Industrial Crops & Products 121 (2018) 338–344

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

High-elevation cultivation increases anti-cancer podophyllotoxin accumulation in *Podophyllum hexandrum*

Mengfei Li[a,b], Li Ge[a], Tianlan Kang[c], Ping Sun[a], Hua Xing[a], Delong Yang[a], Jinlin Zhang[a,d,**], Paul W. Paré[b,*]

[a] Gansu Provincial Key Lab of Arid Land Crop Science/College of Life Science and Technology, Gansu Agricultural University, Lanzhou 730070, PR China
[b] Texas Tech University, Department of Chemistry and Biochemistry, TX 79409, USA
[c] Station of Industrial Crop Promotion of Gansu province, Lanzhou 730000, PR China
[d] College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou 730000, PR China

ARTICLE INFO

Keywords:
Podophyllum hexandrum
High-elevation adaptation
in situ plant growth
Podophyllotoxin accumulation

ABSTRACT

Podophyllum hexandrum, a perennial alpine herb produces the anti-cancer metabolite podophyllotoxin (PPT) that is in large part responsible for the plant's endangered species status. Since PPT commercial production via chemical synthesis, biotechnological intervention and/or cultivation by *ex situ* conditions have yet to meet the ever-increasing demand for this potent anticancer drug, identifying cultivation practices to improve PPT accumulation is essential. While *P. hexandum* is indigenous to the mountainous Himalayan region of Asia, effect of elevations on plant growth and PPT accumulation has not been systematically investigated. Here is reported plant growth and PPT production at two elevations: 2300 and 3300 m. To dissect genetic versus environmental conditions responsible for enhanced growth at the higher elevation, plants adapted to each elevation were transplanted to the alternative site. Aerial and rhizome dry-weight was 1.4- to 2.0-fold and 1.2- to 2.0-fold greater, respectively at the 3300 m versus the 2300-m site for 3- to 5-year-old plants. Other growth parameters including leaf area, rhizome length/diameter, number of petioles, root and fruit per plant and fruit dry weight per plant showed an increased value at the higher elevation. PPT content in the aerial portions and rhizomes for all years studied was greater at the 3300 m with 2.2- to 5.3-fold and 2.2- to 3.5-fold on a per plant basis compared to the 2300-m site. Based on the plant's perennial and fruiting characteristics, a sustainable harvesting scheme that includes the plant's aerial portions, rhizomes and seeds is proposed for improving PPT yield without over harvesting of this endangered species.

1. Introduction

Podophyllum hexandrum (family Berberidaceae), commonly referred to as Himalayan Mayapple, is a perennial rhizomatous species (Fig. S1) distributed in restricted alpine Himalayan regions including China, India, Nepal, Pakistan, Afghanistan and neighboring areas at elevations ranging from 1300 to 4500 m (Alam and Naik, 2009; Guo et al., 2014). Rhizomes are the major source of the highly valued aryltetralin lignan, podophyllotoxin (PPT), which is used worldwide for the treatment of various skin lesions such as warts, non-cervical human papilloma virus and genital infections (Mayeaux et al., 1995; Lv and Xu, 2011). PPT is also employed as a natural product precursor for semi-synthetic production of anticancer chemotherapies including the semi-synthetic derivatives etoposide, etoposide phosphate and teniposide (Canela et al., 2000; Giri and Narasu, 2000). These compounds provide effective treatment for lung, cervical and testicular cancers as well as neuroblastoma, hepatoma and select leukemias (Lv and Xu, 2011; Lau and Sattely, 2015).

P. hexandrum is currently an endangered species, as risk of extinction, in large part because of an indiscriminate uprooting of wild plants to meet the ever-increasing demand of the pharmaceutical industry (Airi et al., 1997; Alam et al., 2009a; Gupta and Dutta, 2011; Kitchlu et al., 2011). Due in large part to the increasing commercial demand for PPT, *P. hexandrum* plant availability has decreased significantly over the last 20 years (Bhadula et al., 1996; Lv and Xu, 2011). High PPT production is limited by irregular plant growth including a long juvenile phase, poor fruit-setting, uneven germination and a narrowly acceptable growth habitat resulting in limited commercial cultivation. As a result, various approaches such as seed germination, vegetative propagation and plant tissue culture have been studied to improve seedling

* Corresponding author.
** Corresponding author.
E-mail addresses: jlzhang@lzu.edu.cn (J. Zhang), paul.pare@ttu.edu (P.W. Paré).

https://doi.org/10.1016/j.indcrop.2018.05.036
Received 14 December 2017; Received in revised form 28 March 2018; Accepted 17 May 2018
0926-6690/ © 2018 Elsevier B.V. All rights reserved.

Table 1
Environmental parameters at two elevations in Gansu Province, China study sites over three years.

Elevations (m)	Year	X_{AJMAT} (°C)	$X_{AJ\text{-}AMAT}$ (°C)	X_{AAAT} (°C)	X_{AAT} (°C)	X_{AP} (mm)	X_{ASHD} (h)
2300	2015	−9.61 ± 2.64	23.44 ± 3.27	6.70	3533.52	576.81	2505.42
	2016	−13.26 ± 3.93	25.34 ± 3.70	6.65	3648.76	605.56	2489.13
	2017	−11.23 ± 1.65	23.97 ± 4.76	6.72	3635.18	583.28	2513.27
3300	2015	−18.74 ± 1.67	19.90 ± 3.42	5.90	2167.12	640.30	2235.25
	2016	−19.00 ± 3.03	21.90 ± 3.18	5.81	2091.20	668.09	2208.34
	2017	−15.84 ± 1.95	20.90 ± 3.96	5.83	2114.06	662.14	2231.64

X_{AJMAT}, average January minimum air temperatures; $X_{AJ\text{-}AMAT}$, average July-August maximum air temperature; X_{AAAT}, average annual air temperature; X_{AAT}, accumulated annual temperature; X_{AP}, annual precipitation; X_{ASHD}, annual sunshine hour duration.

