Sex-related differences in growth and carbon allocation to defence in *Populus tremula* as explained by current plant defence theories

Tendry R. Randriamanana¹,Line Nybakken¹,², Anu Lavola¹, Pedro J. Aphalo³, Katri Nissinen¹ and Riitta Julkunen-Titto¹

¹Natural Products Research Laboratories, Department of Biology, University of Eastern Finland, P.O. Box 111, 80101 Joensuu, Finland; ²Department of Ecology and Natural Resource Management, Norwegian University of Life Sciences, Ås, Norway; ³Department of Biosciences, University of Helsinki, Helsinki, Finland; ⁴Corresponding author (tendry.randriamanana@uef.fi)

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Plant defence theories have recently evolved in such a way that not only the quantity but also the quality of mineral nutrients is expected to influence plant constitutive defence. Recently, an extended prediction derived from the protein competition model (PCM) suggested that nitrogen (N) limitation is more important for the production of phenolic compounds than phosphorus (P). We aimed at studying sexual differences in the patterns of carbon allocation to growth and constitutive defence in relation to N and P availability in *Populus tremula* L. seedlings. We compared the gender responses in photosynthesis, growth and whole-plant allocation to phenolic compounds at different combination levels of N and P, and studied how they are explained by the main plant defence theories. We found no sexual differences in phenolic concentrations, but interestingly, slow-growing females had higher leaf N concentration than did males, and genders differed in their allocation priority. There was a trade-off between growth and the production of flavonoid-derived phenylpropanoids on one hand, and between the production of salicylates and flavonoid-derived phenylpropanoids on the other. Under limited nutrient conditions, females prioritized mineral nutrient acquisition, flavonoid and condensed tannin (CT) production, while males invested more in above-ground biomass. Salicylate accumulation followed the growth differentiation balance hypothesis as low N mainly decreased the production of leaf and stem salicylate content while the combination of both low N and low P increased the amount of flavonoids and CTs allocated to leaves and to a lesser extent stems, which agrees with the PCM. We suggest that such a discrepancy in the responses of salicylates and flavonoid-derived CTs is linked to their clearly distinct biosynthetic origins and/or their metabolic costs.

**Keywords:** aspen, biomass, constitutive defence, dioecious, nitrogen, phenolics, phosphorus, photosynthesis, sexual dimorphism.

**Introduction**

Nitrogen (N) and phosphorus (P) are key nutrient elements and are the main factors limiting the productivity of the biosphere. The driving mechanisms underlying the limitation of N and P often vary geographically, spatially and temporally among sub-habitats (Vitousek and Howarth 1991, Vitousek et al. 2010). Conventionally, the growth of autotrophs is limited by a single mineral nutrient, as stated by Liebig’s law of the minimum. However, there is a growing body of evidence supporting the supposition that elemental nutrients interact with each other and some autotrophs are able to equilibrate their growth and element uptake (Elser et al. 2010, Ågren et al. 2012). Some plants, for instance, are able to do so by secreting phosphatase enzymes, such that they reach an optimum N : P ratio (Güsewell 2004, Marklein and Houlton 2012). By contrast, in other cases, plants’ productivity might be simultaneously...
constrained by multiple resources, a concept known as the ‘multiple element hypothesis’ (Bloom et al. 1985, Rastetter and Shaver 1992, Ågren et al. 2012). In addition, meta-analyses of field experiments manipulating N and P availability reported a uniform and synergistic response of biomass and production to combined N and P enrichment across the biosphere’s major ecosystem types (Elser et al. 2007, Harpole et al. 2011). Such findings point to a preponderant ‘co-limitation’ of these two elemental nutrients as a consequence of closely balanced stoichiometric demand and supply of N and P at the community level of most terrestrial ecosystems, analogously to aquatic and marine ecosystems (Elser et al. 2007, Harpole et al. 2011).

Nitrogen and P are essential components of nucleic acids, phospholipids, ATPs and numerous enzymes, and thus their availability affects ecosystem productivity. Plant secondary metabolites appear to be among the first traits affected by mineral nutrient limitation. For instance, N deficiency induced the expression of the genes involved in phenylpropanoid metabolism, leading to the production of phenolics in tobacco (Fritz et al. 2006). Nitrogen and P depletion were also found to influence the expression level of genes required in the shikimate and phenylpropanoid pathways and to increase the flavonoid content in Arabidopsis (Lillo et al. 2008). Given that secondary metabolites such as phenolics are used by plants for chemical defence, feeding on plants grown under limited mineral nutrients may in turn modify their interaction with other trophic levels such as herbivores (for a review, see for example Chen et al. 2010). Consequently, limitation in N and P may disturb species’ competitiveness and life history traits, and also multistrophic interactions in terrestrial ecosystems.

Due to their biased sex ratio and different growth rates based on gender (Lloyd and Webb 1977, Dawson and Geber 1999, Obeso 2002), dioecious species offer good models for studying plant allocation to defence. European aspen (Populus tremula L.) is a dioecious species and is one of the most widespread broadleaved trees throughout the Eurasian boreal and temperate forests. Natural populations of the European aspen are generally male dominated with a male : female sex ratio of up to 2 : 1 (Myking et al. 2011). So far, the underlying reason behind the predominance of males in Populus populations has not been specifically addressed in the literature. A recent study reported that most insect-pollinated and biotically dispersed trees are male biased (Sinclair et al. 2012). However, P. tremula is wind pollinated and abiotically dispersed, and is therefore an exception. Skewed sex ratios have often been associated with gender differences in reproductive efforts and post-reproductive survival abilities (Lloyd and Webb 1977, Delph 1999, Barrett and Hough 2012). While little is known about gender differences in reproduction costs in P. tremula, it is tempting to think that male catkins might be more expensive to produce in terms of biomass since they are bigger than those of females. However, when considering whole-plant reproduction, unless an extraordinarily high amount of pollen is required during reproduction, females might incur higher total reproductive effort than males because of the long maturation time of catkins into capsules and seeds (Lloyd and Webb 1977, Delph 1999).

Different plant functions compete for the same limited resources, and it is hypothesized that growth, maintenance and sexual reproduction are competing activities. The fact that plants require a minimum age and/or size prior to reproduction argues for the existence of a trade-off between sexual reproduction and vegetative growth (Obeso 2002). It has been suggested that females’ overall greater resource allocation to reproduction would make them less fit than males because of the concomitant decrease in growth rate, longevity and flowering frequency and the delay in reproductive maturity (Delph 1999). In other words, females’ higher reproductive costs would lower their resistance to biotic and abiotic factors, leading ultimately to male-biased sex ratios (Delph 1999, Barrett and Hough 2012 and references therein). The present study involved seedlings that were too young to reproduce; however, studies show that genders often differ in their physiology even in young individuals. In fact, males and females of different Populus species differed often in their morphological and physiological responses to various environmental factors, and in most studies dealing with pre-reproductive plants, females experienced greater negative effects of stress than did males (Xu et al. 2008a, 2008b, 2010, Zhao et al. 2009, 2011, 2012, Zhang et al. 2010, 2011, Peng et al. 2012, Han et al. 2013). Therefore, inequality in reproductive costs might lead to gender differences in morphological and physiological traits, differences that might be inherently present (Delph 1999). This was confirmed by a review including 62 woody species which reported that males outperformed females in size and/or relative growth rate (Obeso 2002).

