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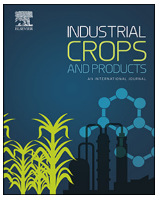

Ontogenetic shifts in resource allocation and potential defense syndromes of a tropical medicinal treelet

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ABSTRACT

Variation of plant traits across ontogeny can play an important role in shaping the vital functions of growth, reproduction and defense, depending on the priorities of resource investment. Plant functional traits in seven ontogenetic stages, following complete trajectories, of a tropical light-demanding evergreen medicinal treelet (*Rauvolfia vomitoria* Afzel.) were examined in a common garden experiment in the field. The highly plastic morph-physiological and potential defense traits showed complex and nonlinear variations with increasing tree height (age), revealing a mostly clear-defined transition at reproductive onset. Leaf photosynthetic rate varied in a unimodal pattern throughout ontogeny, with the highest value occurring at reproductive onset. Except for the oldest plants, priority of resource use in leaves across ontogeny shifted to enhance photosynthetic rate, photosynthetic N and P use efficiencies, and prevention of physical damage (higher leaf thickness, leaf mass per area and lignin concentration), but to decrease carbon-based chemical defense (i.e., total phenolics). Fine root phenolics were relatively stable, but lignin concentration increased throughout ontogeny. With higher levels than fine roots and aboveground parts, the concentrations of an indole alkaloid (i.e. reserpine) increased steadily in coarse roots, especially during the reproductive stages. Whereas, tree growth progressively shifted from the demand of N relative to P, based on the indirect evidence from N:P stoichiometry. Our results demonstrated that, under the conditions of no environmental interference, the pronounced trajectories in functional traits were observed throughout the life cycle of *R. vomitoria* plants due to strong ontogeny-induced shifts in resource allocation. The synergies or tradeoffs of the defense syndromes differed between and within the classes of defense (i.e., physical versus chemical), and also, between and within above- and below-ground tissues across the ontogenetic developmental stages, which could probably be explained by multiple defense hypotheses. The revealed variations of the growth traits and secondary metabolites of *R. vomitoria* plants at different developmental stages will be helpful for maximizing the reserpine yield through the suitable cultivation practices.

1. Introduction

Plants allocate the acquired above- and below-ground resources for the vital functions of growth, resistance, defense and reproduction, to maximize their fitness in the environment. The resource pool being limited, the allocation to one function or structure prevents their use for another one (except in the case of storage), promoting tradeoffs that determine resource allocation constraints (Bazzaz et al., 1987; Züst and Agrawal, 2017). Resources are modulated according to their availability and the plant's need between aboveground (leaf traits for light capture and carbon fixation) and belowground parts (root traits for soil-resource exploration), consequently maximizing the plant's benefit. However, compared with the aboveground counterpart (leaf), root functional traits reveal more complex phylogenetic relationship and a high degree of organization, as belowground environmental conditions and resource acquisition are far more constrained (Freschet et al., 2015). Based on resource availabilities and allocation patterns, priority of these functions changes as the plants grow, particularly predominantly during the stages of seedling establishment and when the plants attain their reproductive maturity (Bazzaz et al., 1987; Barton and Boege, 2017). Leaf mass per area (LMA), which is closely correlated with leaf toughness, along with C/N and other foliar properties, determine the palatability and, hence, physical defense (Coley, 1983). The qualitative and quantitative characteristics of defensive chemicals (i.e., secondary metabolites) also vary as the plant grows. In case of carbon-based secondary metabolites, foliar terpenoids increased while phenolics decreased along with ontogenetic development of *Eucalyptus froggattii* (Goodger et al., 2013). For N-containing secondary

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metabolites, foliar cyanogenic glycoside level decreased in the reproductively mature plants of a tropical tree (*Ryparos akurrangii*; Webber and Woodrow, 2009) and shrub (*Turnera velutina*; Ochoa-López et al., 2015). Interestingly, the environmental influences (i.e., light and N availability) on foliar cyanogenic levels were not detectable in the former species. But total leaf alkaloid concentrations were higher at the reproductive stage than at the vegetative stage of annual herbs and perennial shrubs in the genus *Datura* (Kariñho-Betancourt et al., 2015). Additionally, in many cases, defense traits of plant tissues throughout ontogeny reveal marked differences from whole plant level, implying that variable selective pressures act on tissues and whole plant (Mason and Donovan, 2015; Barton and Boege, 2017).

According to the growth-defense hypothesis, light-demanding fast-growing species should suffer more from herbivores and pathogens since they invest less in defense than shade-tolerant slow-growing species (Herms and Mattson, 1992). But a simple relationship between growth and defense aboveground may not be so clearly reflected belowground because of the involvement of interacting antagonistic and mutualistic organisms, both inside and outside roots (Rasmann and Agrawal, 2008; Lemmermeyer et al., 2015). The growth-differentiation balance hypothesis, on the other hand, predicts that mature plants are better protected because of the resource limitation during early ontogenetic stages (Herms and Mattson, 1992). Since herbivores are the greatest source of seedling's mortality, they potentially act as a strong selection pressure for seedling defense traits in most plant species (Coley, 1983). The optimal defense hypothesis suggests that the plant stage where plants are at maximum risk of herbivory, like the young seedlings, should be most defended (Bryant et al., 1992). It predicts that plant defence allocation should maximize plant fitness (Boege and Marquis, 2005; Ochoa-López et al., 2015; Barton and Boege, 2017). Moreover, the decrease in defense of the vegetative parts is expected with the onset of reproduction, especially for long-lived trees that flower and produce fruit only once per year, where reproduction is prioritized (van Dam et al., 2001; Boege and Marquis, 2005). While, for a given ontogenetic plant stage, herbivore preference varies among species and/or guilds of herbivores, depending on their predation risk, specific adaptations to plant defenses, and on their foraging strategies (Boege and Marquis, 2005; Barton and Boege, 2017). Studies are usually restricted to comparisons of particular plant stages, such as seedlings, or saplings versus mature trees (Barton and Koricheva, 2010; Kariñho-Betancourt et al., 2015), or only focused on early plant ontogenetic stages (Elger et al., 2009), except for a study with complete life cycles of eight perennial grassland species (Del-Val and Crawley, 2005). We know little about how functional traits vary with plant size in woody species across their whole life cycles.