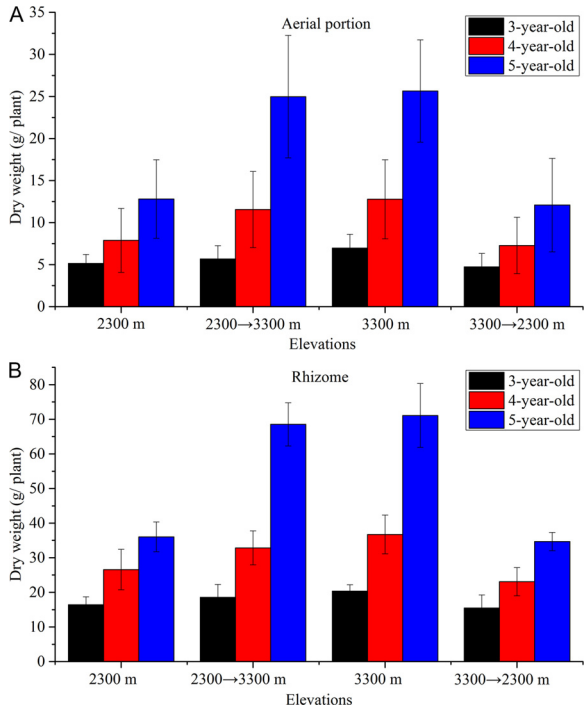

Fig. 1. Biomass accumulations in 3–5-year-old plants grown *in situ* conditions at two elevations. Biomass (without fruit) on a per plant basis (mean ± SD, n = 30).

success (Li et al., 2008; Kharkwal et al., 2008; Nivot et al., 2008; Chakraborty et al., 2010).

PPT chemical synthesis is possible, however production is far from economically feasible due to high synthesis costs (Gordaliza et al., 2004; Kumari et al., 2017). An ancillary approach to increase PPT availability is by biotechnology strategies; methods being investigated include high-yielding PPT cell-culture lines and dissection of the PPT biosynthetic pathway for insertion into transgenic lines (Chattopadhyay et al., 2003; Marques et al., 2013; Rajesh et al., 2013; Rajesh et al., 2014; Lau and Sattely, 2015; Kumari et al., 2017). Identifying alternative plants, such as *P. peltatum*, *Dysosma pleiantha* and *D. versipellis*, that biosynthesize PPT has also been considered for increasing PPT availability (Peng et al., 2006; Kumari et al., 2017); however, accumulation levels are substantially lower than *P. hexandrum* and accordingly are not a commercially viable source (Fay and Ziegler, 1985; Kitchlu et al., 2011).

Studies on the optimization of environmental factors for the domestication, conservation and sustainable utilization of *P. hexandrum* have demonstrated that PPT content is affected by soil nutrients, geographical factors, plant age and harvest stage (Alam and Naik, 2009; Kushwaha et al., 2012; Li et al., 2013; Liu et al., 2015). Cultivation of *P. hexandrum* in glasshouses or *in situ* at lower elevation (1150 m) results in lower PPT levels as compared to wild plants (Sharma et al., 2000; Pandey et al., 2007; Kushwaha et al., 2012; Seegers et al., 2017). Indeed, a positive correlation has been observed between PPT content and increasing elevation (Nadeem et al., 2007; Alam and Naik, 2009; Li et al., 2013; Liu et al., 2015; Pandey et al., 2015). Geographical modeling has predicted that altitude plays a determining role in PPT accumulation with an optimal elevation range of 2800 to 3600 m (Wu et al., 2015). To probe this prediction experimentally, PPT accumulation was examined for plants grown at lower and higher elevations. To confirm the role of elevation over adaptation in PPT accumulation, plants from the two elevations were transplanted to the alternative location. Growth parameters and PPT content for both aerial and rhizome portions of the plant were evaluated at the two sites.

Table 2
Growth parameters in aerial and belowground of 3- to 5-year-old plants grown *in situ* conditions at two elevations.

Elevations (m)	Plant age (Year)	Petiole			Leaf area/plant (cm^2)	Rhizome		
		Number/plant	Height (cm)	Diameter (mm)		Root number	Length of the longest root (cm)	Diameter (cm)
2300	3	1.57 ± 0.73	25.28 ± 3.45	2.83 ± 0.27	83.85	16.28 ± 3.70	13.14 ± 0.91	1.13 ± 0.21
	4	2.40 ± 0.85	35.77 ± 6.60	4.56 ± 0.54	354.14	19.27 ± 5.02	15.74 ± 2.36	1.87 ± 0.27
	5	3.17 ± 1.06	56.23 ± 10.13	5.23 ± 2.23	413.02	21.48 ± 6.21	24.67 ± 4.25	2.25 ± 0.47
2300 → 3300	3	1.73 ± 0.45	22.19 ± 2.19	2.74 ± 0.52	87.67	18.20 ± 2.52	13.26 ± 2.18	1.13 ± 0.14
	4	5.27 ± 1.10	31.47 ± 8.25	4.05 ± 1.86	869.75	22.67 ± 7.60	16.83 ± 4.20	2.03 ± 0.35
	5	14.40 ± 2.67	38.27 ± 6.82	4.77 ± 1.20	2520.70	28.67 ± 6.54	28.86 ± 6.38	3.18 ± 1.09
3300	3	1.87 ± 0.85	22.08 ± 1.25	2.92 ± 0.30	90.15	18.36 ± 3.43	13.67 ± 1.52	1.23 ± 0.18
	4	5.77 ± 1.84	31.57 ± 10.00	4.26 ± 1.37	1015.40	25.27 ± 4.87	20.45 ± 5.00	2.44 ± 0.52
	5	16.10 ± 2.33	38.91 ± 7.63	4.80 ± 2.00	2726.16	29.02 ± 9.10	28.43 ± 6.20	3.23 ± 0.57
3300 → 2300	3	1.60 ± 0.22	24.85 ± 1.08	2.98 ± 0.53	84.10	16.30 ± 2.01	13.30 ± 2.27	1.13 ± 0.43
	4	2.83 ± 0.74	34.87 ± 12.05	4.45 ± 0.88	367.90	18.85 ± 5.62	15.78 ± 3.67	1.92 ± 0.96
	5	3.10 ± 1.12	54.13 ± 7.60	5.25 ± 2.02	433.39	20.09 ± 6.80	22.87 ± 5.98	2.23 ± 0.67

Values are on a per plant basis (mean ± SD, n = 30).

Table 3
Fruit characteristics in 3- to 5-year-old plants grown *in situ* conditions at two elevations.