If European aspen follows the pattern predicted by the resource availability hypothesis (Coley et al. 1985, Endara and Coley 2011), at optimal resources males would have a higher inherent growth rate, enabling them to easily replace the resources lost to herbivores, and they would therefore be expected to invest fewer resources in constitutive defence than do females (Dawson and Ehleringer 1993, Ågren et al. 1999). By contrast, females would grow slowly even under high-resource conditions and, being small, the loss of biomass and elemental nutrients resulting from herbivory or other stress factors would not readily be recovered, so that they would need to invest more in chemical defence for protection. In fact, a meta-analysis assessing the degree of herbivory in dioecious plants confirmed that females were better protected against herbivore damage in comparison with males (Cornelissen and Stiling 2005).

European aspen, like other Salicaceae species, accumulates relatively high levels of carbon-based secondary metabolites, especially salicylates, which are found to be defensive compounds against generalist herbivores (e.g., Ruhola et al. 2001, Heiska et al. 2007). The most prominent theories that are used
to explain resource partitioning to this type of defence are the protein competition model (PCM), which is derived from the carbon-nutrient balance hypothesis, and the growth differentiation balance (GDB) hypothesis (Bryant et al. 1983, Herms and Mattson 1992, Jones and Hartley 1999). The PCM argues that proteins and the production of phenolics compete for a common precursor: phenylalanine (PHE) (Jones and Hartley 1999). Under limited N, growth-related protein synthesis might be more reduced than phenolic synthesis because growth proteins rely not only on PHE but also on other N-containing amino acids, whereas mainly PHE is needed for phenolic synthesis. Accordingly, under conditions of low mineral nutrients, the phenylpropanoid pathway by which phenolics are produced would take priority over growth because the growth rate decreases and so does the likelihood of PHE incorporation into growth-related proteins (Jones and Hartley 1999). Conversely, under non-limiting mineral nutrient conditions, phenolic concentrations will decline due to increased incorporation rate of PHE into growth- and photosynthesis-related proteins. If the PCM is applied to P. tremula, females, which are expected to have slower inherent growth rate and be more vulnerable to nutrient stress, would commit less PHE to growth proteins and thus have more PHE for phenolic synthesis in comparison with males.

In addition, according to the extended PCM as described by Wright et al. (2010), N limitation would also predict different outcomes from P limitation. Reduced N would have a more direct and thus a greater influence on the concentrations of defence-related phenolic compounds than reduced P supply, due to the differential abilities of the plant shikimate pathway to cope with N and P limitation. According to Wright et al. (2010), N limitation would reduce the likelihood of PHE being incorporated into growth proteins, leading to a higher production of phenolic compounds, while P limitation, which mainly affects growth through new cell formation but not through protein production, would not affect phenolic compound production (Wright et al. 2010). Furthermore, phosphorylated intermediates are suggested to have rapid turnover rates and, during P depletion, plants are able to recycle free inorganic phosphate (Pi) produced during the shikimate pathway or from intra- and extracellular P compounds, enabling growth and PHE production to continue independently of the P pool size (De Groot et al. 2003, Plaxton 2004, Lillo et al. 2008, Plaxton and Tran 2011, Maeda and Dudareva 2012).

Therefore, one could expect the same outcome as with the extended PCM, namely that growth and PHE production might not be particularly sensitive to limitation in P supply. By contrast, most of a plant’s N is used in the photosynthetic machinery (e.g., pigment–protein complexes, coupling factors and especially RUBISCO) (Evans 1989), which produces carbohydrates that are necessary for growth and phenolic synthesis. Even though PHE can be recycled and PHE supply may still continue under limited N to support phenolic production (Weaver and Herrmann 1997, Jones and Hartley 1999, Wright et al. 2010), we argue that growth and phenolic biosynthesis can be restricted by N limitation through insufficient synthesis of photosynthetic enzymes and thus lack of carbohydrate production, which is consistent with the GDB hypothesis. GDB predicts a relatively similar pattern to the PCM hypothesis, except that GDB takes into account the interaction between photosynthetic rate and growth as a proxy of carbohydrate surplus (Herms and Mattson 1992). According to GDB, processes that limit growth more than photosynthesis will lead to an accumulation of photosynthates, which can be diverted to differentiation-related processes such as the production of secondary metabolites. The N-driven decrease in carbohydrate production that provides the carbon skeletons for the biosynthesis of amino acids might in turn reduce the availability of PHE. Accordingly, we expect that in addition to PCM predictions, N limitation might also lead to a decrease in the production of phenolics.

Our goal was to study sexual differences in the patterns of carbon allocation to growth and constitutive defence as affected by different levels of N and P availability in males and females of the European aspen. We hypothesized that: (i) N limitation would reduce both growth and phenolic production in the two sexes but to a greater extent in males; (ii) P-supply limitation would affect neither the growth nor the constitutive defence of the two sexes; (iii) demands and use of mineral nutrients will vary between males and females of aspen: males would be growth biased while females, with higher mineral nutrient demands, would invest resources in defence.

Materials and methods

Plant material

During January and February 2011, we collected twigs from 12 (six of each sex) flowering European aspen trees from different locations in eastern and southern Finland (Kaavi 62°54′N, 28°42′E, Liperi 62°41′N, 29°33′E, Loppi 60°43′N, 24°27′E, Pieksamäki 62°18′N, 27°07′E, Polvijärvi 62°52′N, 29°19′E and 62°49′N, 29°20′E, Kontiolahti 62°38′N, 29°41′E). We selected the mother trees from distant locations in order to make sure that they belonged to different genotypes. Axillary buds from each mother tree were aseptically micropropagated using woody plant medium (WPM) supplemented with agar 8.5 g l⁻¹ and benzylaminopurine 1 mg l⁻¹. The plants were maintained in vitro on WPM supplemented with agar 8.5 g l⁻¹ and indole butyric acid 5 mg l⁻¹, at a temperature of 23 ± 0.1 °C, under an 18-h light regime supplied by plant growth lamps (GRO-LUX F36W, Havells Sylvania, Erlangen, Germany) at a photosynthetically active radiation (PAR) of ~70 µmol m⁻² s⁻¹.

Plant acclimation

We carried out the experiment in a greenhouse in Joensuu, Finland (September–December 2011). Prior to the
experiment, 258 micropropagated plantlets were acclimated in the greenhouse and then transferred to 1 l pots filled with non-fertilized 70% peat and 30% vermiculite. The plants were illuminated by a set of 12 high-pressure sodium lamps (400 W, GE Lighting, Cleveland, OH, USA) to yield a PAR of 145–330 μmol m⁻² s⁻¹ at the shoot level and depending on whether the plants were in the centre or slightly in the corner of the greenhouse chamber. A photoperiod of 18 h, a daytime temperature of 26–30 °C, a night-time temperature of 15 °C and an air relative humidity of 41–78% were maintained throughout the experiment.

**Fertilization treatment and experimental design**

We designed the experiment as a full factorial design with two levels of N (NH₄NO₃) and P (NaH₂PO₄): low and optimum P (3.3 and 113 mg l⁻¹ of P, respectively), and low and optimum N (4.2 and 140 mg l⁻¹ of N, respectively). We randomly assigned the 258 seedlings to the following treatments, where O indicates optimum and L indicates low: (OP, ON), (OP, LN), (LP, ON) and (LP, LN).

At the beginning of the experiment, the average mean height was 20.40 ± 0.45 cm for females and 22.05 ± 0.66 cm for males, while the average diameter was 2.70 ± 0.05 mm for females and 2.60 ± 0.05 mm for males. We had six trays per fertilization treatment, each tray including one individual of each of the 12 genotypes. The trays, which were considered as experimental units, were distributed randomly in the greenhouse and were moved around every second day to avoid the effects of differing PAR conditions.