In natural ecosystems, plant size/age is subjected to indistinguishable sets of environmental factors (e.g., abiotic environmental filters, plant competition or potential interactions among them) (Kenzo et al., 2015; Barton and Boege, 2017). For instance, size-dependent variations of plant functional traits were mainly related to differences in light availability in tropical forests (Poorter et al., 2005). Moreover, most of the earlier studies focused mainly on aboveground traits and, except for a few studies, belowground traits were largely ignored (Boege and Marquis, 2005; Webber and Woodrow, 2009; Thomas, 2010; Ochoa-López et al., 2015). Interpretations of these studies depend on the traits evaluated in the particular case and are suitable for a definite system only. Hence, well-controlled experiments including both above- and below-ground traits are needed with minimum and uniform abiotic influence across the whole ontogenetic phases of the studied plants. The present study was conducted on the plants of a tropical light-demanding, fast-growing evergreen medicinal treelet species, *Rauvolfia vomitoria* Afzel. (Apocynaceae), growing in a common garden under the same conditions in the field. Originally from Africa, *R. vomitoria* is cultivated in Southwest China for its alkaloids, especially reserpine, that is used in pharmaceutical industry. Reserpine is an indole alkaloid, that has been historically used in controlling high blood pressure and in relieving psychotic symptoms (Wilkins, 1954). Using the fully-exposed, sparsely cultivated plants, we assessed the effect of real plant ontogeny rather than abiotic and biotic factors, although not directly addressing above- and below-ground herbivory. Encompassing complete ontogenetic trajectories of the experimental plants, we asked the following specific questions: (i) How does resource allocation modulate the above- and below-ground morphological, physiological and putative defense traits in *R. vomitoria* plants throughout its lifecycle? (ii) Do these traits show significant divergences between pre-reproductive and reproductive phases of the plant? (iii) What are the trajectories of secondary metabolites production over ontogeny?

2. Materials and methods

2.1. Plant material and experimental design

As a light-demanding evergreen treelet (Cai et al., 2009), *R. vomitoria* plants attain a maximum height of ca. 5.2 m in the field. They reproduce only once per year producing small seeds (0.0256 g per seed; over 80% seeds germinate within 4 days). Their flowering and fruiting season last in August and September in Xishuangbanna, southwest China. The average annual temperature of Xishuangbanna is 25 °C; the average annual rainfall is about 1500 mm with a distinct wet (from May to October) and dry season (from November to April). The rain-fed *R. vomitoria* plantation is located in Xishuangbanna Tropical Botanical Garden, Chinese Academy of Science (21°09′– 22°33′N, 99°58′–101°50′E; altitude 560 m). The soil (0–20 cm) of this plantation is characterized as acid lateritic red soil (Oxisol) with pH 5.2. Organic matter, total N, P, and K contents of the soil were 28.1, 1.81, 0.62, and 20.6 g kg^{-1}, respectively. Weeding was done bi-monthly. Originated from seeds sown in different months or years in the wet season, *R. vomitoria* plants in each tree height class were cultivated separately in the field. The canopy shading between each studied plant could be avoided, due to the fact that there was larger potential free growing space relative to crown breadth. Thus, there was almost no competition between the plants, even for the largest ones (see Fig. S1 available as Supplementary Data Online). The tree height, stem diameter (5 cm aboveground) and the estimated crown breadth were measured with a ruler. Every three months, the initial flowering and fruiting of the small plants were recorded and were used to assess reproductive maturity. The detailed information of plant size/age was provided in Table S1 available as Supplementary Data Online.

In late September, we selected individual plants of 7 different ages (2.1 months to 7 year-old), which corresponded to distinct tree height classes (ANOVA, $F_{6,75} = 500.1$, $P < 0.001$) ranging from 26.7 cm to 473.4 cm. All plant traits in different tree height classes were measured simultaneously in order to control for confounding environmental factors that could influence plant performance such as photoperiod and temperature. Because our interest was to assess overall variation in plant trait trajectories at the tissue and whole plant levels, we did not assess within-tissue variation (e.g., young, intermediate and old leaf ages) which may add considerable variation in plant physiological and defensive traits (Moles et al., 2013). For leaf anatomical, physiological and chemical measurements, the sun-exposed, fully expanded healthy, and current-year leaves in the canopy were sampled.

2.2. Measurements

2.2.1. Photosynthesis and leaf anatomy

Leaf morphological, physiological and chemical measurements were performed on 1–2 leaves per tree, with minimal herbivore or physical damage, from 3 to 4 individual plants in each tree height class. Light-saturated net CO_2 assimilation was measured between 09:00 and 11:30 h to avoid mid-day stomatal closure, at an ambient condition of CO_2 concentration (about 380 ppm), temperature (26–27 °C) and vapour pressure deficit of the air (ca. 1.1 kPa). Photosynthetic photon flux of the portable infrared gas analyzer (LI-6400, Li-Cor) with a built-in LED-B light source was set at the saturating levels (1600–1800 μmol $m^{-2}s^{-1}$; Cai et al., 2009) in the open-system mode. Light-saturated photosynthesis (A_a) and stomatal conductance (g_s) were recorded, and instantaneous water-use efficiency (WUEi = A_a/g_s) was calculated. For a subset of fresh leaf samples, hand-cut transverse sections were prepared from the mid-lamina region of the leaf for anatomical measurements with a microscope (Olympus BX41-P, Olympus Corp., Kyoto, Japan). Thickness of the lamina, palisade mesophyll and spongy mesophyll were measured; the percentage of thickness in each type of leaf tissue was then calculated. Stomatal density and guard cell lengths were measured using impressions made with clear nail polish in the abaxial leaf surface; five nail polish peels per leaf from each of the three leaves taken from each plant height category were used. No stomata were found at the adaxial surfaces of leaves.

2.2.2. Nutrient and chemical traits

Nitrogen and P concentrations in leaves and roots (both fine roots with diameter $\leq$ 2 mm and coarse roots with diameter > 2 mm) were determined by the semi-micro Kjeldahl analysis using a wet digestion procedure and by inductively coupled plasma atomic-emission spectrometry (iCAP6300, Thermo Fisher Scientific, USA), respectively. Photosynthetic N- and P-use efficiency (PNUE, PPUE) were calculated as A_a/ leaf N and P concentration.

Total phenolic concentrations (mg g^{-1}, dry weight) in leaves and fine roots were determined as described by Salminen and Karonen (2011), in technical triplicates, using a Folin-Ciocalteu assay with gallic acid monohydrate (Sigma-Aldrich, St. Louis, MO, USA) as the quantification standard. Based on the standard curve, sample absorbance for total phenolic concentration was estimated and expressed as gallic equivalents. To determine total lignin concentration, we followed the procedure described by Barber and Ride (1988), in which application of thioglycolic acid leads to the formation of benzyl-alcohol thioesthers, the chemical groups found typically in lignin (Hatfield and Fukushima, 2005). Because extracted lignin has its absorbance read at 280 nm, we determined the lignin concentration relative to the alkali lignin standard 2-hydroxypropyl ether (Sigma-Aldrich), using a spectrophotometer (UV—B 2501, Shimadzu, Japan). As one of the most economically important secondary metabolites in the genus *Rauvolfia*, reserpine concentration is extremely low in fine roots, leaves and stems of *R. vomitoria* plants (Feng and Li, 1965; Court, 1983; Cai et al., 2009; Zhu et al., 2011). Thus, reserpine concentrations of only coarse roots were considered in the present study. The concentrations were detected by HPLC (Waters 600 HPLC: 600 Controller, 717 plus Auto-sampler, 2487 Dual Absorbance Detector, M32 Chromatography work-station), following the procedure described by Cai et al. (2009). In brief, after dissolving in 25 ml methanol, dry-root tissues were extracted in 30 min by ultrasonic process; the solution was then filtered through 0.45 μm membrane filter. Reserpine concentrations of the filtrates were analyzed by using reserpine (National Institute for the Control of Pharmaceutical and Biological products, China) as an external standard. Reserpine was determined with a spectrophotometer at 268 nm. The peak area ratios of reserpine were calculated with reference to the external standard and substituted in the respective regression equations to calculate the amount of its concentrations in the coarse roots.

