensity is, therefore, a question needing exploration for successful *ex situ* conservation or efficient commercial plantation of each endangered plant.

Dysosma versipellis (Hance) M. Cheng ex Ying (Berberidaceae) is a rare and vulnerable, perennial herb endemic to China [8]. A total of 30 populations are known to inhabit disjunct stands of warm-temperate, deciduous, montane forest across central and eastern China [9]. The plant's rhizome has long been applied for the treatment of snake bites,

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parotitis, and rheumatism, in traditional medicine [10]. In recent decades *D. versipellis* has attracted the interest of the pharmaceutical industry due to the presence of podophylotoxin (PTOX), a lignan used as a leading compound in the synthesis of several popular anti-tumor drugs [11,12]. Excessive medicinal exploitation coupled with natural habitat loss has rendered *D. versipellis* at a higher risk of extinction, necessitating immediate protection [13,14]. To conserve this endangered species, we established successful systems for both *ex situ* conservation and pre-commercial cultivation [15–17]. However, its physioecology, including the effects of light intensity on its growth and PTOX production, remains poorly evaluated.

The general biosynthetic pathway of PTOX has been outlined previously [18]. Deoxypodophylotoxin has been thought to be the common precursor of both PTOX and β -peltatin; these can be further synthesized into PTOX-glucoside and β -peltatin-glucoside, respectively. Despite a body of research into PTOX production at the cellular level *in vitro* and relating to levels of specific compounds in the plant tissues *in vivo*, no assessment has yet been performed on PTOX dynamics *in vivo* in light of PTOX biosynthesis.

We therefore addressed the following questions: (i) How do *D. versipellis* plants respond to a light gradient in terms of morphology, biomass yield, and photosynthesis? and (ii) What is the response of PTOX accumulation to a light gradient *in vivo*? Our results will improve the understanding of plant responses to variation in irradiance to facilitate optimal light intensity for both *ex situ* conservation and commercial cultivation.

1 Materials and methods

1.1 Study station and plant materials

The field experiment was conducted at Linfeng Farm of Medicinal Plants, Pan'an County, Zhejiang Province, China (120°32'–56"E, 29°1'–25"N, 835 m a.s.l.) in a typical subtropical zone with mild seasonal temperature variation and plentiful precipitation. The climate is mainly characterized by average annual air temperature: 13.9–17.4°C, extremely low temperature in January: 2°C, extremely high temperature in July: 28.8°C, average annual precipitation: 1409.8–1527.8 mm, and average precipitation in July and August: 208.3–246.5 mm.

Plantlets of *D. versipellis* produced via tissue culture were transplanted to the Medicinal Plant Farm in April, 2008, and survivals with a uniform size (ca. 10 cm in height measured in August 2008) were marked and introduced to experimental blocks on April 1, 2009. *D. versipellis* is a typical understory herb in habitats with a light intensity of 10%–40% (occasionally up to 58%) full sunlight. It can survive full sunlight after introduction 4 natural habitats (data not shown). Accordingly, four irradiance levels (10%, 30%, 50% and 100% sunlight) were simulated using poly-

propylene shading cloth. Each treatment included 7 plants. Based on our phenological observations in the Botanical Garden of Zhejiang University, these perennial plants with 5–7 rhizomatous nodules introduced from the wild produced shoots in early March, bloomed in early April, subsequently set fruit, senesced in August, and perished in late September (data not shown). The plants regenerated via tissue culture, however, presented no flowers during the first 3 years, and displayed longer annual vegetative growth, growing until October. Plant materials were harvested on September 1, prior to leaf senescence, for analysis.

1.2 Leaf photosynthesis, plant morphology, growth and biomass analyses

Gas exchange measurements were made on the centers of fully expanded, healthy leaves using a portable LI-6400 photosynthesis system (LI-COR, Lincoln, USA) in open system mode, during the morning (07:00–11:00) of a sunny day (August 28, 2009). Three replications were performed for each light gradient. Net photosynthetic rate (P_n) – photosynthetic photon flux density (PPFD) response curves were generated based on the assumption of natural conditions (approximately 380 $\mu\text{mol mol}^{-1} \text{CO}_2$, relative humidity of 40%–60%, 500 $\mu\text{mol s}^{-1}$ air flow, and temperature of $(30 \pm 3)^\circ\text{C}$) at irradiance gradients of 1800, 1600, 1400, 1000, 600, 300, 100, 60, 30, 10 and 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$, using a built-in LED-B light source.