In order to meet the plants’ mineral requirements, we prepared Ingestad’s basic nutrient solution, recommended for a range of different species (Ingestad 1962). The stock nutrient solution used for fertilizing the plants contained the following macronutrients, CaCl₂ · 6H₂O 65.7 g l⁻¹, MgSO₄ · 7H₂O 49.2 g l⁻¹ and KCl 33.5 g l⁻¹, and the following micronutrients, FeCl₃ · 6H₂O 1.4 g l⁻¹, MnCl₂ · 4H₂O 0.18 g l⁻¹, H₃BO₃ 0.29 g l⁻¹, ZnCl₂ 0.012 g l⁻¹, CuCl₂ · 2H₂O 0.015 g l⁻¹ and NaMoO₄ · H₂O 0.0022 g l⁻¹. The stock solution was diluted 100 times and the pH was adjusted to 5.6.

We began the first fertilization on 11 November 2011. At the beginning of every week, we watered each individual pot with 150 ml of diluted stock solution. To ensure that the seedlings were well watered, they were supplied with 200 ml of tap water every second day (except on fertilization days). The pots and trays were provided with bottom holes in order to drain excess water and fertilization solution.

The fertilization treatment lasted for 6 weeks.

**Gas exchange and chlorophyll content index measurements**

During the third and fifth week after the first fertilization treatment, we randomly selected four seedlings of each clone from each treatment (n = 192). We measured the gas exchange parameters on the youngest fully expanded leaf using LC-PRO+ (ADC BioScientific Ltd, Hertfordshire, UK). We carried out measurements from around 9 am to 12 noon and from 2 to 4 pm for 3–5 days, choosing randomly one tray at a time. Leaves’ gas exchange parameters were measured by enclosing them in a square cuvette (6.25 cm² of the leaf surface) with a leaf chamber temperature of 26 ± 0.5 °C and a saturating PAR of 800 μmol m⁻² s⁻¹, which we determined from a light response curve. We recorded the following parameters: net assimilation rate (A), transpiration rate (E), stomatal conductance of water vapour (gs) and intracellular CO₂ concentration (C). Water-use efficiency (WUE) was calculated as the ratio of A/E. We measured chlorophyll content index (CCI) using CCM-200 (Opti-Sciences, Hudson, NH, USA), and we used the same leaves for the two time-point measurements of gas exchange parameters and CCI. For approximation, the relationship between CCI and the actual chlorophyll content (mg cm⁻²) is explained in the case of paper birch according to the following equation (r² = 0.958, n = 100): total chlorophyll a and b content = −2.20 × 10⁻³ + 3.09 × 10⁻³ CCI − 5.63 × 10⁻⁵ CCI² (Richardson et al. 2002).

**Growth measurements**

We measured the height growth and basal diameter of all seedlings (1 cm above the root collar) once a week. We harvested the plants on 13 December 2011, and after drying the biomass samples, we weighed the plant parts separately. The roots were washed and dried at room temperature. We measured leaf area (cm²) using a portable leaf area meter LI-3000C (LI-COR, Lincoln, NE, USA) and calculated specific leaf area (cm² g⁻¹) as the ratio of leaf area to its dry weight.

**Sampling for chemical analyses**

From each individual in all treatments, we selected leaves, stems and roots for phenolic compound analysis. At the end of the experiment, we sampled 10-cm-long stem internodes, 5 cm away from the shoot apex and then divided them longitudinally into two parts. We sampled two mature leaves in order to analyse the concentration of N and phenolics in leaves. The leaves and stems for phenolic concentration measurements were stored in paper bags and dried in a drying room (at a relative humidity of 10%) according to published methods (Julkunen-Titto and Sorsa 2001). In order to obtain root powder for phenolic extraction, we collected two pieces of the main root (3 cm long each), 0.5 cm below the root collar. The root pieces were cut into small pieces and ground with a Precellys® 24 (Bertin Technologies, Île-de-France, France). The dried samples were stored at −20 °C until the analyses were performed.

We measured the N concentration of all the leaves sampled for phenolic analysis using a nitrogen analyser LECO® FP-528 (Leco Corporation Svenska AB, Uplands Väsby, Sweden) on
15 mg leaf discs (without veins) and according to the manufacturer’s directions. We used corn flour 1.7% N as a reference.

**Phenolic analyses**

The analysis of phenolics followed earlier published methods (Ruuhola et al. 2011, Nybakken et al. 2012). Leaf discs of ~5 mg (without veins and taken from the margins), 20 mg of stems cut into small pieces and 15 mg of root powder, respectively, were weighed and extracted separately using 600 µl of cold methanol 100% (v/v). The samples were homogenized for 30 s at 5500 rpm using a Precellys® 24 (Bertin Technologies, Île-de-France, France) and incubated in an ice bath for 15 min. The mixture was then centrifuged at 13,000 rpm for 3 min at +4 °C (Eppendorf® Centrifuge 5415R, Hamburg, Germany) and the supernatant was collected. The residue was re-extracted twice with an incubation time of 5 min in an ice bath. The three consecutive supernatants were pooled together, and the extract was evaporated to dryness using an Eppendorf® concentrator and stored in a deep freezer until high-pressure liquid chromatography (HPLC) analysis. The residues from stem and root phenolic extraction were collected and used for the measurements of methanol-insoluble condensed tannins (CTs).

Prior to the HPLC analyses, the dried extract was re-suspended in 600 µl methanol–water (50 : 50, v/v). A volume of 20 µl from each sample was injected and separated by HPLC (1100 series, Agilent, Santa Clara, CA, USA) equipped with an ALS autosampler (G1329A), a binary pump (G1312A), a vacuum degasser (G1322A), a diode array detector (G1315B), a column compartment (G1315B) and a C18 reverse-phase column (Zorbax SB-C18, 4.6 × 75 mm, particle size 3.5 µm, Agilent). The column and injector temperatures were kept at 30 and 22 °C, respectively. The mobile phase consisted of two solvents: 0.25% o-phosphoric acid and 1.5% tetrahydrofuran in Milli-Q ultrapure water (Merck Millipore, Darmstadt, Germany), and methanol 100% (v/v) with a flow rate of 2 ml min⁻¹.

We used the acid butanol assay for proanthocyanidins, as described by Hagerman (2002), to measure CTs. We determined methanol-soluble and non-soluble CT in stems and roots from an aliquot of dissolved HPLC samples and dried residues of extracted plant material, respectively. For reference, we built a standard curve from purified crude tannin extracts of *P. tremula* leaves, stems and roots, respectively (Hagerman, 2002).