2.2.3. Plant morphology and biomass allocation

At the end of the experiment, for each tree height class, 4–8 individual plants were harvested and washed free of soil particles with tap water. The plant parts were separated into leaves, stems, coarse roots and fine roots. Subsamples of leaf and fine roots were scanned on a desktop scanner. The acquired images were analyzed for leaf area, root length and average diameter, using DT-SCAN image analysis software (Delta-T Devices, Cambridge, UK) at 400 dots per inch. All samples were dried at 70∘C to a constant mass and then weighed. Leaf mass per area (LMA, g leaf m^{-2} leaf), leaf area ratio (LAR, cm^2 leaf g^{-1} plant), leaf mass fraction (LMF, g leaf g^{-1} plant), stem mass fraction (SMF, g stem g^{-1} plant), coarse root mass fraction (CRMF, g coarse root g^{-1} plant), fine root mass fraction (FRMF, g fine-root g^{-1} plant) and specific fine root length (SRL, m fine-root g^{-1} fine-root mass) were then calculated.

2.3. Statistical analysis

For the morphological and physiological variables, differences among tree height (age) classes were analyzed using one-way ANOVA, followed by Tukey post-hoc test. Significant differences (at $P < 0.05$) among the different ontogenetic stages are indicated by different letters. The ANOVAs revealed a significant plant height (age) effect in most analyses (Table S2), highlighting the ontogenetic variations of the measured parameters. We first assessed simultaneously for both plant tissues (leaves vs. roots), and both above- and below-ground secondary metabolites. Because phenolics and lignins are correlated and their concentrations are also potentially correlated between leaf and root tissues, we used multivariate analysis of variance (MANOVA) to examine variation in these chemical variables due to tree height. When significant effects were detected by MANOVA, we followed up with univariate ANOVA for each of these variables. Lastly, we did a one-way ANOVA in order to test overall variation in chemicals among tree height classes. Before analysis, data were checked for normality and homogeneity of variables, and were $\log_{10}$ or square-root transformed when necessary to satisfy the assumption of ANOVA. Relationships among plant traits were explored by linear (Pearson) correlation and polynomial regression analyses. Statistical analysis was performed with SPSS 22.0 software for Windows (SPSS, Chicago, IL).

3. Results

3.1. Leaf stomata, anatomy and photosynthetic traits

Leaf stomatal density showed a unimodal pattern of variation that increased rapidly during vegetative stages but decreased once after reproductive onset, while guard cell length showed a U-shape pattern with increasing tree height (Fig. 1A, B). Anatomically, leaf thickness and mesophyll proportion increased during the vegetative stages, but remained stable during the reproductive stages (Fig. 1C, D). The proportion of other tissues like palisade parenchyma, abaxial and adaxial epidermis initially decreased as the plants transform from seedlings to young mature treelets after which they remained constant (Fig. 1D).

Both the photosynthetic rate based on leaf area (A_a) and stomatal conductance (g_s) showed unimodal patterns with increasing tree height, having the highest values during transition from the vegetative to reproductive stage but decreased sharply in the oldest plants (Fig. 2A, B). In general, the instantaneous water-use efficiency (WUEi) increased with increasing tree height (Fig. 2C). Photosynthetic N- and P-use efficiency (PNUE, PPUE) increased with increasing tree height in the vegetative stage and remained relatively high levels in young mature treelets, but they decreased sharply in the oldest plants (Fig. 2D, E). Across all plant ontogenetic stages, A_a was positively correlated with g_s ($r = 0.91$, $P < 0.001$), but not with leaf N concentration (N_m) or mesophyll thickness (both $P > 0.05$); while PNUE but PPUE was negatively related to WUEi, although not significant ($r = -0.36$, $P = 0.11$).

3.2. Leaf and fine-root nutrition, morphology and biomass allocation

During the ontogenetic development of *R. vomitoria* plants, except for the very young seedlings, leaf N_m did not reveal much variations while P concentration (P_m) decreased continuously, especially after plants reached reproductive maturity (Fig. 3A, B). In case of fine roots, both N_m and P_m increased across the vegetative stages, while N_m subsequently remained stable but P_m decreased continuously during the reproductive stages (Fig. 3D, E). N:P ratios of leaves and fine-roots,