Elevations (m)	Plant age (Year)	Fruit number	Length (cm)	Diameter (cm)	Dry weight (g)	Seed number	Seed dry weight (g/thousand seeds)
2300	3	nd					
	4	1.13 ± 0.34	6.01 ± 0.83	3.65 ± 0.70	7.63 ± 2.14	139.90 ± 22.40	42.52 ± 1.68
	5	1.50 ± 0.51	5.92 ± 0.56	3.73 ± 0.64	10.10 ± 3.01	183.22 ± 23.24	42.66 ± 0.64
2300 → 3300	3	nd					
	4	1.30 ± 0.47	5.42 ± 1.40	3.26 ± 1.10	5.54 ± 2.10	79.67 ± 20.00	31.28 ± 1.06
	5	2.50 ± 0.50	5.59 ± 0.92	3.28 ± 0.95	11.23 ± 3.23	141.34 ± 24.48	31.60 ± 1.37
3300	3	nd					
	4	1.33 ± 0.48	5.45 ± 0.37	3.28 ± 0.74	5.73 ± 1.75	76.97 ± 16.55	31.53 ± 1.53
	5	2.47 ± 0.48	5.43 ± 0.52	3.33 ± 0.48	11.35 ± 2.82	136.31 ± 22.18	31.52 ± 1.17
3300 → 2300	3	nd					
	4	1.17 ± 0.38	6.23 ± 1.15	3.81 ± 1.42	7.83 ± 1.56	142.31 ± 21.53	42.56 ± 1.49
	5	1.40 ± 0.50	5.90 ± 0.80	3.73 ± 1.27	9.47 ± 2.91	167.70 ± 37.25	42.83 ± 1.40

Values are on a per plant basis (mean ± SD, n = 30). nd, not detected.

2. Materials and methods

2.1. Plant material

2.1.1. Seeds collection

Fully mature fruit of *P. hexandrum* were collected in September, 2012 from alpine and sub-alpine regions located in Gannan Tibetan Autonomous Prefecture (higher elevation, 3250–3350 m; 34°42′53”-34°43′40“N, 102°53′12”-102°55′46“E) and Weiyuan (lower elevation, 2250–2350 m; 34°58′8”-34°59′36“N, 104° 04′52”-104° 09′01“E) of Gansu province, P R. China. Seeds were separated from mature red berries, washed in water, air dried at room temperature and stored at 4 °C in air-tight bags in the dark.

2.1.2. Seed germination and seedling establishment

Approximately 2000 seeds were pre-treated in May, 2013 with 0.01% Bavistin (carbendazim; $C_9H_9N_3O_2$) for 10 min, and then immersed in sterile 500 mg/L GA_3 for 48 h; seeds were then sown at a soil depth of 2.0-2.5 cm at the two elevation sites in which soil and sun exposure (south facing and slope 15–20°) were similar. Germination rate after 25 d was measured by visual inspection to be 85%. After 2 months, seedlings had advanced to the cotyledon stage (Fig. S2A).

2.1.3. Seedling transplantation

In May 2014, 500 two-year-old seedlings (Fig. S2B) were transplanted into the different elevation environment (plants adapted to 2300 m were moved to 3300 m and 3300 m plants moved to 2300 m). No additional fertilizer was applied before or after the transplant. Soil nutrients from different elevations ranging from 2200 to 3600 m were analyzed to probe possible correlations between elevation and soil nutrient. The coefficient of determination (R^2) was calculated to define elevation-nutrient correlations. With all nutrients measured, the R^2 value was less than 0.3 (Fig. S3). These correlation coefficients indicate little if any (linear) correlation as defined by Richard and Gordon (2009), indicating that elevation is not correlated with soil nutrients.

2.1.4. Samples collection and growth analysis

Plant samples were collected in September for three consecutive years (2015–2017). From each site representative samples (30 plants) of three age groups (3-, 4- and 5-year-old, Fig. S2 C-E) were randomly harvested for recording growth characteristics (leaf area, petiole height/diameter, rhizome length/diameter, fruit length/diameter, number of petiole, root and fruit per plant). For harvest, plants were rinsed in water, separated into aerial and rhizome components and dried at 60 °C in a convection oven (HK-350A+, Hangzhou Xuzhong Machinery Equipment Co., Ltd., China).

2.2. Podophyllotoxin (PPT) extraction and HPLC quantification

Plant samples from different tissue components, age and elevation were collected, dried and finely powdered. Tissue material was solvent extracted for PPT based on a previous reported protocol (Sharma et al., 2000; Nadeem et al., 2007; Yang et al., 2016). Sample aliquots (2.0 g) suspended in ethanol (95% v/v, 30 mL) were agitated in the dark for 12 h at 35 °C; samples were then centrifuged at 8000 rpm for 10 min at 4 °C. Following exhaustive extraction (× 3), the upper portion was pooled and partitioned thrice with a water and dichloromethane partition (1:1, v/v; 30 mL each time). The dichloromethane fractions were combined and dried *in vacuo* at 35 °C. Dried residue was re-dissolved in ethanol (95% v/v, 5.0 mL) and filtered (0.22 µm durapore membrane; Millipore, Sigma, USA).

Samples (20 µL) were analyzed using a Nova Pack C_{18} column (250 × 4.6 mm, 5 µm; Shimadzu, Japan) based on a previously reported protocol (Sharma et al., 2000; Yang et al., 2016). Acetonitrile:H_2O:MeOH (37:58:5, v/v/v) was the mobile phase (1.0 mL/min). Compounds were detected at 230 nm with quantification based on peak area comparison with a reference standard curve using PPT (P-4405, Sigma, USA). Plant samples from pooled tissue were extracted in triplicate and each extract was analyzed a single time by HPLC (LC-10A, Shimadzu, Japan).

2.3. Monitoring environmental factors

Environmental factors were collected over three years (2015–2017) from a local meteorological bureau and included: average monthly minimum and maximum air temperatures (January and July-August, respectively); average annual air temperature; accumulated annual temperature above 10 °C; annual precipitation; and the annual sunshine hour duration.

2.4. Statistical analysis

Statistical analysis was performed via a one-way analysis of variance (ANOVA) and Duncan multiple comparison tests. Statistical Product and Service Solutions (SPSS 22.0) was the software package used with $P < 0.05$ as the basis for statistical differences.