**Compound identification**

We identified compounds according to their retention time, their ultraviolet spectra and by mass spectrometry (MS) using a UHPLC-DAD (1200 series, Agilent) equipped with a quadrupole time-of-flight mass spectrometer (Q-TOF/MS) (6340 series, Agilent). Ultra-HPLC (UHPLC) separation was achieved using a C₁₈ column (2.1 × 60 mm, particle size 1.7 µm, Agilent) as the stationary phase and 1.5% tetrahydrofuran and 0.25% acetic acid in Milli-Q ultrapure water (Eluent A) and 100% methanol (Eluent B) as the mobile phase. We used the following gradient for Eluent B: 0% (0–1.5 min), 0–15% (1.5–3 min), 10–30% (3–6 min), 30–50% (6–12 min), 50–100% (12–20 min) and 100–0% (20–22 min). The flow rate was 0.4 ml min⁻¹ and the injection volume was 0.2 µl. The UHPLC injector and oven temperatures were set at 22 and 30 °C, respectively. The Q-TOF/MS spectra were collected at ESI positive ion mode according to the following parameters: mass range 100–3000 m/z; temperature of the drying gas and sheath gas 350 °C; flow rate respectively 12–11 l min⁻¹; nebulizer pressure 35 psi; capillary voltage 3500 V; nozzle voltage 1000 V; fragmentor voltage 80 V; skimmer voltage 65 V; octopole voltage 750 V. The mass-to-charge ratio m/z 922.0098 was used as a reference for accurate mass measurements. The mass accuracy or error term (in ppm) was calculated as follows: 10⁶ × (monoisotopic mass – accurate mass)/accurate mass. The compounds we were able to identify with UPLC-DAD-MS are listed in Table S1 available as Supplementary Data at Tree Physiology Online. There was one compound that we were unable to identify and therefore did not include in our analyses.

We quantified flavonoids at 220 and 320 nm, salicylates at 220 and 270 nm, and phenolic acids at 320 nm. The concentrations were calculated on the basis of the following standards: salicin for diglucosides salicyl alcohol, salicin, trichocarpin, gentisic acid derivative, acetyl salicin derivative, and lignan; neochlorogenic acid for neochlorogenic acid; p-OH-cinnamic acid for cinnamoyl salicortin, cinnamoyl salicylate, p-OH-cinnamic acid and their derivatives; (+)-catechin for (+)-catechin, galloallocatechin and (−)-epigallocatechin gallate; salicortin for salicortin, HCH-salicortin and disalicortin; hyperin for quercetins and their derivatives; kaempferol-3-glucoside for kaempferols and monocoumaroyl astragalin der; apigenin-7-glucoside for apigenin-7-glucoside; tremulacin for tremulacin and its derivatives; tremuloidin for tremuloidin; chlorogenic acid for chlorogenic acid; 2-O’-acetyl-salicortin for 2-O’-acetyl-salicortin; salireposide for salireposide; and procyanidin B2 for procyanidin.

**Graphic vector analysis**

In order to study the effects of N and P limitation and their interaction on phenolic production and biomass accumulation simultaneously, we carried out a graphic vector analysis (GVA) according to published methods (Haase and Rose 1995, Koricheva 1999, Veteli et al. 2007). For each plant organ, we built vector diagrams based on the relative values of the concentration (y) and content (x) of each phenolic group and the dry weight (z) of each phenolic group, according to the following equation: \( x = f(y, z) \). We determined phenolic content as the amount of phenolics in each plant organ. We then calculated the relative values according to the following formula: treated

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mean × 100 + reference mean. Mean values under optimal N and P nutrition (OP, ON) were used as reference means. The GVA plots were built using SigmaPlot 12.3 (Systat Software, Inc., Chicago, IL, USA).

Statistical analyses
We conducted all the statistical analyses with IBM® SPSS® Statistics 19 (Armonk, NY, USA).

All the data were tested for normality. When needed, non-normal datasets were transformed using square root (e.g., for total root phenolic acid concentration) or logarithmic function (log$(10)$) for CCI, total phenolic acid and flavonoid concentrations in leaves, stems and roots, and total root salicylates and HPLC phenolics and few individual phenolic compounds, which yielded a better model, as inferred from restricted maximum likelihood (REML). The experimental layout is described in the section ‘Fertilization treatment and experimental design’. The data were analysed using the linear mixed-effects model with N, P, sex and genotype nested within sex as fixed factors. The variable ‘trays’ was nested within N and P and was used as a random factor. For datasets with two time points, we used the ‘repeated-measures’ option in the linear mixed-effects model and we specified ‘time’ as a repeated variable. We used the initial values of the seedlings’ height and diameter as covariates. We also used the REML estimation method to select the adequate covariance structure of the random term. When the main and interaction effects were significant, mean comparison and pairwise contrasts were carried out using the least significant difference (LSD) method. When more than one leaf was measured (e.g., leaf area, N concentration), the average mean was taken for statistical tests.

In this paper, low-molecular-weight phenolics, which are the sum of the concentrations of individual phenolic compounds identified with HPLC, are referred to as ‘HPLC phenolics’. The correlation between A and $g_s$, and between A and $E$ was tested using Spearman’s correlation test. A principal component analysis (PCA) was carried out in order to detect which of the dependent variables were most affected by N and P limitation and in order to detect any correlation between the dependent variables. Principal component analysis was undertaken using SIMCA-P+ (Umetrics AB, Umeå, Sweden) on 21 variables: N concentration, height, diameter, CCI (5th week), leaf area, assimilation rate (fifth week), root : shoot ratio, shoot biomass, root biomass, and concentrations of leaf CT, stem CT, root CT, leaf salicylates, leaf flavonoids, leaf phenolic acids, stem salicylates, stem flavonoids, stem phenolic acids, root salicylates, root flavonoids and root phenolic acids.

Results
Gas exchange measurements and CCI
Overall, N limitation significantly reduced A by 13–33%, $E$, $g_s$ and CCI by 18–25%, while P limitation only reduced CCI (Figure 1a–c and f). The assimilation rate was positively correlated with $g_s$ ($r = 0.904$, $P < 0.001$, $n = 381$) and $E$ ($r = 0.913$, $P < 0.001$, $n = 381$) across all treatments and genotypes. Intercellular CO$_2$ concentration and WUE were not affected by any of the nutrient limitation and did not differ between males and females (Figure 1d and e). Females had a significantly higher (12–15% increase) CCI than did males (Figure 1f). Genotypes varied significantly in their A, $E$, C, $g_s$ and CCI (Figure 1a–f). Significant interactions of the treatments with time were also found (Figure 1a–f).

Total leaf N concentration, height, diameter, biomass and leaf area
Nitrogen limitation reduced foliar N concentration (%N), which was also significantly affected by sex and genotype (Table 1). Females had significantly higher N concentration than did males ($P = 0.012$, Table 1). Although not significant, the effect of N level on N concentration was slightly higher for males under low P (33% increase) than for males under optimum P (31% increase). We detected the largest height and diameter increment under (OP, ON) for both sexes. The main effects of genotype and N level on height and diameter were significant, while that of P was not (Table 1). Phosphorus limitation reduced the height of females only, which results in a significant sex × P interaction ($P < 0.001$, Table 1). Seedlings of both sexes were up to 20% taller under ON than under LN, while the sex-based difference in height growth was greater under ON, which explains sex × N ($P = 0.042$, Table 1). Irrespective of P and N levels, males were up to 16% taller than females, and sex-based differences were greater under LP, which resulted in a significant sex × P interaction ($P < 0.001$, Table 1). Sex-based differences in diameter were only significant under (OP, LN) and (LP, ON), in which females had 4–7% thicker diameters than did males, which results in a significant sex × N × P interaction (Table 1). Nitrogen limitation significantly decreased the seedlings’ diameter, with the exception of females under OP, and the effect of N level was greatest for females under low P, which also results in a significant sex × N × P interaction ($P = 0.009$, Table 1).

