# Metabolite variation in ecologically diverse black cottonwood, *Populus trichocarpa* Torr. & A. Gray

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**Abstract**

Black cottonwood (*Populus trichocarpa* Torr. & A. Gray) is mass productive tree species native to the Pacific Northwest of North America. Gas chromatography - mass spectrometry was used to study the metabolic profiling of leaves from multiple genotypes to investigate the presence of clinal trends in metabolite levels and to determine if relationships with geo-climatic variables and date of bud set exist. In the late summer (September 3rd) of 2008, young leaves were collected from the species' range and represented by 106 clones grown in a common garden established in Vancouver, British Columbia, Canada. The results validity was verified through the use of two independent canonical correlation analyses (CCA) that were performed on the intensity of the detected 104 compounds, including 40 known metabolites. Principle Component Analysis (PCA) was performed for original variables reduction and to determine the principle components accounting for most of the variation (the first ten PCAs accounted for 63% of the variation). The first analysis utilized the metabolites associated with the first ten principal components to determine the relationship between the original metabolites and geography, climate and date of bud set, while the second was based on the first ten principal components themselves. Both analyses yielded strong to moderate trends but the correlations (ranging from 0.45 to 0.97) were not statistically significant most likely due to the small sample size used. Based on the analyses conducted, it appears that *P. trichocarpa* ecotypes are preconditioned to suite their location-origin and the observed differences in metabolites reflected the genotypic variability among the studied trees.

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**Dedication**

I dedicate this thesis to my parents for their encouragement and support. Special thanks go to my husband for being there when I needed help, my children for their patience and my brothers for motivating me to attain my dream.

# 1 Introduction

Black cottonwood, *Populus trichocarpa* Torr. & A. Gray, a member of the family *Salicaceae,* is the largest and fastest growing hardwood tree in western North America and the largest of the American poplars (DeBell 1990; Bassman and Zwier 1991). *P. trichocarpa* covers vast areas of the Pacific Northwest region, spanning 31 latitudinal degrees (31º - 62° N). It expands northeast from Kodiak Island along Cook Inlet, southeast in southeastern Alaska, and British Columbia to the forested areas of Washington and Oregon, then to the mountains in southern California and northern Baja California with many scattered small populations in southeastern Alberta, eastern Montana, western North Dakota, western Wyoming, Utah, and Nevada (Figure 1.1) (DeBell 1990).

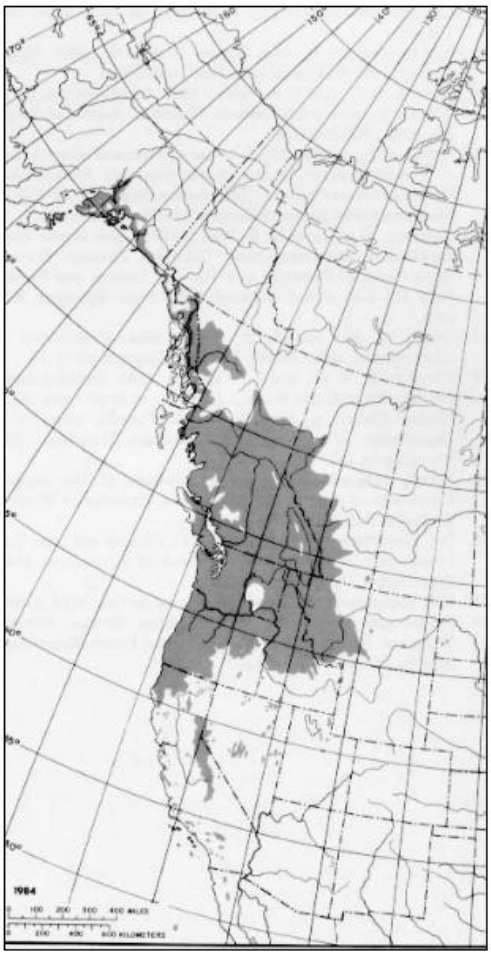

**Figure 1.1** *Populus trichocarpa* geographic range (DeBell 1990).

As a deciduous forest tree, *P. trichocarpa* has substantial economic value as a raw material for wood products (plywood) and pulp and paper (Taylor 2002) and environmental importance as an ecosystem component that harbours many plants and fauna (Jansson and Douglas 2007). Currently, it is used for windbreaks and shelterbelts as well as industrial plantations (DeBell 1990; Braatne *et al.*, 1996; Stettler *et al*., 1996). The species harbours extensive natural phenotypic and genotypic variation, is easily propagated and genetically transformed, and more importantly is characterized by a relatively small genome size (Taylor 2002; Ma *et al*., 2004; Tuskan *et al*., 2006; Street *et al*., 2006). Consequently, *P. trichocarpa* became the first forest tree species to be chosen as a model system for plant biology with established molecular genetics and physical maps and a sequenced genome. The species offers a unique opportunity to investigate biological interactions, wood formation and seasonality; attributes not easily found in other model plants (Tuskan *et al*., 2006; Jansson and Douglas 2007). Moreover, black cottonwood permitted comparative studies with Arabidopsis and the description of a wood transcriptome in a perennial species (Taylor 2002; Quesada *et al*., 2008). As a promising bioenergy crop, *P. trichocarpa* has become of interest as a source of renewable biofuel (Ragauskas *et al*., 2006; Li *et al*., 2008; Rubin 2008), specifically lines that produce greater biomass for biorefining (Ragauskas et al., 2006; Rubin 2008). More recently, *P. trichocarpa* has worked as a good system for answering essential biological and ecological questions using metabolomic techniques (Wullschleger *et al*., 2002).