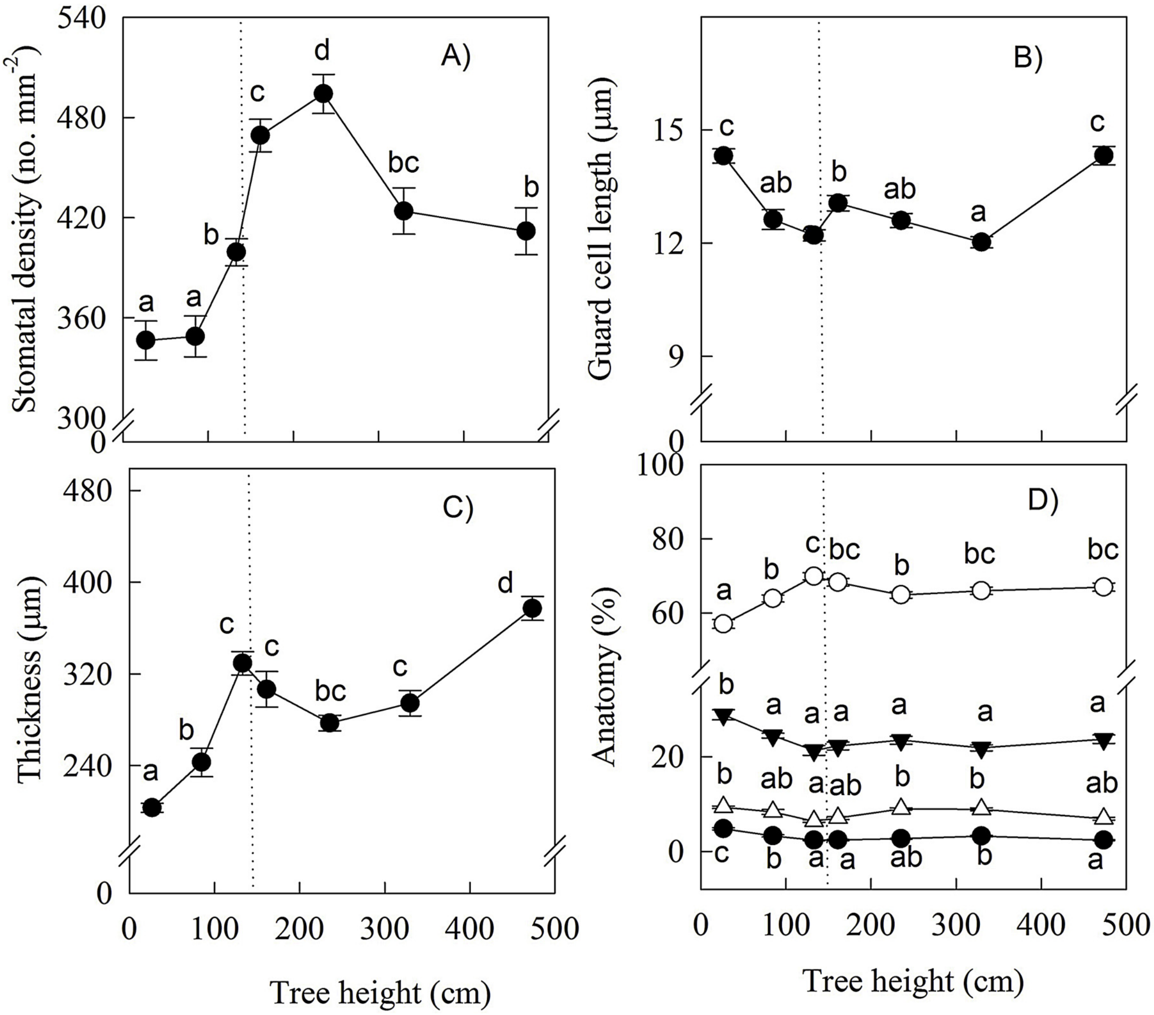

Fig. 1. Tree height-dependent variations in leaf stomata and anatomical traits (mean ± SE, n = 8–10) of *R. vomitoria* plants. In panel D, the symbols of "●, ▼, ○, and △" represent the percentage of thickness of abaxial epidermis, palisade, mesophyll, and adaxial epidermis, respectively. Dotted lines represent the transition from the vegetative to reproductive stage. Significant differences (at $P < 0.05$) among the different ontogenetic stages are indicated by different letters.

ranging from 6.2 to 24.3 and 7.1 to 19.1, respectively, remained constant during the vegetative stages but increased greatly once after reproductive onset (Fig. 3C, F). N_m, P_m and N:P ratio in coarse roots did not change greatly (Fig. 3G, H, I).

With increasing in tree height, leaf mass per area (LMA) increased but leaf area ratio (LAR) sharply decreased, while specific fine-root length (SRL) decreased steadily and fine-root diameter increased (Fig. 4A-D). A steady and rapid increase and decrease in shoot mass fraction (SMF) and leaf mass fraction (LMF) was found, respectively (Fig. 4E), while both coarse and fine root mass fractions (CRMF and FRMF) decreased (Fig. 4F).

3.3. Secondary metabolites in leaves and roots

Total phenolic concentration in leaves decreased sharply as the plants started growing from seedling stage (Fig. 5A). However, fine-root phenolics remained constant in both young and adult plants except for the significant higher level in the oldest plants (Fig. 5C). Lignin concentration remained stable before reproduction and increased afterward in both leaves and fine roots (Fig. 5B, D). Compared with the leaves, fine roots had lower phenolic concentration but higher lignin concentrations (ANCOVA with tissue mass as covariate, both $P < 0.05$). For coarse roots, both reserpine concentration and reserpine/N ratio remained stable during the vegetative stages and then increased significantly as the plants started to flower, with the highest values in the oldest plants (Fig. 5E, F). Regression analysis indicated that the relationship between reserpine concentration and tree height was best expressed by a quadratic equation ($R^2 = 0.89$, $P < 0.001$), namely: $Y = 0.61–6.40 \times 10^{-4}H + 5.42 \times 10^{-6}H^2$; where Y was the reserpine concentration (mg g^{-1}), H was the tree height (cm). The regressions indicated that the highest reserpine concentration was attained at or near the maximum of tree height. Across the different ontogenetic stages, LMA was negatively correlated to leaf phenolic concentration, but had a positive relationship with leaf lignin concentration (Fig. 6A). Leaf vs. fine-root phenolic and lignin concentration was significantly negatively and positively correlated, respectively (Fig. 6B), whereas total phenolics in leaves and reserpine in coarse roots was negatively correlated (Fig. 6C).

4. Discussion

To the best of our knowledge, it is the first study to reveal the functional trajectories throughout a woody plant's life cycle under the conditions of no environmental interference. We demonstrated that, at both plant-tissue and whole-plant levels, plant traits related to growth and potential defense syndromes of *R. vomitoria* plants are highly plastic throughout their life cycle (seedling, sapling, reproductive maturity, post-reproductive plants), and most of them do not vary in a linear fashion (c.f. del-Val and Crawley, 2005; Webber and Woodrow, 2009; Ochoa-López et al., 2015). The pronounced non-linear ontogenetic

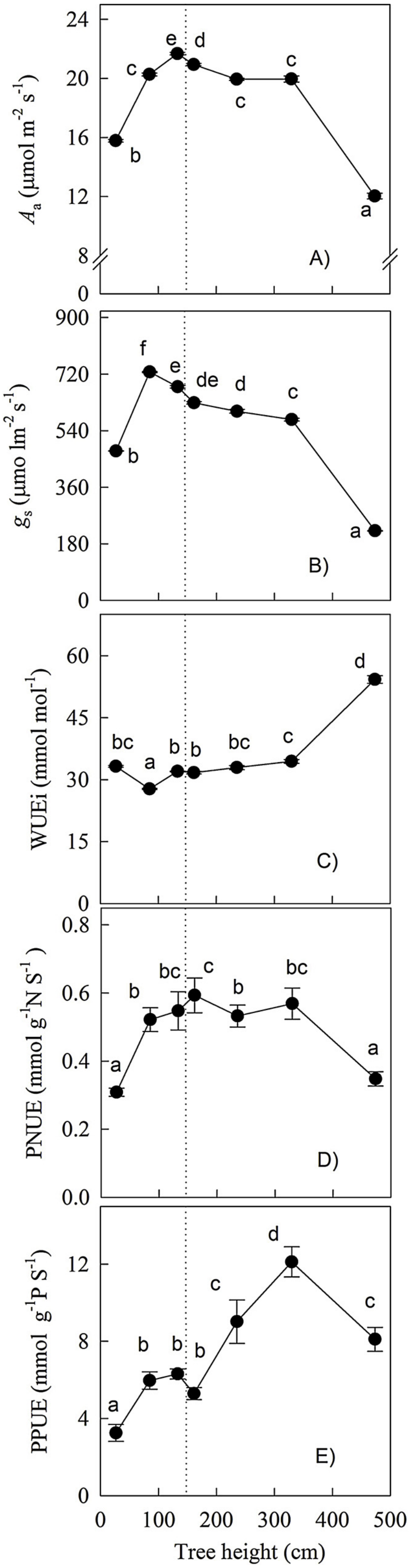

Fig. 2. Tree height-dependent variations in leaf photosynthetic traits (mean ± SE, n = 4–5) of *R. vomitoria* plants. A_a, light-saturated net photosynthetic rate based on leaf area; g_s, stomatal conductance; WUEi, instantaneous water use efficiency; PNUE, photosynthetic N-use efficiency; PPUE, photosynthetic P-use efficiency. Dotted lines represent the transition from the vegetative to reproductive stage. Significant differences (at $P < 0.05$) among the different ontogenetic stages are indicated by different letters.