Plant height, leaf blade diameter, and petiole diameter were measured on all individuals each month during the studied growing season. We compared the data generated on August 21 (closest to when photosynthesis was measured – August 28) with that generated when the plant materials were harvested (September 1). Finally, 3 of the 7 individuals in each treatment were harvested, separated into leaves (blades plus petioles), rhizomes and roots. All samples were dried at 40°C for 48 h, and then weighed.

1.3 Lignan determination

The dried rhizomes were ground using a MM400 ball mill (Retsch, Germany), and ultrasonically extracted with methanol (0.1 g:2 mL) for 30 min. The extraction was centrifuged at 10000 r/min for 10 min preceding filtering of the supernatant through a 0.22- μm membrane following a procedure modified from that previously described [19]. All solutions were analyzed using a Varian Prostar 210 HPLC system, consisting of a ProStar 335 diode array detector, a manual injector, a column oven, and a data system (Galaxie Workstation). Chromatographic separation was performed on an Agilent HC C18 column (250 mm \times 4.6 mm, 5 μm) using a gradient of methanol (A) and 0.01% aqueous phosphoric acid (B). The gradient program was designed as follows: 0–15 min, 15%–45% A; 15–45 min, 45% A; 45–55 min, 45%–70% A; 55–75 min, 70%–75% A; with flow rate of 1 mL min^{-1} . Column temperature was maintained at 35°C,

and the detection wavelength was set at 254 nm. Five lignans were qualified and quantified with corresponding reference compounds: PTOX (purity $\geq 99\%$, National Institute for Food and Drug Control), deoxypodophyllotoxin (purity $\geq 98\%$, provided by Professor Xuan Tian, Lanzhou University, China), 4'-podophyllotoxin-glucoside (purity $\geq 95\%$), β -peltatin (purity $\geq 94\%$) and β -peltatin-glucoside (purity $\geq 94\%$). We prepared the latter three from the rhizomes of *D. versipellis*.

1.4 Statistical analyses

Significance of difference of each parameter among treatments was evaluated by one-way ANOVA followed by a LSD post hoc test with a significance level of 0.05, using the SPSS software (version 16).

2 Results

2.1 Growth, morphology and biomass allocation

Light significantly affected the leaf morphology of *D. versipellis*. Leaves were severely damaged under full sunlight in mid-summer (July), although they grew vigorously in the spring (from April to June). Following leaf death from over-irradiance, new leaves emerged in August. Plants growing under the other 3 light levels displayed vigorous, green leaves; those under 30% sunlight appeared relatively darker in color. Plant height, leaf blade diameter and petiole diameter differed significantly between the 4 light treatments. Generally, plants were shorter with smaller leaves as sunlight intensity increased (Figure 1). There were no significant differences in petiole diameter with increasing light gradient except that plants under 50% sunlight had thinner petioles. All plants produced similar total biomass (no statistical differences), as well as similar allocation patterns (Figure 2). *D. versipellis* allocated an average of 85% of its biomass to underground organs (rhizomes plus roots) and the remaining 15% to the aerial plant parts.

2.2 Leaf photosynthesis

Regardless of the irradiance treatment, P_n values rose rapidly as PPFD increased to $400 \mu\text{mol m}^{-2} \text{s}^{-1}$, and then increased slowly to a maximum followed by a general leveling off until the greatest PPFD of $1800 \mu\text{mol m}^{-2} \text{s}^{-1}$ was reached (Figure 3). The light compensation point (LCP) of plants in full sunlight ($72.48 \pm 26.07 \mu\text{mol m}^{-2} \text{s}^{-1}$) was significantly higher than of those under 50% ($24.60 \pm 9.95 \mu\text{mol m}^{-2} \text{s}^{-1}$), 30% ($8.46 \pm 4.00 \mu\text{mol m}^{-2} \text{s}^{-1}$) and 10% ($12.01 \pm 2.95 \mu\text{mol m}^{-2} \text{s}^{-1}$) sunlight. All test plants displayed similar light saturation points (LSPs) of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$. Plants under 30% sunlight treatment showed the highest maximum P_n (P_{max}) while those under full sunlight exhibited the lowest, and those under either 10% or 50% sunlight gave intermediate values.