Seedlings under ON also had significantly higher leaf, stem, root and total biomass than under LN in both sexes (Table 1). Overall, males had significantly higher leaf and stem biomass, up to 13% higher total biomass and up to 24% lower root : shoot ratio than did females (Table 1). We also found a significant effect of genotype on leaf, stem, root, total biomass and root : shoot ratio (Table 1). We detected no significant effect of P level on root : shoot ratio. On the other hand, we found that under ON, P limitation significantly increased root : shoot ratio, and the effect of N level on root : shoot ratio was highest under OP, which explains the significant N × P interaction (Table 1). As with females under ON, males (irrespective of their N level) allocated up to 45% of their total biomass to roots.
biomass to leaves, then to roots and least to stems, while females under LN favoured root biomass allocation (which was up to 43% of the total biomass) over leaf and stem biomass allocations (Table 1).

We found no significant effect of P level on leaf area (Table 1) but seedlings under ON had up to 23% greater leaf area than did seedlings under LN ($P < 0.001$, Table 1). Males had up to 2–9% greater leaf area and specific leaf area than did females (Table 1). Leaf area and specific leaf area differed significantly between genotypes ($P < 0.001$, Table 1). Seedlings under combined ON and OP had significantly greater leaf specific area than that of other treatment combinations, thus the significant N × P interaction (Table 1).

**Phenolic compound concentrations**

Nitrogen limitation significantly affected the concentrations of 14 out of the 33 individual compounds in leaves, and P limitation affected seven compounds (see Table S2 available as Supplementary Data at Tree Physiology Online); P also affected almost all the identified compounds in roots (see Table S6 available as Supplementary Data at Tree Physiology Online), but it only affected seven out of 27 identified compounds in stems (see Table S4 available as Supplementary Data at Tree Physiology Online). Overall, genotype had a significant effect on leaf, stem and root total HPLC phenolics, salicylates, flavonoids and phenolic acids (see Tables S2, S4 and S6 available as Supplementary Data at Tree Physiology Online).

**HPLC phenolics in leaves**

We found that N limitation significantly decreased leaf total concentrations of HPLC phenolics, salicylates and phenolic acids, but P level had no significant effect (see Table S2 available as Supplementary Data at Tree Physiology Online). Leaf total flavonoid concentrations were considerably lower under combined ON and OP than other treatment combinations.
Table 1. Average means of all growth-related variables: height, diameter, total, root, leaf, stem, shoot biomass, root : shoot ratio, leaf area, specific leaf area (SLA) and N concentration (N%) of leaves within each combination level of N and P in males (M) and females (F) of aspen (n = 248).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sex</th>
<th>Diameter (mm)</th>
<th>Height (cm)</th>
<th>Leaf biomass (g)</th>
<th>Stem biomass (g)</th>
<th>Shoot biomass (g)</th>
<th>Root biomass (g)</th>
<th>Total biomass (g)</th>
<th>Root : shoot ratio</th>
<th>Leaf area (cm²)</th>
<th>SLA (cm² g⁻¹)</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(LP, LN)</td>
<td>F</td>
<td>3.73 ± 0.06</td>
<td>28.66 ± 0.74</td>
<td>2.46 ± 0.17</td>
<td>1.36 ± 0.10</td>
<td>3.82 ± 0.27</td>
<td>2.79 ± 0.20</td>
<td>6.61 ± 0.42</td>
<td>0.82 ± 0.08</td>
<td>52.42 ± 2.24</td>
<td>189.70 ± 5.22</td>
<td>1.41 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>3.76 ± 0.08</td>
<td>36.73 ± 0.97</td>
<td>3.15 ± 0.24</td>
<td>1.68 ± 0.14</td>
<td>4.83 ± 0.38</td>
<td>2.93 ± 0.25</td>
<td>7.76 ± 0.59</td>
<td>0.62 ± 0.04</td>
<td>62.15 ± 3.43</td>
<td>194.83 ± 8.60</td>
<td>1.27 ± 0.04</td>
</tr>
<tr>
<td>(OR LN)</td>
<td>F</td>
<td>3.94 ± 0.08</td>
<td>29.72 ± 0.66</td>
<td>2.61 ± 0.15</td>
<td>1.45 ± 0.10</td>
<td>4.05 ± 0.25</td>
<td>3.06 ± 0.18</td>
<td>7.11 ± 0.37</td>
<td>0.80 ± 0.05</td>
<td>53.57 ± 2.65</td>
<td>188.04 ± 5.48</td>
<td>1.32 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>3.66 ± 0.07</td>
<td>33.05 ± 1.05</td>
<td>2.72 ± 0.23</td>
<td>1.40 ± 0.14</td>
<td>4.12 ± 0.37</td>
<td>2.69 ± 0.19</td>
<td>6.73 ± 0.56</td>
<td>0.65 ± 0.04</td>
<td>57.86 ± 3.84</td>
<td>198.46 ± 8.65</td>
<td>1.30 ± 0.06</td>
</tr>
<tr>
<td>(LP, ON)</td>
<td>F</td>
<td>4.18 ± 0.09</td>
<td>33.93 ± 0.87</td>
<td>4.20 ± 0.24</td>
<td>2.41 ± 0.15</td>
<td>6.61 ± 0.38</td>
<td>3.68 ± 0.28</td>
<td>10.30 ± 0.55</td>
<td>0.59 ± 0.04</td>
<td>64.61 ± 3.23</td>
<td>192.96 ± 6.74</td>
<td>2.00 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>4.06 ± 0.09</td>
<td>43.62 ± 1.43</td>
<td>5.23 ± 0.32</td>
<td>2.81 ± 0.20</td>
<td>8.04 ± 0.50</td>
<td>3.62 ± 0.32</td>
<td>11.67 ± 0.71</td>
<td>0.50 ± 0.05</td>
<td>74.31 ± 5.14</td>
<td>209.73 ± 10.24</td>
<td>1.90 ± 0.07</td>
</tr>
<tr>
<td>(OP, ON)</td>
<td>F</td>
<td>4.18 ± 0.09</td>
<td>38.13 ± 1.09</td>
<td>4.30 ± 0.28</td>
<td>2.52 ± 0.19</td>
<td>6.82 ± 0.45</td>
<td>3.30 ± 0.23</td>
<td>10.12 ± 0.58</td>
<td>0.54 ± 0.04</td>
<td>74.49 ± 3.76</td>
<td>217.52 ± 8.91</td>
<td>2.01 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>4.04 ± 0.10</td>
<td>45.24 ± 1.47</td>
<td>5.30 ± 0.33</td>
<td>2.96 ± 0.21</td>
<td>8.26 ± 0.53</td>
<td>3.39 ± 0.33</td>
<td>11.66 ± 0.80</td>
<td>0.42 ± 0.03</td>
<td>78.99 ± 3.54</td>
<td>236.69 ± 9.28</td>
<td>1.89 ± 0.09</td>
</tr>
</tbody>
</table>

Asterisks (*) denote P-values: *P < 0.05; **P < 0.01; ***P < 0.001; $F_{sex}$ tests the effects of sex for each measured variable, $F_N$ tests the effects of N, $F_P$ tests the effects of P, $F_{clone}$ tests the effects of clone, $F_{time}$ tests the effects of time, $F_{sex \times N}$, $F_{sex \times P}$, $F_{sex \times time}$, $F_{N \times P}$, $F_{N \times time}$, $F_{P \times time}$, $F_{sex \times N \times P}$, $F_{sex \times P \times time}$, $F_{sex \times N \times time}$, $F_{sex \times N \times P \times time}$ and $F_{sex \times N \times P \times time}$ are their interaction effects. Values are average means ± 1 SE.
We found no sex-related differences in leaf total flavonoid, salicylate and total HPLC phenolic concentrations (Figure 2a, see Table S2 available as Supplementary Data at *Tree Physiology* Online), but females had more concentrated leaf phenolic acids under LN but not under ON (significant N × sex, *P* = 0.039, see Tables S2 and S3 available as Supplementary Data at *Tree Physiology* Online). Leaf total HPLC phenolics represented 15–21%, salicylates 10–15%, flavonoids 3–5% and phenolic acids 0.3–0.5% of the leaf dry weight (see Table S3 available as Supplementary Data at *Tree Physiology* Online).