trajectories, with a mostly well-defined transition at the reproductive onset, are due to real ontogeny-induced shifts in resource allocation. As for the secondary metabolites mainly functioning as defense syndromes throughout plant ontogeny, contrasting directions of changes of some traits were found. Additionally, in most cases, the physiological and defensive patterns of the oldest reproductively mature plants are generally distinct from either seedlings, saplings or younger mature reproductive treelets. This may be expected that the steadily accumulated pathogens and specific herbivores through tree development are likely to have direct physiological consequence (e.g., decreases in xylem conductance and photosynthesis), but to favor increased investment in constitutive defenses, at least prior to senescence (Boege and Marquis, 2005).

4.1. Ontogenetic shifts in resource use and allocation, especially during vegetative vs reproductive stages

Plant size-dependent changes in leaf photosynthetic rate (A_a), g_s, stomatal density, PNUE and PPUE of *R. vomitoria* plants showed unimodal patterns, increasing rapidly in the vegetative phase but decreasing in the matured reproductive plants (Figs. 1 and 2). Size-dependent patterns of A_a and g_s were consistent with the results found in temperate deciduous trees (Thomas, 2010), in contrast to the monotonic decreases with tree size/age in forests as reported by Kenzo et al. (2015). Size-related trends in a range of leaf physiological traits of two conifers (*Picea abies* (L.) H. Karst. and *Pinus sylvestris* L.) across northern Europe also revealed a unimodal pattern of variation (Niinemets, 2002). However, a meta-analysis of tree ontogeny indicated that the variations of A_a depended on the leaf form and leaf phenology: it decreased in coniferous trees, increased in deciduous broadleaved trees but changed slightly in tropical evergreen trees (Thomas and Winner, 2002). Size-dependent variation in A_a or g_s was suggested to be due to the fact that as tree grows taller, the increases in both the resistance to water flow in the xylem and the hydrostatic tension produces a limit above which sufficient water cannot be delivered to the leaves for net photosynthetic gain to occur (Bond, 2000; Ryan et al., 2006). Such hydraulic limitation in tall conifers induced reduction in A_a (Kolb and Stone, 2010). However, size-dependent water access and transport are unlikely to influence the observed patterns in our studied treelets in the wet season. Size-related changes in A_a of *R. vomitoria* plants can be, in part, attributed to g_s, rather than solely on changes in leaf N or mesophyll thickness. These results suggested that the direct effects of either hydraulic path length or leaf turgor were insufficient to explain the observed changes in net photosynthetic rate in ontogeny of these plants; the ontogenetic pattern seemed to be the effect of age-related changes on the genetic expression (Day et al., 2002), rather than the simple biophysical mechanism. However, it was found that plant size, not cellular senescence (a direct genetic or age-related control), accounted for the age-related declines in the relative growth rates and net assimilation rates of four temperate deciduous tree species (Mencuccini et al., 2005). The highest A_a value occurring on the transition from pre-reproductive to reproductive stage (Fig. 2A), which was sometimes observed (Maherali et al., 2009; Bond, 2000; Thomas and Winner, 2002), might be a constitutive change that resulted from the necessity to increase endogenous carbohydrate concentration acting as a signal required for this transition in woody plants (Lastdrager et al., 2014). Although not significantly, a negative trend observed between WUEi and PNUE, but not PPUE, across the ontogenetic developmental stages agreed with a general consensus that a trade-off existed between water- and N-use efficiency under limited resource conditions (Geng et al., 2017). The increase in PNUE was probably due to the high partition of N to photosynthetic enzymes (such as Rubisco) and/or low mesophyll resistance during the vegetative stages, shifting from nutrition-acquisitive to nutrition-conservative strategies as the plants matured.

Shifts in morphology and growth patterns at the whole-plant level occur throughout a plant's life cycle because of limited resource (e.g.,