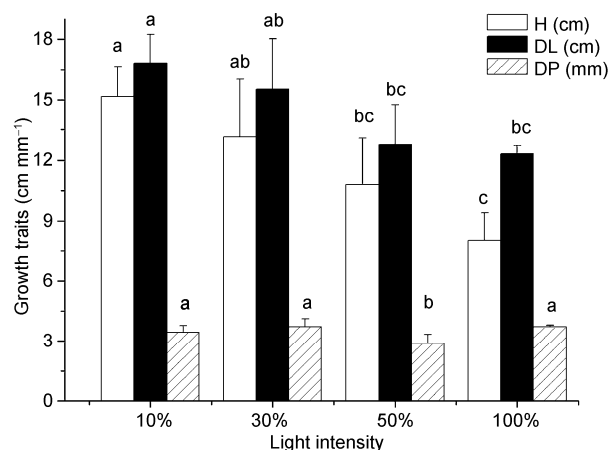


Figure 1 Comparisons of vegetative growth traits of *D. versipellis* under different light conditions. H, plant height; DL, leaf blade diameter; DP, petiole diameter. Lowercase letters indicate significant differences ($\alpha=0.05$).

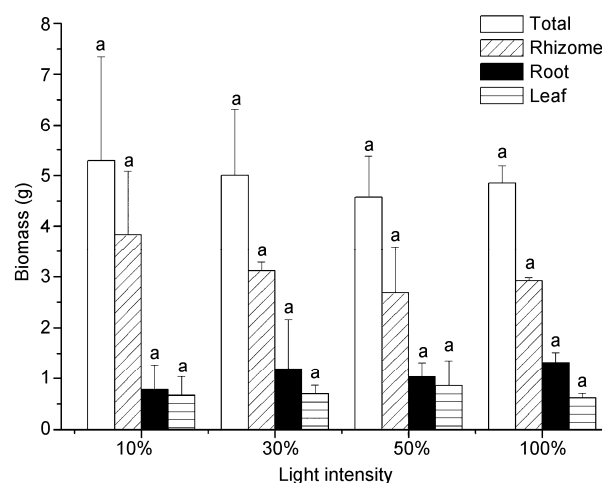


Figure 2 Production and allocation of vegetative biomass of *D. versipellis* under different light conditions. Total, total biomass; rhizome, biomass in rhizomes; root, biomass in roots; leaf, biomass in leaves. Lowercase letters indicate significant differences ($\alpha=0.05$).

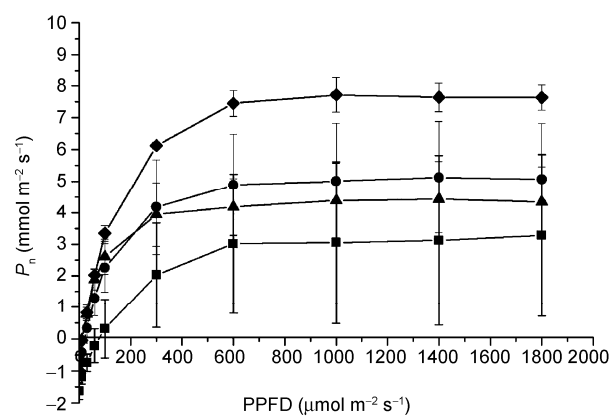


Figure 3 Photosynthetic light response curves of *D. versipellis* under different light conditions. Irradiance rate: 10% (▲), 30% (◆), 50% (●), and 100% (■).

Both LCP and P_{\max} values varied significantly ($P < 0.05$) across the light gradient. The greatest value of P_{\max} and the lowest value of LCP were observed in plants under 30% sunlight.