Salicortin, which represented up to 58% of leaf total salicylates, and tremulacin (31%) were the most abundant leaf salicylate compounds in leaves (see Table S3 available as Supplementary Data at *Tree Physiology* Online). Salicin, salicortin and tremulacin concentrations were significantly lowered by N limitation (see Table S2 available as Supplementary Data at *Tree Physiology* Online). The most abundant leaf phenolic acid was chlorogenic acid (~38% of leaf total phenolic acids), and this compound was up to 48% more concentrated in females than in males (see Table S3 available as Supplementary Data at *Tree Physiology* Online). The most abundant flavonoids in leaves were kaempferol-3-glucuronide and (+)-catechin (up to 56% of leaf total flavonoids, see Table S3 available as Supplementary Data at *Tree Physiology* Online), and P limitation increased the concentrations of leaf flavonoids under ON but not under LN, which explains the significant N × P interaction (see Table S2 available as Supplementary Data at *Tree Physiology* Online).

![Figure 2](https://academic.oup.com/treephys/article-abstract/34/5/471/2332729/34-5-471-12332729)
HPLC phenolics in stems
We found that N limitation increased stem total HPLC phenolic, phenolic acid and flavonoid concentrations (Figure 2c, see Tables S4 and S5 available as Supplementary Data at Tree Physiology Online). Nitrogen limitation also increased stem salicylate concentrations, but only in females ($P = 0.008$, see Table S4 available as Supplementary Data at Tree Physiology Online). Males had significantly more stem salicylates than did females under ON ($P = 0.023$, see Table S4 available as Supplementary Data at Tree Physiology Online). Phosphorus limitation also increased stem phenolic acid and flavonoid concentrations, but had no significant effect on total HPLC phenolic and salicylate concentrations (see Table S4 available as Supplementary Data at Tree Physiology Online). Stem HPLC phenolics represented 10–13% of stem biomass, salicylates 9–10%, flavonoids 0.2–0.5% and phenolic acids 0.09–0.18% (see Table S5 available as Supplementary Data at Tree Physiology Online).

Salicortin was the main salicylate compound in stems (up to 80%, see Table S5 available as Supplementary Data at Tree Physiology Online). Nitrogen limitation increased stem total phenolic acid concentration irrespective of the P level, but the difference between ON and LN was greater under OP, which explains the significant N × P interaction (see Table S4 available as Supplementary Data at Tree Physiology Online). By contrast, P limitation increased stem total phenolic acids only under ON ($P = 0.002$), but not under LN, which also explains the significant N × P interaction (see Table S4 available as Supplementary Data at Tree Physiology Online). The most abundant phenolic acid in the stem was p-OH-cinnamic acid derivatives (see Table S5 available as Supplementary Data at Tree Physiology Online), and females had up to 37% more phenolic acids in the stems than did males ($P < 0.001$). We found that total stem flavonoids and especially (+)-catechin concentrations (with 30–50% of the total) were significantly increased by N and P limitation (see Tables S4 and S5 available as Supplementary Data at Tree Physiology Online). We found no significant sex-related differences in total stem flavonoids; however, males had more (+)-catechin under LN but not under ON ($P < 0.001$, N × sex interaction), whereas females had more (−)-epigallocatechin gallate ($P < 0.001$) (see Tables S4 and S5 available as Supplementary Data at Tree Physiology Online). Moreover, these two compounds were both increased by N limitation, but the increase was more pronounced in males than in females, which explains the significant N × sex (see Table S4 available as Supplementary Data at Tree Physiology Online).

HPLC phenolics in roots
The main effect of N and P level on root phenolics was significant, except for that of flavonoids (see Table S6 available as Supplementary Data at Tree Physiology Online). In males, N limitation increased root HPLC phenolic ($P < 0.001$) and salicylate ($P < 0.001$) concentrations under OP and ON but not under LP or LN, thus the significant N × P interaction (see Table S6 available as Supplementary Data at Tree Physiology Online). Moreover, under LN, males’ roots had more total HPLC phenolics and salicylates than did females, which explains the significant N × sex interaction (see Table S6 available as Supplementary Data at Tree Physiology Online).

Root total HPLC phenolics represented 8–12%, salicylates 7.5–11%, flavonoids 0.3–0.4% and phenolic acids 0.1–0.2% of the root dry weight. Salicortin represented up to 70% of root total salicylates (see Table S7 available as Supplementary Data at Tree Physiology Online), and that compound was significantly increased by both N and P limitation ($P < 0.001$). Salicortin concentration in roots was 16–25% higher in males than in females ($P < 0.001$). Both N and P limitation decreased root total phenolic acid concentrations ($P < 0.001$), and the increase attributable to P limitation was greater than that of N limitation, which explains the significant N × P interaction ($P < 0.05$, Figure 2e, see Table S6 available as Supplementary Data at Tree Physiology Online).

The most abundant phenolic acid in roots was p-OH-cinnamic acid derivatives (see Table S7 available as Supplementary Data at Tree Physiology Online). The males’ roots had 2–13% more phenolic acids than those of females (see Table S7 available as Supplementary Data at Tree Physiology Online). Nitrogen limitation significantly increased all individual flavonoid compounds except for (−)-epigallocatechin gallate (see Tables S6 and S7 available as Supplementary Data at Tree Physiology Online). The most abundant flavonoid in roots was (+)-catechin, and the concentration of (+)-catechin was significantly higher in females than in males (see Tables S6 and S7 available as Supplementary Data at Tree Physiology Online). Under OP but not under LP, root total flavonoids were significantly more concentrated in females than in males ($P < 0.001$), which explains P × sex interaction (see Table S6 available as Supplementary Data at Tree Physiology Online).

Condensed tannin concentrations
Both N and P levels had a significant effect on leaf, stem and root CT concentrations (see Tables S2, S4 and S5 available as Supplementary Data at Tree Physiology Online, Figure 2b, d and f). Combined N and P limitation significantly increased leaf CT concentration by 51–62% and stem CT by 17–55% when compared with the other three N and P treatment combinations (see Tables S2 and S4 available as Supplementary Data at Tree Physiology Online, Figure 2b and d). Nitrogen limitation significantly increased but P limitation marginally lowered the concentration of total CT in roots, which explains the significant N × P interaction (Figure 2f, see Table S6 available as Supplementary Data at Tree Physiology Online). Nitrogen limitation significantly increased the concentration of soluble CT in roots (see Tables S6 and S7 available as Supplementary Data at Tree Physiology Online).
Likewise, N limitation decreased the concentration of root insoluble CT but in females only, which explains the significant N × sex interaction (see Table S6 available as Supplementary Data at Tree Physiology Online). We found no significant effects of sex, while genotype had a significant effect on leaf, stem and root CT (see Tables S2, S4 and S6 available as Supplementary Data at Tree Physiology Online).