3. Results

3.1. Environmental conditions at two elevations

To link plant growth and PPT accumulation with the environmental conditions of temperature, light duration and precipitation for the two study sites, daily meteorological data (governmental reports) was averaged on an annual basis and reported over a three-year period

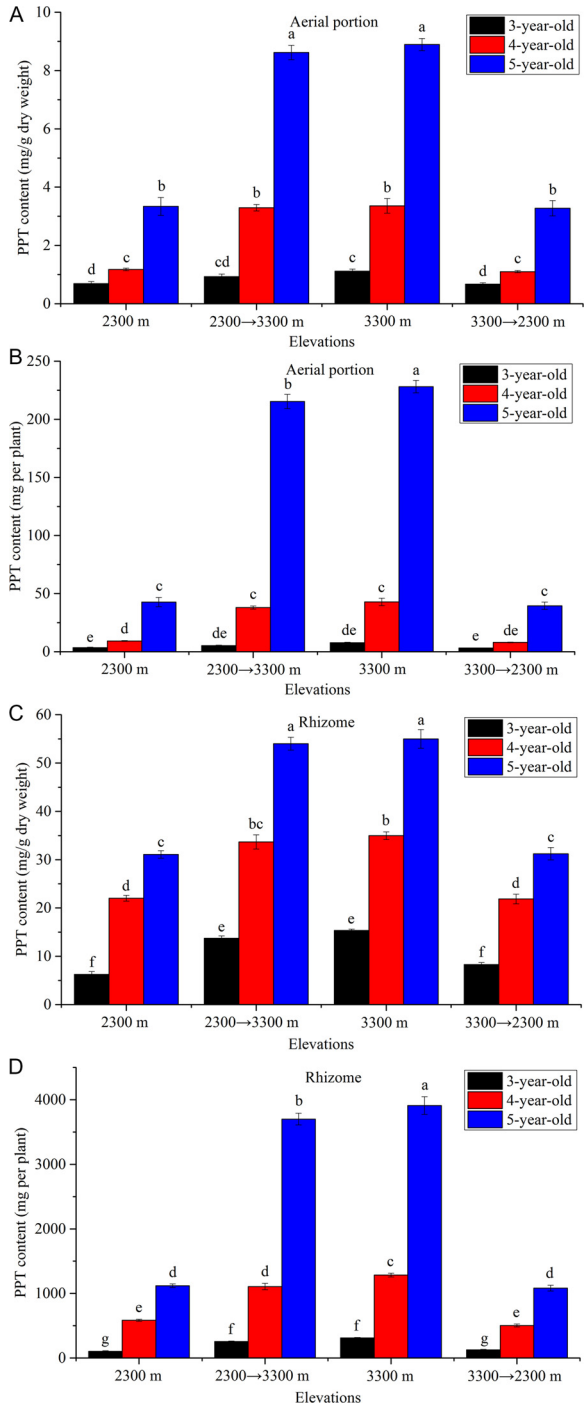

Fig. 2. PPT content in 3–5-year-old plants grown *in situ* conditions at two elevations. PPT amount (without fruit) on a per plant basis (mean ± SD, n = 30). Different letters signify $p < 0.05$ based on SPSS analysis.

(Table 1). Although average annual air temperature (X_{AAAT}) for the two sites varied by less than 1 °C, the average January minimum air temperature (X_{AJMAT}) and average July-August maximum air temperature ($X_{AJ\text{-}AMAT}$) exhibited much greater variation for the two sites. Plant growth occurs between April (germination) and October (leaf senescence), while the X_{AJMAT} defines if the plant will over winter and the number of rhizome buds that will germinate; in addition, the $X_{AJ\text{-}AMAT}$ impacts plant growth and PPT accumulation. Average minimum and maximum air temperatures in January (X_{AJMAT}) and July-August ($X_{AJ\text{-}AMAT}$) were −17.8 °C and 20.9 °C, respectively at 3300 m for the three years recorded; these averages were 4.6–9.1 °C and 3.1–3.5 °C lower in winter and summer, respectively than that of 2300 m. The X_{AAAT}, accumulated annual temperature (X_{AAT}) and annual sunshine hour duration (X_{ASHD}) at 3300 m were also lower as compared to that of 2300 m. Annual precipitation (X_{AP}) at 3300 m was 60 mm more than that of 2300 m for the three years. This agrees with an observed decrease in atmospheric temperature with an increase in elevation, commonly referred to as the lapse rate. In general, the lapse rate decreases 0.6 °C with elevation increases of 100 m from sea level to 11 km elevation (Jacobson, 2005; Ahrens, 2006).

3.2. Elevation-dependent plant growth

Aerial dry-weight for 3-, 4- and 5-year-old plants was greater at the 3300 m higher elevation with a 1.4-, 1.6- and 2.0-fold increase compared to the 2300-m site (Fig. 1A). Rhizome dry-weight exhibited a similar trend with biomass greater at the higher elevation with a 1.2-, 1.4- and 2.0-fold increase observed (Fig. 1B). While young plants will typically put on only 1 bud/year, older plants can put on many new buds to substantially increase plant size in later growing seasons as was observed in Table 2. While leaf size (*i.e.* area and petiole length/diameter) at 3300 m was smaller than for plants grown at 2300 m, overall plant size at the higher elevation site was greater due to larger total leaf number. Other growth parameters including leaf area, rhizome length/diameter, number of petioles, roots and fruit per plant and fruit dry weight per plant also showed increased values with the higher elevation site (Tables 2 and 3).

While this establishes that plants present at the higher elevation favor more robust plant growth, to dissect the impact of environmental conditions versus genetic factors on growth, plants adapted to low and high elevation were moved to the alternative environmental condition. When plants were shifted from 2300 m to the higher elevation site, aerial dry-weight biomass for 3, 4- and 5-year-old plants was greater with a 1.1-, 1.5- and 2.0-fold increase compared to plants that were maintained at the lower elevation. While plants transplanted from 3300 m to the lower elevation exhibited a biomass reduction of 1.5-, 1.8- and 2.1-fold for years 3, 4 and 5, respectively compared to the high elevation site (Fig. 1A). Rhizome dry-weight also exhibited a similar trend with biomass greater at the 2300 → 3300 m transplant with a 1.1-, 1.2- and 1.9-fold increase compared to the 2300-m site and the transplant from 3300 m to the lower elevation site exhibited a 1.3-, 1.6- and 2.1-fold reduction for years 3, 4 and 5, respectively compared to the 3300-m site (Fig. 1B).