2.3 Lignan dynamics

Levels of 5 analyzed lignans varied significantly in the rhizomes of *D. versipellis*. Deoxypodophyllotoxin was the most abundant ($16.00 \pm 3.70 \mu\text{mol g}^{-1}$); 4.2 times the level of PTOX and 12.7 times the level of β -peltatin (Table 1). PTOX levels were higher than those of β -peltatin. Both of these two aglycones were at far lower levels than their corresponding glycosides, leading to significantly higher levels of total glycoside. Total levels of PTOX and its glycoside were higher than those of β -peltatin and its glycoside, and the ratio of β -peltatin-glycoside to β -peltatin was significantly higher than that of PTOX-glycoside to PTOX.

PTOX and its derivatives varied remarkably with increasing light gradient. PTOX was at higher levels under 30% and full sunlight, than under 10% and 50% sunlight. β -peltatin only exhibited a rise under full sunlight (Table 1). Their common precursor, deoxypodophyllotoxin, maintained relatively stable levels except for a significant decrease under 50% sunlight. PTOX-glycoside showed the same trend as deoxypodophyllotoxin. β -peltatin-glycoside was the most variable lignan analyzed; it was at its highest concentrations under middle irradiance (30% and 50% sunlight) and was significantly reduced with either an increase or a decrease in irradiance. β -peltatin-glycoside was also the only metabolite showing increased levels under 50% sunlight. Total glycoside levels appeared highest under 30% sunlight, and fell with either an increase or a decrease in irradiance. The ratio of β -peltatin-glycoside to β -peltatin was quite close to the ratio of PTOX-glycoside to PTOX under extremely high (100%) or low (10%) irradiance, whereas the former was ca. 4.5 times the latter under middle

irradiance (30% and 50%).

3 Discussion

Irradiance levels greatly affected plant growth. As expected, the tested *D. versipellis* plants growing under decreasing irradiance produced increasingly larger leaves and taller plants. This is consistent with the trend observed in many other shade-tolerant plant species, especially in *Podophyllum peltatum* [20], a North American Berberidaceae species that is famous for its PTOX. We found that larger leaves, however, did not possess greater leaf mass, and consequently the light gradient did not influence total leaf mass in addition to total mass.

D. versipellis, however, appeared to respond to changes in irradiance by changing its photosynthetic capacity. Plants exhibited the strongest photosynthetic capability in terms of P_n under 30% sunlight, and showed significant reductions under irradiance of both flanking spectra. This parallels the patterns found in another shade-tolerant plant, *Tetrastigma hemsleyanum* [21]. The trends in the light compensation point (LCP) coincided with those in P_n in *D. versipellis*, and were in contrast to those in *T. hemsleyanum*, in which LCPs were reduced under full and 50% sunlight. The LCP of our studied plant under 10% sunlight was similar to that under 30% sunlight with smallest value; this may indicate shade adaptation [22]. Plants acclimated to a high light environment tend to display increased respiration and LCP [22]. This could explain why the highest LCP of the emerged leaves of *D. versipellis* was under full sunlight, suggesting acclimation to strong sunlight. LCP and LSP are considered effective indicators of the light requirements of plants, and sciophytes generally exhibit lower values than heliophytes [23]. *D. versipellis* demonstrated smaller LCPs ($8.46\text{--}72.48 \mu\text{mol m}^{-2} \text{s}^{-1}$) and greater LSPs (ca. $600 \mu\text{mol m}^{-2} \text{s}^{-1}$) than *Sinopodophyllum hexandrum* (synonyms: *Podophyllum*

Table 1 Levels of podophyllotoxin and its derivatives in rhizomes of *D. versipellis* under different light conditions ($\mu\text{mol g}^{-1}$)