**Principal component analysis**

Principal component analysis showed a clear demarcation between seedlings under optimum and limited N, but not for seedlings under OP and LP (Figure 3a). Overall, seedlings under ON had higher N concentration, leaf area, CCI, A, shoot biomass, height and diameter, and higher leaf salicylate and phenolic acid concentrations, but lower root : shoot ratio, lower concentrations of stem and root phenolic acids, and lower leaf, stem and root flavonoid and CT concentrations than did seedlings under LN. The model explained 52.7% of the dataset’s total variance, which was loaded into three components. The first component (24.4%) was strongly influenced by shoot biomass, height, diameter, leaf area, and leaf salicylate and phenolic acid concentrations, which were negatively correlated with root : shoot ratio, stem CT, salicylate, phenolic acid and flavonoid concentrations and root flavonoid and CT concentrations (Figure 3b). The second component (14.8%) was strongly influenced by N concentration, CCI, A and leaf salicylate concentrations, which were negatively correlated with root salicylate and phenolic acid concentrations, leaf and stem CT concentrations, and leaf and stem flavonoid concentrations (Figure 3b). The third component (13.4%) was mainly influenced by root biomass, diameter, salicylate and phenolic acid concentrations of stems and roots, CCI, A, leaf area, shoot biomass and root : shoot ratio, which were all positively correlated.

**Graphic vector analysis**

Concentration values are suitable indexes for plant quality in relation to herbivores; however, chemical compounds in terms of amounts are more relevant for the study of plants’ allocation to defence, as plants produce molecules in quantities, not in concentrations. We used GVA to investigate N- and P-induced shifts in plants’ contents and concentrations as a function of plant biomass. Phosphorus limitation significantly increased the synthesis of phenolic acids in stems and roots as well as the synthesis of flavonoids and CTs in leaves to a greater extent in females than in males (Figure 4e, f, g and j). In stems and roots, P limitation had only minor effects on flavonoids and CT synthesis, except for males under LP in which P limitation decreased root CT content by 60% (Figure 4h, i, k and l). In most cases, the effect of N limitation alone on HPLC phenolics and CTs was similar to that of combined N and P limitation. For instance, N and combined N and P limitation reduced the synthesis of leaf salicylates and phenolic acids, which means that both their concentration and content were decreased in leaves (Figure 4a and d). Combined N and P limitation also decreased stem salicylate content by up to 50% in male seedlings (Figure 4b). By contrast, N and P limitation slightly increased salicylate, phenolic acid and flavonoid synthesis in roots and phenolic acid synthesis in stems (Figure 4c, e, f and i). In stems of female seedlings,
an increase in salicylate concentration and a decrease in salicylate content were paralleled with a decrease in stem dry weight, leading to a concentration effect (Figure 4b). Due to N limitation, there was also a concentration effect of leaf phenolic acids (Figure 4g) and stem CT in males (Figure 4k). In both sexes, N and combined N and P limitation increased leaf and stem flavonoid as well as CT concentrations by up to 150% and contents by 50–100% (Figure 4g, h, j and k).

Discussion
We inferred from PCA that N had more influence on growth and phenolic concentrations of European aspen seedlings than P and from GVA that P had more influence on the accumulation of leaf flavonoid-derived phenylpropanoids than N. The low irradiance under which we conducted our experiment might have increased leaf N concentration and might have reduced seedling photosynthesis and growth rates.
However, when transferred outdoors, seedlings of the same age and genotypes achieved similar growth increments as in the present study (unpublished data), which confirms that seedling growth was not too much affected by light limitation. Consequently, the low irradiance might have increased the carbon surplus between photosynthesis and growth, thus increasing the availability of phenolic precursors and seedling allocation to phenolics, but this should be tested further. In addition, the present study, which dealt with seedlings, should be interpreted with caution because when maturing, *Populus* trees may experience age-related changes in phenolic concentrations (Donaldson et al. 2006b) and in growth and photosynthetic rates. This would suggest the need to undertake further studies concerning gender differences of adult *Populus* trees in terms of resource allocation to growth and chemical defence. Besides, there is a high variability in leaf N concentrations found in *Populus* sp. depending on various factors such as growth rate, seedling age, genotype or season (Donaldson et al. 2006b, Stevens et al. 2007, Harding et al. 2009, Holeski et al. 2012, Luo et al. 2013). The leaf N concentration of our seedlings falls within the average of what has been found in several studies dealing with *Populus* sp. (1–2.6%) (Häikiö et al. 2007, 2008, Nikula et al. 2010, Calder et al. 2011, Coll et al. 2011, Kosonen et al. 2012, Way et al. 2013). In addition, it is worth mentioning that even though the root growth was greatly increased by N limitation, roots did not appear to be restricted by the pots. Moreover, as in other species of *Populus*, a reduced N concentration in leaf was associated with an increase in allocation to below-ground biomass and a decline in photosynthetic rate, chlorophyll content and specific leaf area (Coleman et al. 1998, 2004, Reich et al. 1998, 1999, Ripullone et al. 2003, Cooke et al. 2005).

### The distribution and roles of constitutive defence in European aspen seedlings

Despite their substantial vulnerability to herbivory and their fitness value to the plant, stem and especially root chemical defences are rarely considered in plant defence theories. To our knowledge, this is the first study analysing the production of root phenolics in the European aspen. Our data showed that after N was reduced, leaf and stem salicylate content decreased and that of roots increased (Figure 4a–c). We also found that the effect of N and P limitation on salicylate content depended on the organ and probably reflected differences in tissue gene expression and/or source–sink relationships. In *Populus* species, the local syntheses of phenolic glycosides and tannins were connected with long-distance transport of carbohydrates, suggesting a sink strength effect on the levels of constitutive phenolics (Arnold et al. 2004). In addition, the leaves, stems and roots of *Populus trichocarpa* L. seedlings had different metabolite profiles and responded differently to N and P limitation, which supports the existence of an organ-specific shikimate pathway and phenylpropanoid function for salicylate production (Tsai et al. 2006).

Salicylates are the main phenolics in European aspen seedlings and they seem to have important functions in *Populus* and in their close relatives in the Salicaceae family (Julkunen-Titto 1986, Boekelker et al. 2011). Salicylate concentrations are known to reduce vole feeding in willows (e.g., Heiska et al. 2007) and also to impair the performance of gypsy moth larvae (Lymantria dispar) (Osier et al. 2000, Donaldson and Lindroth 2007) and reduce leaf mining damage (Young et al. 2010) in quaking aspens. We found that the leaves of our aspen seedlings contained more salicylates than did stems and roots, which fit the optimal defence theory predictions that, in order for plants to maximize their fitness, they should distribute their defences to the organ at the highest risk for predation and having the highest fitness value (Stamp 2003 and references therein). Thus, as reflected in their higher salicylate concentrations, leaves would be most likely to be attacked by generalist herbivores and would have the highest fitness value, followed by stems and roots.

The role of CTs in *Populus* is not yet very clear, and they do not seem to affect the performance of herbivorous insects such as gypsy moths in quaking aspens (Osier et al. 2000, Osier and Lindroth 2006, Donaldson and Lindroth 2007). Tannins are known to have astringent and anti-digestive properties, especially for mammalian herbivores (Barbehenn and Constabel 2011), and it is suggested that they protect plants against photodamage (Close and McArthur 2002, Mellway and Constabel 2009). It has also been suggested that CTs affect the dynamics of the microbial communities that are responsible for nutrient cycling and thus regulate nutrient dynamics in terrestrial ecosystems (Schweitzer et al. 2008). For instance, a high correlation between fine-root production and leaf CT concentrations has been detected in *Populus fremontii* S. Watson and *Populus angustifolia* James (Fischer et al. 2006). Since CTs are known to decelerate N mineralization, to retard microbial activity and thus to slow down litter decay, it was suggested that there is a dynamic feedback between leaf CT and fine-root production in order to compensate for slow nutrient cycling and thus to meet the plants’ nutrient requirements (Fischer et al. 2006, Madritch et al. 2007, Schweitzer et al. 2008). This corroborates our result as we found that under low N, an increase in root biomass was associated with high CT concentrations in leaves, especially in females.