3.3. Higher elevation increases PPT accumulation

While a direct correlation between elevation and PPT content has been reported (Nadeem et al., 2007; Alam and Naik, 2009; Li et al., 2013; Liu et al., 2015; Pandey et al., 2015), to confirm that plants that exhibit higher biomass contain a relative or absolution increase in PPT amounts, PPT levels were monitored in plants grown at both elevations. Indeed, PPT content in the aerial portion for all years studied was greater at the 3300-m site, with a 1.6-, 2.8- and 2.7-fold increase on a dry weight and a 2.2-, 4.6- and 5.3-fold increase on a per plant basis compared to the 2300-m site (Fig. 2A and B). Within the rhizome, increases of 2.5-, 1.6- and 1.8-fold as well as 3.0-, 2.2- and 3.5-fold on a dry weight and per plant basis, respectively, were observed (Fig. 2C and D).

When plants were shifted from 2300 m to the higher elevation site, PPT content in the aerial portion for 3, 4- and 5-year-old plants was 1.3-, 2.8- and 2.6-fold higher on a dry weight basis and 1.5-, 4.1- and 5.0-fold on per plant basis compared to plants that were maintained at the lower elevation. In contrast plants transplanted from 3300 m to the lower elevation exhibited a content reduction of 1.7-, 3.0- and 2.7-fold on a dry weight, 2.4-, 5.3- and 5.8-fold on per plant basis for years 3, 4 and 5, respectively compared to maintaining at the high elevation site (Fig. 2A and B). PPT in rhizomes also exhibited greater amounts with

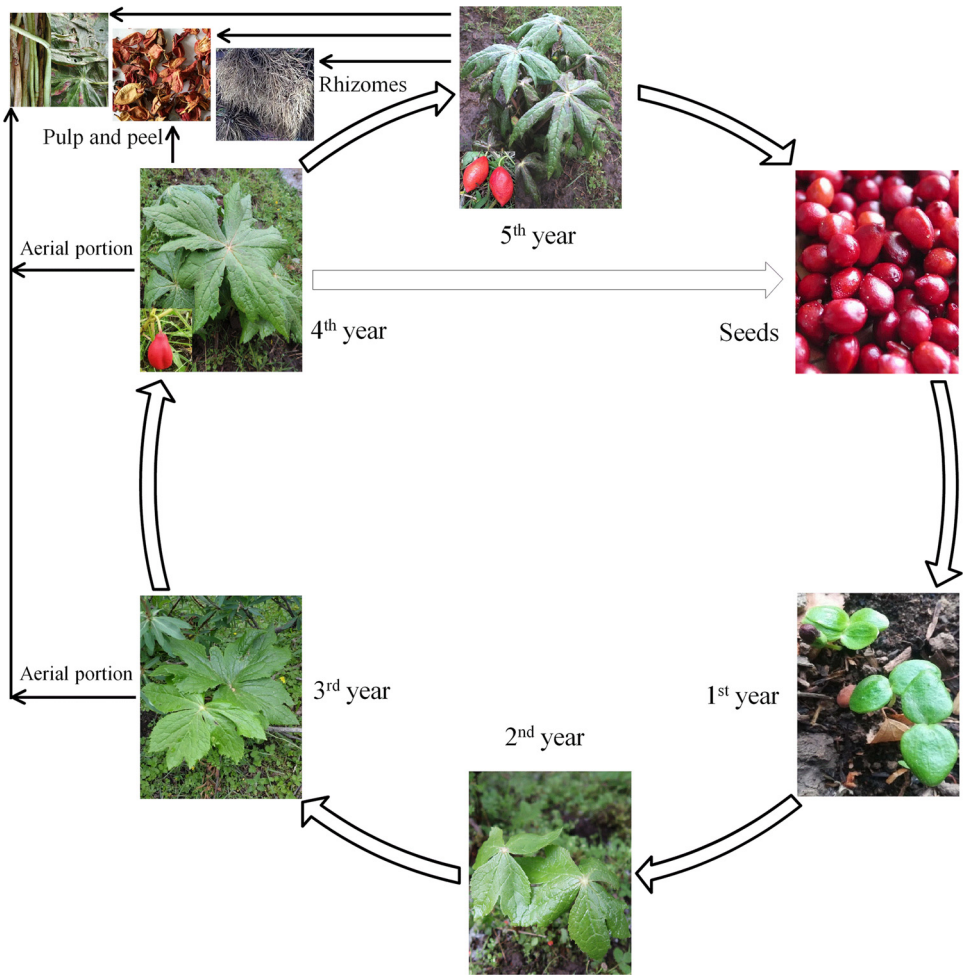

Fig. 3. A proposal approach for sustainable cultivation-collection under *in situ* conditions for optimized PPT production.

the 2300 → 3300 m shift: 2.2-, 1.5- and 1.7-fold increase on a dry weight basis and 2.5-, 1.9- and 3.3-fold increase on a per plant basis compared to maintaining at the 2300-m site. Transplanting from 3300 m to the lower elevation site reduced PPT accumulation by 1.8-, 1.6- and 1.8-fold on a dry weight basis and 2.4-, 2.5- and 3.6-fold on per plant basis for years 3, 4 and 5, respectively compared to remaining at the 3300-m site (Fig. 2C and D).

4. Discussion

A great demand for the biological active metabolite podophyllotoxin (PPT) has pushed *P. hexandrum* towards extinction (Gupta and Dutta, 2011; Kitchlu et al., 2011). While previous studies have established the role of low temperature in stimulating transcriptional induction of PPT biosynthesis and accumulation under controlled laboratory conditions (Kumari et al., 2014; Yang et al., 2016; Seegers et al., 2017), high PPT accumulation under specific environmental conditions has not been established. Here is shown that greater PPT accumulation occurs for plants collected from both low and high elevation sites when such plants are grown at a high elevation.