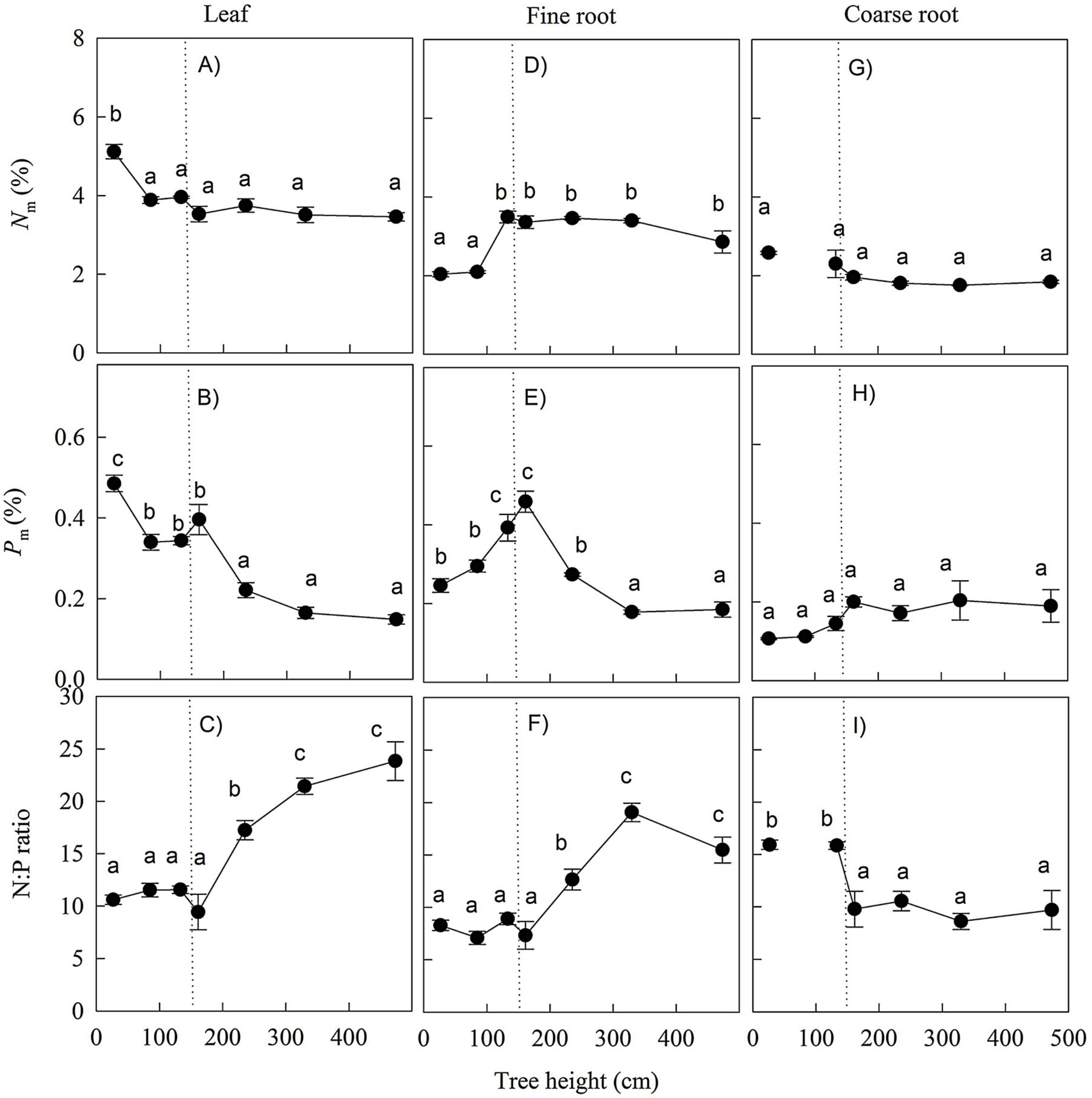

Fig. 3. Tree height-dependent variations in leaf and root nutrition (mean ± SE, n = 3–4) of *R. vomitoria* plants. N_m, Nutrient concentration; P_m, phosphorus concentration. Dotted lines represent the transition from the vegetative to reproductive stage. Significant differences (at $P < 0.05$) among the different ontogenetic stages are indicated by different letters.

water and nutrition) availability (Kolb and Stone, 2010). Shoot mass fraction (Fig. 4E) and shoot:root ratio (data not shown) greatly increased with increasing tree height (age), which agreed with a common phenomenon that biomass allocation of shoots increased in the woody plants with steady growth. Higher LAR (due to low LMA and high LMF) was found to be more prominent before the onset of reproduction of *R. vomitoria* plants (Fig. 4), leading to higher growth rate of seedlings and saplings (Paine et al., 2012). The high specific fine-root length, fine-root mass fraction and small fine-root diameter, particularly in the vegetative stages (Fig. 4 A,B), enable plants to acquire more water and nutrients from soil, thus inherently increasing their growth rate (Cai et al., 2009). In addition, N and P concentrations and the N:P stoichiometry in plant tissues, especially in leaves, has been used to evaluate whether N or P is more limiting for plant biomass (growth) (Güsewell, 2004). Specially, P concentrations in both leaves and fine roots of *R. vomitoria* plants reduced steadily once after reproductive onset (Fig. 3B,E). While more resources are required with increasing tree size in tropical P-limited soil, reproductive parts perhaps become more P demanding (DiManno and Ostertag, 2016), resulting in a decrease in resource allocation in the vegetative parts. In adult trees (*Fagus crenata* Blume), isotope tracer study indicated that fruiting led to greater N uptake from soil and finely-tune growth phenology alleviated N limitation (Han et al., 2017). This is attributed to the observed higher fine-root N concentrations in reproductive stages than vegetative plants (Fig. 3D). Reproduction-related variations in nutrient uptake and allocation determine foliage and fine-root qualities and, thus, besides change in leaf palatability particular late in tree ontogeny, is likely to be a consequence of selective pressure and direct physiological changes with increasing tree size and reproductive effort (Mason and Donovan, 2015; Barton and Boege, 2017). Moreover, N:P ratios in leaves and fine roots of *R. vomitoria* plants ranged respectively from 6.2 to 24.3 and 7.1 to 19.1 (Fig. 3C,F). The limiting factor of plant growth, hence, shifted from N to P throughout their ontogenetic developmental stages, as leaf N:P ratio of < 14.0 and > 16.0 generally suggests N and P limitation, respectively (Tessier and Raynal, 2003). Meanwhile, extensive differences among studies regarding thresholds of N and/or P limitation of plant growth existed; N:P ratios may not always precisely reflect plant nutrient requirements (Güsewell, 2004; Sullivan et al., 2014). More