Irradiance rate	10%	30%	50%	100%	Mean ^{a)}
β -peltatin-glycoside	4.27±0.40 b	7.66±0.79 a	7.91±0.13 a	1.75±0.60 c	5.40±2.70 bc
β -peltatin	1.07±0.11 b	1.21±0.03 b	1.17±0.07 b	1.58±0.06 a	1.26±0.21 d
Deoxypodophyllotoxin	18.66±5.45 a	16.84±0.75 a	11.89±1.23 b	16.59±2.43 a	16.00±3.70 a
Podophyllotoxin	2.33±0.21 b	4.88±0.80 a	2.98±0.24 b	5.14±0.05 a	3.84±1.31 c
4'-podophyllotoxin-glycoside	7.59±0.56 a	6.72±1.35 a	4.57±0.64 b	8.15±1.41 a	6.76±1.69 b
Total	33.93±6.74 a	37.32±3.73 a	28.51±2.31 b	33.23±4.54 a	
Total glucosides	11.86±0.52 b	14.38±0.86 a	12.48±0.53 b	9.91±0.82 c	12.16±1.77 a
β -peltatin and its glucoside	5.35±0.30 b	8.87±0.82 a	9.08±0.07 a	3.33±0.65 c	6.66±2.57 b
PTOX and its glucoside	9.92±0.51 b	11.60±1.52 ab	7.55±0.79 c	13.30±1.38 a	10.59±2.42 a
Ratio of peltatin-glycoside to peltatin	4.03±0.81 b	6.35±0.48 a	6.81±0.52 a	1.10±0.34 c	4.57±2.41 a
Ratio of PTOX-glycoside to PTOX	3.27±0.44 a	1.40±0.36 b	1.53±0.19 b	1.59±0.28 b	1.95±0.85 b

a) Italic lowercase letters indicate significance of vertical comparison, and non-italic indicate significance of horizontal comparison ($\alpha=0.05$).

hexandrum and *P. emodi*); the latter showed LCPs of 19.30–104.00 and LSPs of 211.00–452.00 $\mu\text{mol m}^{-2} \text{s}^{-1}$ [24]. Because *S. hexandrum* prefers sunlight and tolerates shade, *D. versipellis* seems to have adapted to greater variation in irradiance. This could explain our observations of irradiance in the natural habitat ranging from 10% to 58% sunlight, and also the survival of *D. versipellis* under full sunlight (data not shown). However, the highest value of P_{max} and vigorous growth under 30% sunlight indicated its preference for shade. This is also consistent with the most frequent light intensity in natural habitats of about 30% sunlight (data not shown).

Similarly to its relative, *P. peltatum* [20], and other reported plant species [6,7], *D. versipellis* responded prominently to the light gradient in terms of its production of secondary metabolites of the five lignans including PTOX. Deoxypodophyllotoxin, the common precursor of both PTOX and β -peltatin, showed a high and stable concentration with increasing light gradient. It seemed that the allocation of deoxypodophyllotoxin differed between the two shunt pathways, and was further affected by changes in light intensity. The shunt pathway of PTOX significantly preceded that of β -peltatin in terms of the total concentration of aglycone and the corresponding glycoside. The concentration peak in the former pathway was produced under either 30% or 50% sunlight, in contrast to that in the latter pathway, produced at 100% or under 30% sunlight. Generally, 30% sunlight benefited high production in both pathways. Interestingly, the dynamic trend in the total glycosides (PTOX-glucoside and β -peltatin-glucoside) paralleled that of photosynthetic capacity (P_n) (Figure 3 and Table 1). However, the two shunt pathways showed inconsistent responses. It appeared that the β -peltatin pathway was more sensitive to changes in light intensity because fluctuation was noticeable in the levels of β -peltatin or its glycoside; their ratio indicating the degree of glycosylation. Increased glycosylation may be one of the consequences of increasing photosynthesis and assimilation. On the contrary, β -peltatin-glucoside was strongly deglycosylated when photoinhibited. PTOX glycosylation remained relatively stable except for a significant response to the weakest irradiance (10% sunlight).

4 Conclusion

D. versipellis responded to a light gradient in terms of plant height, leaf size, leaf photosynthesis and PTOX accumulation. The plant demonstrated optimal vegetative growth and photosynthetic capability as well as PTOX production under a light intensity of 30% full sunlight. Half full sunlight led to the lowest production of PTOX despite no remarkably different effects on growth. Full sunlight was observed to lead to leaf death in 2-year-old plantlets in mid-summer

(July), although these plantlets differed insignificantly in growth and biomass production before July and new leaves emerged in August. *D. versipellis* is thus proposed to be a shade-tolerant plant with well adapted to variation in irradiance. Irradiance of 10%–50%, particularly ca. 30% full sunlight is strongly recommended for *ex situ* conservation and commercial cultivation.

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