P. hexandrum growth is temperature dependent with a 4–10 °C average range ideal for growth (Kushwaha et al., 2008; Yang et al., 2016). Growth parameters that were promoted include: stem and rhizome diameter, length and biomass. Aerial and rhizome dry weight also increased over 3-fold with a 10 °C decrease in temperature starting at 22 °C (Yang et al., 2016). Starting at 25 °C rhizome dry weight was 2-fold higher with a 10 °C drop (Seegers et al., 2017). The current study also showed greater growth with lower temperature and by having the plants grown at two differing elevations, provided a realistic/natural growth environment. This uncontrolled condition introduces ancillary environmental variables such soil carbon and nitrogen, moisture level and pH that may also impact growth. However up to this point, the growth impact of such environmental factors has not been reported. Surprisingly when comparing greenhouse grown *P. hexandrum* plants with 500 μmol $m^{-2} s^{-1}$ PAR to field grown controls with > 2000 μmol $m^{-2} s^{-1}$ PAR, differences in leaf morphology and leaf dry mass were not observed (Pandey et al., 2006).

While our observation that PPT content increases with plant age is consistent with literature reporting (Pandey et al., 2007), with a large jump up in PPT levels as the plant matures, the major finding of this study is that elevation can serve as a driver of PPT accumulation. One component of this higher elevation is lower temperature which is observed to correlate with increased PPT accumulation (Fig. 2). This is consistent with previous reports in which PPT content at 4 versus 22 °C is 5-fold higher on dry weight basis in rhizomes and 2-fold on per plant basis at 15 °C compared to 25 °C (Yang et al., 2016; Seegers et al., 2017). This 5-fold induction is correlated with over expression of key genes related to PPT biosynthesis. Another environmental factor that regulates PPT accumulation was soil nutrients with elevated Fe^{2+} and Mn^{2+} increasing PPT accumulation in rhizome seedling by enhancing the enzyme activity of phenylalanine ammonia lyase, cinnamyl alcohol-dehydrogenase and deoxypodophyllotoxin 6-hydroylase (Li et al., 2013). Ammonium, nitrate and phosphate supplements have also been shown to increase PPT accumulation in suspension cultures (Chattopadhyay et al., 2003).

Unlike a previous study in which plants were chemically analyzed

as a fixed elevation to identify the role of changing environmental conditions on PPT accumulation (Guo et al., 2017), environmental and genetic factors were considered jointly by transplanting individuals, adapted to either lower and higher elevations to an alternative site. Since genetic diversity is high among *P. hexandrum* genotypes from different geographic locations (Alam et al., 2009b; Paul et al., 2013; Liu et al., 2016), switching location can begin to differentiate between environmental and genetic control of PPT production. With high genetic diversity reported, the strongly dominate influence of localized environmental conditions on growth and PPT accumulation was unexpected. For both low and high elevation adapted plants, growth and PPT content was greater at higher elevation thereby minimizing the role of genetic diversity in growth and PPT content.

While PPT content in rhizomes was observed to be up to ten times greater than in aerial parts of the plant, leaves can provide a continuous nondestructive source of PPT under timed leaf removal from *P. hexandrum* (Pandey et al., 2013) and *P. peltatum* (Cushman et al., 2005; Cushman et al., 2006). From the aerial part of 3- to 5-year-old plants grown at the higher elevation, PPT content was 0.1-0.9% and 0.8-23% on dry weight and per plant basis, respectively. These above-ground PPT values are nearly equal to rhizome PPT content for plants cultivated outdoors at 1150 m (Pandey et al., 2007) or in a glasshouse (Kushwaha et al., 2012; Seegers et al., 2017). With cultivation at 3300 m, PPT content in rhizomes was 1.5-5.5% on a dry weight basis, which is comparable to wild plants with a range of 1.5-10% (Alam and Naik, 2009; Sharma et al., 2000; Li et al., 2013; Pandey et al., 2015). Moreover, plant dry weight biomass as well as PPT accumulation was found to increase with plant age (Figs. 1 and 2) and consistent with previously reported results (Purohit et al., 1999; Pandey et al., 2007).

Here is proposed a sustainable cultivation-collection approach to improve PPT yield while reducing the impact of destructive harvesting on endangered plant populations (Fig. 3). This strategy utilizes multiple harvests of the regenerable, aerial plant portions, seed harvesting for greater plant cultivation and a final destructive harvest for high PPT yield from the rhizome. This proposed strategy begins with an autumn seed collection from the fruit of wild or previously cultivated *P. hexandrum.* Seeds are sown during the following spring at a suitable elevation (2800–3600 m) (Wu et al., 2015). Aerial parts including petioles and leaves are harvested during the fall of the 3rd-5th year for PPT extraction, while fruit can be collected in the 4th-5th year for seed collection; fruit pulp and peel can also be utilized for herbal medicine production (Kong et al., 2010; Sun et al., 2014). Since after five years, root growth reaches a steady state (preliminary data), plants are destructively harvested for rhizome collection and PPT extraction. This cultivation strategy allows for the greatest possible PPT collection while minimizing damage to this endangered species.

5. Conclusion

From the above observations, higher elevation increases growth and PPT accumulation when *P. hexandrum* are grown *in situ* conditions. Additional studies will be required to identify the role of temperature, light and nutrient conditions in more robust growth. Based on these results, a sustainable cultivation-collection approach has been proposed for sustainable PPT harvesting. The adaptation mechanism of this plant to alpine conditions will be examined via future proteomics, genomics and metabolomic investigations.

Acknowledgments

We are grateful for financial support from National Natural Science Foundation of China (81560617), Ministry of Agriculture and Animal Husbandry of Gansu Provincial China (GYC14-03, GNCX-2016-12) and ‘Fuxi’ Project of Gansu Agricultural University (Gaufx-02J04).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.indcrop.2018.05.036.