### Effect of N and P on phenolic production and its implication for plant defence hypotheses

We used GVA to describe plants’ allocation to constitutive defence, as it gives an unambiguous estimate of the actual amount of secondary metabolites that is produced by the plants (e.g., Koricheva 1999). In order to compare our results with current plant defence theories, we hereafter discuss how...
the amounts of salicylates, phenolic acids, flavonoids and CTs in aspen seedlings are affected by N, P or combined N and P limitation. In terms of CTs and flavonoids, our results were in general consistent with the PCM predictions that N limitation should increase the production of phenolics, although this was not the case for leaf and stem salicylates. In contrast to the extended PCM, which predicts that N limitation affects phenolic production more than does P limitation, we found that both N and P limitation increased flavonoid and CT production in most plant parts. Interestingly, P limitation had an even stronger effect on leaf flavonoid and CT accumulation than did N, especially in females (Figure 4g and j), which suggests that the synthesis of (+)-catechin, a precursor of flavonoids and CTs, probably needs more P or that the decreasing effect of P limitation on the production of growth proteins in leaves would be greater than what we expected, leaving room for CT production. Moreover, we found that foliar N concentration was negatively correlated with flavonoid and CT concentrations in leaves and stems and to a smaller extent in roots. Such findings suggest that when foliar N concentration is high, seedlings grow more, and consequently more PHE is committed into growth-related proteins, resulting in a lower availability of PHE and thus a lower production of flavonoids and CTs. Likewise, N limitation increased CT concentrations in Populus tremuloides Michx., but it often causes a decrease in leaf salicylates (Osier and Lindroth 2001, 2006, Donaldson et al. 2006a). Phosphorus had only minor effects on phenolic compound concentrations (Keski-Saari and Julkunen-Titto 2003a), while N limitation increased leaf total HPLC phenolic and CT concentrations in birches (Keski-Saari and Julkunen-Titto 2003b).

In Populus, salicylates, which are produced via a partially distinct metabolic pathway from that of flavonoids and CTs (Tsai et al. 2006), are probably less sensitive to the P pool and appear to match the GDB hypothesis. As predicted by GDB, we found that N availability limited both growth and photosynthesis, which would presumably lead to a decrease of surplus carbohydrates for salicylate production, and thus the decrease of their amounts in leaves and stems. Since root growth was increased under limited N availability, roots would probably have greater access to carbohydrates, which would explain their increased synthesis of salicylates under low N and P. Hence, it is very likely that in aspen seedlings, salicylate synthesis is more dependent on carbon surplus, as predicted by GDB, while flavonoid and CT synthesis is more limited by PHE availability, as predicted by the PCM. When growth was not limited (under ON and OP), high-molecular-weight flavonoid and CT appeared to be expensive to produce and were thereby reduced, while salicylate synthesis continued. This was probably due to their higher metabolic costs in comparison to salicylates and phenolic acids (Gershenzon 1994).

As in our case, many studies of the Populus species have reported that seedlings with high CT had low phenolic glycoside content and vice versa (Donaldson et al. 2006b, Kosonen et al. 2012). It has been suggested that the contrasting responses of growth and HPLC phenolic vs CT concentrations to nutrient limitation are linked with the fact that HPLC phenolic concentrations in aspen are thought to be mainly determined by the genotype, while CT concentrations are more plastic and depend more on the treatment and the environment (Osier and Lindroth 2001, 2006). An attempt has been made to elucidate such a trade-off by feeding Populus cell cultures with salicylic alcohol (a precursor and aglycone form of salicin) and other precursors, and it has been suggested that glycosylation of salicylic alcohol appears to regulate carbon partitioning between salicylates and CTs (Payyavula et al. 2009).

Sex-related differences in nutrient requirement, growth and allocation costs to chemical defence

As expected, males had a higher inherent growth rate (taller, greater leaf area and higher shoot biomass) than did females while, interestingly, females had higher leaf N concentration, greater stem diameter and higher chlorophyll content than did males. It can be argued that, although no significant sex-related differences in A were found, males grew better because they had a higher leaf weight ratio and greater leaf area and thus could photosynthesize better than females. This confirms our hypothesis that males would be more growth oriented than females. Genders did not differ much in their allocation to phenolic defence but they seemed to differ in their allocation priority. Under P limitation, females produced slightly more foliar flavonoids and CTs and thus probably have higher costs of chemical defence than males. This corroborates our hypothesis that slow-growing females would commit more PHE into phenolic synthesis than into growth proteins. Even under optimum nutrient conditions, females' slower growth and higher leaf N concentration when compared with males might be some sort of strategy for maximizing PHE availability in order to produce more CTs. Therefore, there is a trade-off between growth and the production of flavonoid-derived phenylpropanoids, and investing in vegetative growth appeared to be the priority in males while investing in chemical defence appeared to be more important than growing fast in females.

Given that females have a higher N concentration than males, the reasons underlying females’ lower growth are uncertain. On one hand, one may assume that since the foremost plant functions (growth, sexual reproduction and defence) compete for the same limited resources, in order to adapt to most ecological situations, plants have to make trade-offs between these three life history traits. Past studies suggested that N is more limiting to reproduction than is carbon (McDowell et al. 2000, Oseso 2002), and carbon rarely limits tree growth (Millard et al. 2007). Consequently, one could argue that females, having higher reproduction costs than males, are more demanding in terms of N, and thus females invest more in mineral nutrient
uptake and store N for future reproduction. This hypothesis, however, needs to be tested by conducting further experiments with mature plants and taking reproductive costs into account. In either case, it can be inferred that females would be less oriented to growth and more oriented to chemical defence. At the population level, producing females would probably be more expensive because they incur higher costs in chemical defence and/or possibly higher total reproductive costs relative to males. In the context of a preponderant co-limitation of N and P to terrestrial ecosystems, this corroborates the conventional sex ratio theory stating that in order to maximize their fitness, populations of dioecious species would favour the sex with the lower cost, which in the case of Populus is male.

Conclusions

We could infer from our study that females invested more in mineral nutrient acquisition and flavonoid and CT production while males invested more in vegetative growth, especially in above-ground biomass. If we compare our findings with the results expected from the PCM scenario, under limited N, growth should have decreased for the benefit of salicylate production, while in our case both growth and salicylate production were limited by the lack of N. Salicylates rather followed the prediction pattern of GDB, as their amounts in leaves and stems were reduced by N limitation. Differences in salicylates between leaves, stems and roots would probably reflect differences in the regulation of shikimate and phenylpropanoid pathways and/or differences in the amount of available carbohydrates, and they might be a means to optimize defence. Flavonoids and CTs, however, were negatively correlated with N concentration and followed the prediction of the PCM. The P level affected height growth in females, root : shoot ratio, the concentrations of some individual salicylates as well as the production of leaf flavonoids and CTs in both genders. To conclude, despite the frequent assumption that carbon availability is the main factor limiting carbon-based secondary metabolites, it is very likely that N and P limitation affects plants’ constitutive defence more than does carbon, at least within the current context of increasing CO₂ availability as induced by climate change.

Supplementary data

Supplementary data are available at Tree Physiology online.

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Conflict of interest

None declared.

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