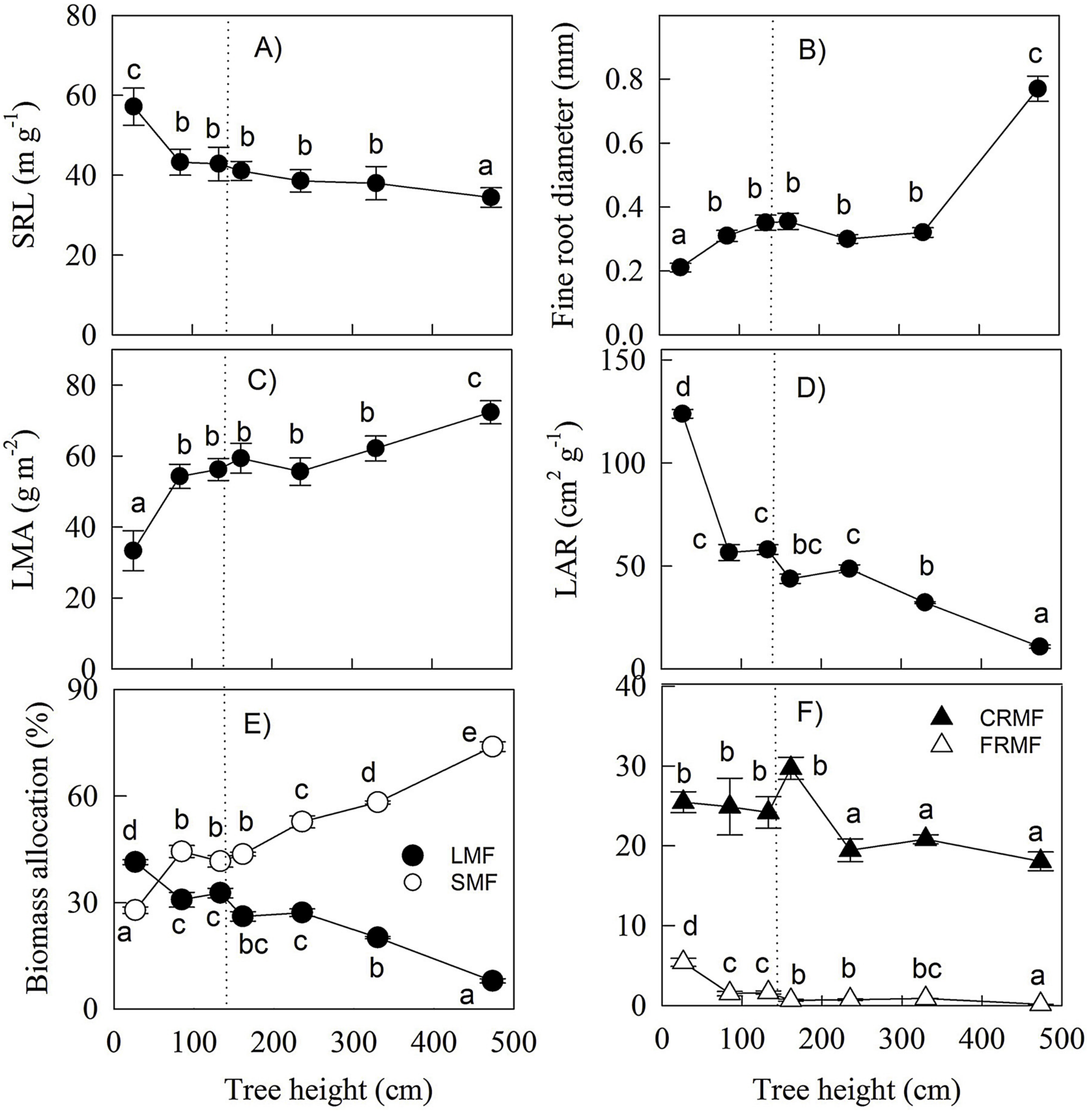

Fig. 4. Tree height-dependent variations in leaf and fine-root morphology and biomass allocation (mean ± SE, n = 4–8) of *R. vomitoria* plants. SRL, specific fine-root length; LMA, leaf mass per area; LAR, leaf area ratio; LMF, leaf mass fraction; SMF, stem mass fraction; CRMF, coarse root mass fraction; FRMF, fine root mass fraction. Dotted lines represent the transition from the vegetative to reproductive stage. Significant differences (at $P < 0.05$) among the different ontogenetic stages are indicated by different letters.

evidence coming from organismal indicators of nutrient limitation, soil nutrient supply and lab-based experiments and nutrient depletions, are needed to confirm plant nutrient limitation and to discuss their relative importance in determining plant growth and productivity (Sullivan et al., 2014).

4.2. Ontogenetic shifts in the potential physical and chemical defenses

It was reported that essential oil (C-based secondary metabolites) content in leaves of the cultivated perennial herbs (*Artemisia absinthium* L. and *Agastache rugosa* (Fisch. & C.A. Mey) Kuntze) increased with increasing plant size (Zielińska et al., 2017; Nguyen et al., 2018); the higher value was also found in the post-reproductive stage than the vegetative stage of the former species (Nguyen et al., 2018). However, the plant size-dependent expression of secondary metabolites was quite variable and seldom followed a linear pattern because of resource availability and priorities of resource allocation (Boege and Marquis, 2005; Ochoa-López et al., 2015). In *R. vomitoria* plants, some defense traits even showed opposite directions of changes, depending on the ontogenetic stage and plant tissue concerned. Lignin concentration in both leaves and fine roots increased across ontogeny (Fig. 5), rendering them tougher, consequently decreasing herbivory risk. Reproduction being more resource demand should result in the decreased defense allocation, especially that in leaves, once the plants reach reproductive maturity (Boege and Marquis, 2005). Compared with the vegetative plants, leaves in the reproductive *R. vomitoria* plants generally showed lower C-based chemical defense (i.e., total phenolics) and nutritional quality (i.e., lower N and P concentrations), but had higher physical defense (i.e., higher leaf thickness, LMA and lignin); while fine roots had higher phenolics, lignin and N nutrition, and coarse roots had higher N-containing chemical defense (i.e., reserpine) (Figs. 3–5). As predicted by the growth–differentiation balance hypothesis, the larger plants are prone to be less palatable and more resistant to herbivory as they have the higher levels of physical defense than the smaller ones. In contrast, leaf phenolic tolerance had a decreasing trajectory throughout ontogeny (Fig. 5A), which is consistent with the optimal defense theory.

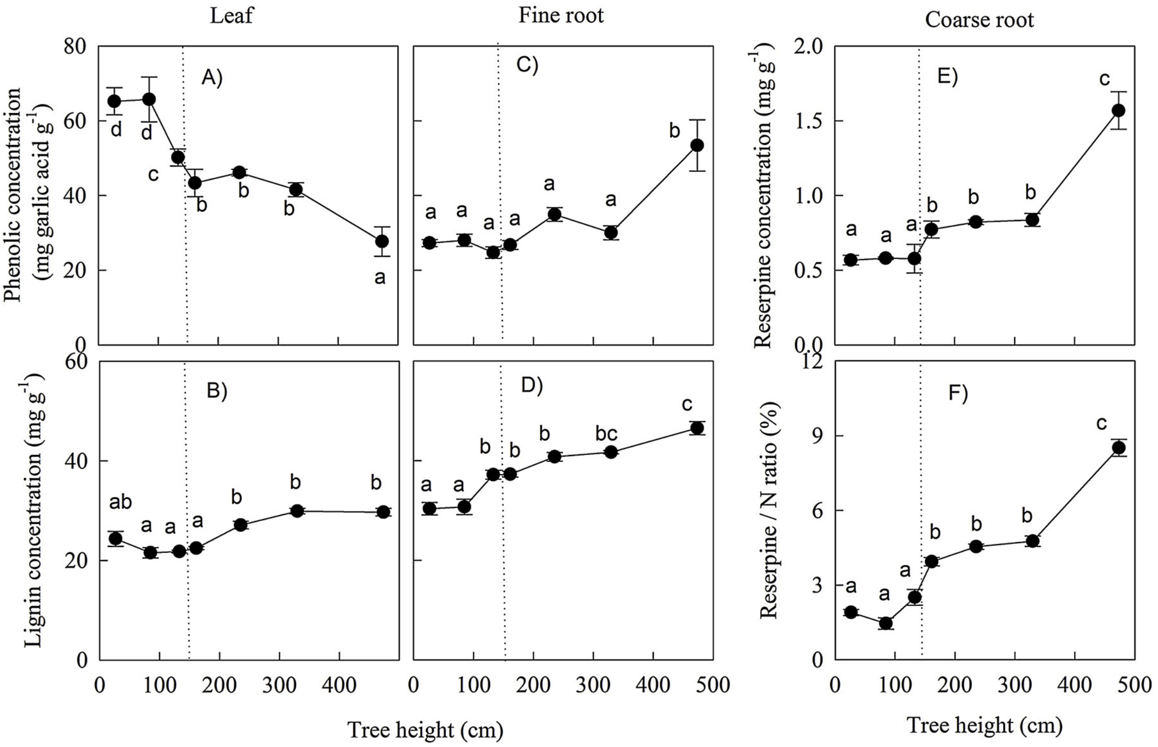

Fig. 5. Tree height-dependent variations in the secondary metabolites (mean ± SE, n = 3–4; dry weight) in leaves and roots of *R. vomitoria* plants. Dotted lines represent the transition from the vegetative to reproductive stage. Significant differences (at $P < 0.05$) among the different ontogenetic stages are indicated by different letters.