References

Ahrens, C.D., 2006. Meteorology Today, 8th ed. Brooks/Cole Publishing.
Airi, S., Rawal, R.S., Dhar, U., Purohit, A.N., 1997. Population studies on *Podophyllum hexandrum* Royle a dwindling medicinal plant of the Himalaya. Plant Genet. Resour. Newsl. 110, 20–34.
Alam, M.A., Naik, P.K., 2009. Impact of soil nutrients and environmental factors on podophyllotoxin content among 28 *Podophyllum hexandrum* populations of north-western Himalayan region using linear and nonlinear. Commun. Soil Sci. Plan. 40, 2485–2504.
Alam, M.A., Gulati, P., Gulati, A.K., Mishra, G.P., Naik, P.K., 2009a. Assessment of genetic diversity among *Podophyllum hexandrum* genotypes of the North-western Himalayan region for podophyllotoxin production. Indian J. Biotechnol. 8, 391–399.
Alam, M.A., Naik, P.K., Mishra, G.P., 2009b. Congruence of RAPD and ISSR markers for evaluation of genomic relationship among 28 populations of *Podophyllum hexandrum* Royle from Himachal Pradesh, India. Turk J. Bot. 33, 1–12.
Bhadula, S.K., Singh, A., Lata, H., Kuniyal, C.P., Purohit, A.N., 1996. Genetic resources of *Podophyllum hexandrum* Royle an endangered medicinal species from Garhwal Himalaya, India. Plant Genet. Resour. Newl. 106, 26–29.
Canela, C., Moraesb, R.M., Dayana, F.E., Ferreira, D., 2000. Molecules of interest: podophylllotoxin. Phytochemistry 54, 115–120.
Chakraborty, A., Bhattacharya, D., Ghanta, S., Chattopadhyay, S., 2010. An efficient protocol for *in vitro* regeneration of *Podophyllum hexandrum*, a critically endangered medicinal plant, Indian. J. Biotechnol. 9, 217–220.
Chattopadhyay, S., Mehra, R.S., Srivastava, A.K., Bhojwani, S.S., Bisaria, V.S., 2003. Effect of major nutrients on podophyllotoxin production in *Podophyllum hexandrum* suspension cultures. Appl. Microbiol. Biotechnol. 60, 541–546.
Cushman, K.E., Maqbool, M., Lata, H., Bedir, E., Khan, I.A., Moraes, R.M., 2005. Podophyllotoxin content and yield of American mayapple leaves in sun and shade. Hortic. Sci. 40, 60–63.
Cushman, K.E., Moraes, R.M., Gerard, P.D., Bedir, E., Silva, B., Khan, I.A., 2006. Frequency and timing of leaf removal affect growth and podophyllotoxin content of American mayapple in shade. Hortic. Sci. 41, 582–585.
Fay, D.A., Ziegler, H.W., 1985. Botanical source differentiation of *Podophyllum* resin by high performance liquid chromatography. J. Liq. Chromatogr. 8, 1501–1506.
Giri, A., Narasu, M.L., 2000. Production of podophyllotoxin from *Podophyllum hexandrum*: a potential natural product for clinically useful anticancer drugs. Cytotechnology 34, 17–26.
Gordaliza, M., García, P.A., del Corral, J.M., Castro, M.A., Gómez-Zurita, M.A., 2004. Podophyllotoxin: distribution, sources, applications and new cytotoxic derivatives. Toxicon 44, 441–459.
Guo, Y.L., Wang, W.Y., Lu, C.Y., Zhang, H.L., Gu, W., 2014. Predictions of potential geographical distribution of *Sinopodophyllum hexandrum* under climate change. Chin. J. Plant Ecol. 38, 249–261.
Guo, Q., Zhang, J., Wang, P.Y., Gong, X.F., Peng, Y.N., Yang, H., Du, W.B., 2017. Correlation between podophyllotoxin content in *Sinopodophyllum hexandrum* (Royle) Ying from different populations in Gansu province and environmental factors. Chin. J. Ecol. 36, 1315–1321.
Gupta, M.L., Dutta, A., 2011. Stress-mediated adaptive response leading to genetic diversity and instability in metabolite contents of high medicinal value: an overview on *Podophyllum hexandrum*. OMICS 15, 873–882.
Jacobson, M.Z., 2005. Fundamentals of Atmospheric Modeling, 2nd ed. Cambridge University Press.
Kharkwal, A.C., Kushwaha, R., Prakash, O., Ogra, R.K., Bhattacharya, A., Nagar, P.K., Ahuja, P.S., 2008. An efficient method of propagation of *Podophyllum hexandrum*: an endangered medicinal plant of the Western Himalayas under *ex situ* conditions. J. Nat. Med. 62, 211–216.
Kitchlu, S., Ram, G., Koul, S., Koul, K., Gupta, K.K., Ahuja, A., 2011. *Podophyllum* lignans array of Podophyllum hexandrum Royle populations from semi-desert alpine region of Zanskar valley in Himalayas. Ind. Crop Prod. 33, 584–587.
Kong, Y., Xiao, J.J., Meng, S.C., Dong, X.M., Ge, Y.W., Wang, R.F., Shang, M.Y., Cai, S.Q., 2010. A new cytotoxic flavonoid from the fruit of Sinopodophyllum hexandrum. Fitoterapia 81, 367–370.
Kumari, A., Singh, H.R., Jha, A., Swarnkar, M.K., Shankar, R., Kumar, S., 2014. Transcriptome sequencing of rhizome tissue of < *it* > *Sinopodophyllum hexandrum* at two temperatures. BMC Genomics 15, 871.
Kumari, A., Singh, D., Kumar, S., 2017. Biotechnological interventions for harnessing podophyllotoxin from plant and fungal species: current status challenges, and opportunities for its commercialization. Crit. Rev. Biotechnol. 37, 739–753.
Kushwaha, R., Pandey, S., Chanda, S., Bhattacharya, A., Ahuja, P.S., 2008. Temperature-dependent growth and emergence of functional leaves: an adaptive mechanism in the seedlings of the western Himalayan plant *Podophyllum hexandrum*. J. Plant Res. 121, 299–309.
Kushwaha, R., Bhattacharya, A., Singh, B., Singh, R.D., 2012. Factors affecting podophyllotoxin yield in the *ex situ* grown *Podophyllum hexandrum*, an endangered alpine native of the western Himalayas. J. Nat. Med. 66, 1–7.
Lau, W., Sattely, E.S., 2015. Six enzymes from may apple that complete the biosynthetic pathway to the etoposide aglycone. Science 349, 1224–1228.
Li, C.D., Li, W., Li, M.F., Sun, P., Wang, W.T., Song, Y.Y., 2008. Seed dormancy and

germination in endangered plant *Podophyllum hexandrum* Royle. Bull. Bot. Res. 28, 618–621.
Li, M.F., Li, W., Yang, D.L., Zhou, L.L., Li, T.T., Su, X.M., 2013. Relationship between podophyllotoxin accumulation and soil nutrients and the influence of Fe^{2+} and Mn^{2+} on podophyllotoxin biosynthesis in *Podophyllum hexandrum* tissue culture. Plant Physiol. Biochem. 71, 96–102.
Liu, W., Liu, J., Yin, D., Zhao, X., 2015. Influence of ecological factors on the production of active substances in the anti-cancer plant *Sinopodophyllum hexandrum* (Royle) T.S. Ying. PLoS One 10, e0122981.
Liu, W., Wang, J., Yin, D.X., Yang, M., Wang, P., Han, Q.S., Ma, Q.Q., Liu, J.J., Wang, J.X., 2016. Genetic diversity and structure of the threatened species Sinopodophyllum hexandrum (Royle) Ying. Genet. Mol. Res. 15 (gmr.15028130).
Lv, M., Xu, H., 2011. Recent advances in semisynthesis, biosynthesis, biological activities, mode of action, and structure-activity relationship of podophyllotoxins: an update (2008–2010). Mini Rev. Med. Chem. 11, 901–909.
Marques, J.V., Kim, K.W., Lee, C., Costa, M.A., May, G.D., Crow, J.A., Davin, L.B., Lewis, N.G., 2013. Next generation sequencing in predicting gene function in podophyllotoxin biosynthesis. J. Biol. Chem. 288, 466–479.
Mayeaux, E.J., Harper, M.B., Barksdale, W., Pope, J.B., 1995. Noncervical human papillomavirus genital infections. Am. Fam. Phys. 52 (1137–1146), 1149–1150.
Nadeem, M., Palni, L.M.S., Kumar, A., Nandi, S.K., 2007. Podophyllotoxin content, above- and belowground biomass in relation to altitude in *Podophyllum hexandrum* populations from Kumaun region of the Indian central Himalaya. Planta Med. 73, 388–391.
Nivot, N., Olivier, A., Lapointe, L., 2008. Vegetative propagation of five northern forest understory plant species from either rhizome or stem sections. Hortic. Sci. 43, 1531–1537.
Pandey, S., Kumar, N., Kushwaha, R., 2006. Morpho-anatomical and physiological leaf traits of two alpine herbs, *Podophyllum hexandrum* and Rheum emodi in the Western Himalaya under different irradiances. Photosynthetica 44, 11–16.
Pandey, H., Nandi, S.K., Kumar, A., Palni, U.T., Palni, L.M.S., 2007. Podophyllotoxin content in *Podophyllum hexandrum* Royle plants of known age of seed origin and grown at a lower altitude. Acta Physiol. Plant. 29, 121–126.
Pandey, H., Nandi, S.K., Palni, L.M.S., 2013. Podophyllotoxin content in leaves and stems of *Podophyllum hexandrum* Royle from Indian Himalayan region. J. Med. Plants Res. 7, 3237–3241.
Pandey, H., Kumar, A., Palni, L.M.S., Nandi, S.K., 2015. Podophyllotoxin content in rhizome and root samples of *Podophyllum hexandrum* Royle populations from Indian Himalayan region. J. Med. Plants Res. 9, 320–325.
Paul, S., Nandi, S.K., Palni, L.M.S., 2013. Assessment of genetic diversity and interspecific relationships among three species of *Podophyllum* using AFLP markers and podophyllotoxin content. Plant Syst. Evol. 299, 1879–1887.
Peng, Y., Chen, S.B., Liu, Y., Chen, S.L., Xiao, P.G., 2006. A pharmacophylogenetic study of the Berberidaceae (s. l.). Acta Phytotaxon. Sin. 44, 241–257.
Purohit, M.C., Bahuguna, R., Maithani, U.C., Purohit, A.N., Rawat, A.N., Rawat, M.S.M., 1999. Variation in podophylloresin and podophyllotoxin content in different populations of *Podophyllum hexandrum*. Curr. Sci. 77, 1078–1080.
Rajesh, M., Jeyaraj, M., Sivanandhan, G., Subramanyam, K., Mariashibu, T.S., Mayavan, S., Dev, G.K., Anbazhagan, V.R., Manickavasagam, M., Ganapathi, A., 2013. Agrobacterium-mediated transformation of the medicinal plant *Podophyllum hexandrum* royle (syn. *P.* emodi wall. ex hook.f. & thomas). Plant Cell Tissue Organ Cult. 114, 71–82.
Rajesh, M., Sivanandhan, G., Jeyaraj, M., Chackravarthy, R., Manickavasagam, M., Selvaraj, N., Ganapathi, A., 2014. An efficient in vitro system for somatic embryogenesis and podophyllotoxin production in *Podophyllum hexandrum* Royle. Protoplasma 251, 1231–1243.
Richard, B., Gordon, S., 2009. Statistical Methods in Practice: for Scientists and Technologists. Wiley, Chichester, U.K, pp. 95–96.
Seegers, C.L.C., Setroikromo, R., Tepper, P.G., Horvatovich, P., Peters, R., Quax, W.J., 2017. Methyl jasmonate treatment increases podophyllotoxin production in *Podophyllum hexandrum*, roots under glasshouse conditions. Plant Soil 417, 117–126.
Sharma, T.R., Singh, B.M., Sharma, N.R., Chauhan, R.S., 2000. Identification of high podophyllotoxin producing biotypes of *Podophyllum hexandrum* Royle from North-Western Himalaya. J. Plant Biochem. Biotechnol. 9, 49–51.
Sun, Y.J., Sun, Y.S., Chen, H., Hao, Z.Y., Wang, J.M., Guan, Y.B., Zhang, Y.L., Feng, W.S., Zheng, X.K., 2014. Isolation of two new prenylated flavonoids from *Sinopodophyllum emodi:* fruit by silica gel column and high-speed counter-current chromatography. J. Chromatogr. B 969, 190–198.
Wu, A.L., Li, M., Zhang, S.W., Zhao, J.F., Liu, X., Wang, C.H., Wang, X.Y., Zhong, G.Y., 2015. Effect of topographical factors on podophyllotoxin content in *Sinopodophyllum hexandrum* and study on ecological suitability. China J. Chin. Mater. Med. 40, 2299–2303.
Yang, D.L., Sun, P., Li, M.F., 2016. Chilling temperature stimulates growth, gene overexpression and podophyllotoxin biosynthesis in *Podophyllum hexandrum* Royle. Plant Physiol. Biochem. 107, 197–203.