To prioritize growth and establishment, resource-limited young seedlings possessing small photosynthetic area and shallow roots, tended to allocate more resources to chemical defense (Barton and Koricheva, 2010). Thus, resource allocation of seedlings is predicted to be prioritized towards higher growth rate to outcompete neighbours at the expense of the relatively low-cost chemical defense (Boege and Marquis, 2005). The leaves of *R. vomitoria* plants, hence, switched ontogenetically from chemical resistance in seedlings to physical resistance in mature plants after weak developmental constraints. The lower leaf physical defense of the vegetative plants is probably compensated by their inherent fast growth rate, thus escaping herbivory at younger stages in space. Physical defense in leaves is compromised again at the onset of flowering when reproductive parts become more resource demanding and is prioritized over growth (Boege and Marquis, 2005; Barton and Boege, 2017).

On the other hand, understanding the physiological and biochemical mechanisms for how alkaloid metabolism and accumulation respond to ontogenetic variations is important. This will also help to establish effective agricultural methods to increase the quantity and quality of the indole alkaloid, e.g. reserpine, produced in *R. vomitoria* plants. Reserpine concentration and reserpine:N ratio in coarse roots increased throughout ontogeny (Fig. 5E,F). The detected reserpine concentration in coarse roots of *R. vomitoria* plants were found to be much higher (ca. 7–10 times) than those in fine roots (Cai et al., 2009) and aboveground parts (stem and leaf; Feng and Li, 1965; Court, 1983; Zhu et al., 2011). Such high levels of reserpine in coarse roots than other vegetative tissues corroborated with the results of indole glucosinolates, the most important constitutive defensive chemicals of plants in Brassicaceae. The taproot, which is more vulnerable and valuable than in lateral and fine root, had the highest indole glucosinolates concentrations (Tsunoda et al., 2017). In a perennial herb (*Panax ginseng* C.A. Mey) cultivated in the shade conditions, higher alkaloid ginsenoside content was also found in the main root than the rootlets (Zhang et al., 2018). Compared with the older roots, fine roots, having higher N turnover rates, are costly to produce expensive N-containing secondary metabolites (Rasmann and Agrawal, 2008). Moreover, the production of indole glucosinolates was higher in roots than in shoots (van Dam et al., 2009), probably because of the selection pressures

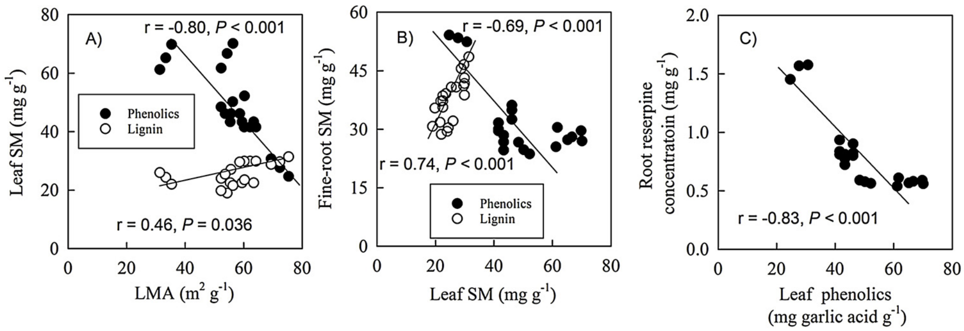

Fig. 6. Relationships between and within the potential physical and chemical defense traits across the ontogenetic development stages of *R. vomitoria* plants. SM, secondary metabolites.

exerted by insect herbivores independently in roots and shoots of Brassicaceae (Tsunoda et al., 2017). Combined with our results, these may reflect the optimal defense allocation patterns, because most root herbivores occurring just under the soil surface near the main root are less diverse than shoot herbivores and are least likely attack roots (Zangerl and Rutledge, 1996). Natural selective pressure exerted by herbivores on plants may, thus, alter defense allocation over that of the plant tissues (Zangerl and Rutledge, 1996; Barton and Boege, 2017).

In agreement with the hypotheses about tradeoffs and synergism, we found evidence of both negative and positive correlations between and within potential physical and chemical defensive traits. As expected, the increased leaf lignin concentration with increased LMA across the whole life cycle of *R. vomitoria* plants suggested that a synergistic interaction led to the coordinated developmental evolution of these physical defensive traits. The negative relationship between leaf phenolic concentration and LMA (Fig. 6A) indicated that the overall leaf physical and C-based chemical defenses were generally not compatible (Moles et al., 2013; Kariñho-Betancourt et al., 2015). The negative relationship between phenolics in leaves and reserpine in coarse roots (Fig. 6C) suggested that a finite pool of resources was constrained on the simultaneously constitutive expression of the production of proteins and phenolic compounds (*sensu* carbon/nutrient balance hypothesis; Bryant et al., 1983). There were also contradictions regarding a certain category of chemical defense among above- and below-ground parts, since a negative relationship between leaf and fine-root phenolics was found (Fig. 6B). These correlations may reflect allocation costs of traits or redundancy in their ecological functions, which was typically interpreted as a strong physiological cost constraint within plants (Barton and Koricheva, 2010; Züst and Agrawal, 2017). Plant defenses throughout ontogeny are composed of multiple traits organized into coadapted complexes (Kariñho-Betancourt et al., 2015; Ochoa-López et al., 2015), resulting from variable selective pressures deploying different combinations of defenses to coexist (Barton and Boege, 2017).

5. Conclusions

The ontogenetic variations of the measured traits of *R. vomitoria* plants are highly plastic, showing non-linear patterns with a mostly well-defined transition at the reproductive onset. At the leaf level, priority of resource use shifted to enhance the photosynthetic rate, nutrition conservation and prevention of physical damage, but to decrease C-based chemical defense (total phenolics) during the transition from vegetative to reproductive phase. With higher levels than fine roots and aboveground parts, the N-containing secondary metabolite (reserpine) in coarse roots did not vary greatly in the vegetative stages but increased significantly in the reproductive stages. At the whole-plant level, plant growth shifted from N- to P-limited conditions throughout the ontogenetic development. There were overall trade-offs between leaf physical and chemical defenses across ontogeny of *R. vomitoria* plants, but phenotypic expression of potential defensive traits differed between above- and below-ground parts. Providing synergies or tradeoffs existed between defensive mechanisms, it is important to understand the expression of multiple defenses across ontogeny to perceive lifetime plant defensive strategies. Additional assessment of how herbivory affects plant growth and fitness and the genetic control of plant's resistance, at different developmental stages of *R. vomitoria* plants growing under different environmental conditions, may shed light on the adaptation of ontogenetic variation and on the evolution of defense trajectories, which may be helpful in maximizing the secondary metabolites' yield through the suitable cultivation practices.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.06.013.

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