Metabolomics represents the comprehensive analysis used for the quantification and identification of the metabolome which refers to the full array of small-molecular-weight metabolites which outline an elaborate network of metabolic reactions (Fiehn 2002; Sumner et al., 2003; Daskalchuk et al., 2006). Metabolites are the intermediates of the metabolic pathways leading to and including the final end products of an organism's gene expression (Oliver *et al*., 1998; Sumner *et al*., 2003; Morreel *et al*., 2006; Goodacre *et al*., 2004). Metabolomics has the ability to discover genetic and physiological alteration through analyzing biological samples, and is being used as a novel and powerful technology for the development and discovery of biomarkers (Harrigan and Goodacre 2003; Sabatine *et al*., 2005; Kell 2007; Meyer *et al*., 2007; Boudonck *et al*., 2009). Unlike genomics, transcriptomics and proteomics, metabolomics (metabolite profiling) acts as a functional genomics tool that provides biochemical signatures for plants, although it is still in the developmental stage (Oliver *et al*., 2002; Fiehn 2002; Harrigan and Goodacre 2003; Bino *et al*., 2004; Dettmer and Hammock 2004; Morris *et al*., 2004; Saito

*et al*., 2007). Conditional alterations at the plant transcriptome, proteome and metabolome caused by specific treatment or seasonal differences offers the opportunity to successfully infer relationships between metabolite pools, genotype and phenotype.

Metabolome analyses have been classified depending on the objectives of a particular study into targeted and non-targeted analyses. Targeted analysis focuses on quantifying changes in the presence or concentration of specific compounds. Targeted analysis is mainly used for screening purposes, for example, phenotypic biomarkers. Conversely, non-targeted analysis seeks to identify and qualify the full complement of soluble small molecular weight metabolites in metabolically active tissues using libraries of the spectral and retention index which are metabolically or chemically related for classification of samples (Fiehn and Weckwerth 2003). Here, the relative relationship between the metabolites to one another is an important consideration. Metabolome-trait relationships have most often been characterized in individual species, or individual families, or clonal lines to make the particular plant system a fixed factor in the analyses. For example, metabolomic analysis of a specific phenotypic trait across a set of genetic backgrounds (i.e., hybrids, ecotypes, species, etc.) could aid in identifying and describing broadly applicable relationships. Non-targeted analysis is commonly used in phenotypic selection.

Because of their secondary metabolism, plants have a diverse range of metabolites. Sample preparation is an important consideration based on the analysis purpose (Dettmer *et al*., 2006). The metabolites extracted will be those that are highly soluble in the chosen solvent (e.g., methanol or chloroform). Because of the high diversity of physico-chemical properties and abundance of metabolites, sample preparation for comprehensive non-targeted metabolomics has frequently employed multi-solvent extraction systems including water as a very polar solvent and at least one less-polar solvent (e.g. chloroform) (Fiehn 2001).

Metabolomics has been used effectively on different plant genera for assessing the changes caused by genetic engineering (Gall *et al*., 2003; Tretheway 2004; Schauer and Fernie 2006), phenotype prediction (Fiehn *et al*., 2000a; Roessner *et al*., 2001a and b; Morris *et al*., 2004), plant breeding (Stitt and Fernie 2003; Robinson *et al*., 2005; Meyer *et al*., 2007), investigations of wood properties (Morris *et al*., 2004; Robinson and Mansfield 2009), ecological studies (Stitt and Fernie 2003), and as a diagnostic tool (Kell 2004; Dieterle *et al*., 2006; Schauer and Fernie 2006). Recently, metabolite profiling has been extensively used to study the influence of biotic (Broeckling *et al*. 2005; Desbrosses *et al*. 2005; Hamzehzarghani *et*

*al*., 2005) and abiotic factors to gain greater understanding of plant metabolic networks during growth and development (Kaplan *et al*., 2004; Baxter *et al*., 2007; Huang *et al*., 2008; Vasquez-Robinet *et al*., 2008; Korn *et al*., 2009).

Because of the widespread interest in fast-growing trees for fiber and biofuel production, the genus *Populus* is currently under investigation using a wide array of effective metabolite profiling techniques. Changes in metabolome have been used to discriminate among closely related poplar species (Robinson *et al*., 2005; Morse *et al*., 2007), detect biochemical changes during wood formation (Andersson-Gunnerås *et al*., 2006), study sink-source relationships in developing leaves of aspen (Jeong *et al*., 2004), illustrate the genetic control of bud formation and dormancy in poplar (Ruttink *et al*., 2007), discover and identify candidate genes involved in controlling different biological processes as well as specific functions such as growth and lignin biosynthesis in hybrid aspen (Bylesjö *et al*., 2009), examine growth and phenolic reserves in hybrid cottonwood for trait selection or manipulation (Harding *et al*., 2005), and describe responses to abiotic stresses in poplar (Brosché *et al*., 2005). Furthermore, the availability of the *P. trichocarpa* genome sequence coupled with metabolite profiling has provided an ideal opportunity to examine changes in the transcriptome in response to biotic and abiotic stresses (Guignard *et al*., 2005; Morreel *et al*., 2006) and a new selection tool for breeding and genetic engineering (Morse *et al*., 2007; Robinson and Mansfield 2009).

Due to the inherent diversity in chemical and physical properties of metabolite groups, broad metabolome analysis cannot be achieved by one single analytical technology (Fiehn 2001; Saito *et al*., 2007). A combination of methods such as gas chromatography and mass spectrometry (GC-MS) and liquid chromatography and mass spectrometry (LC/MS) are often used to provide highly sensitive and more robust detection (Fiehn *et al*., 2000a; Roessner *et al*., 2000; Fiehn 2002; Daskalchuk *et al*., 2006). Additionally, sophisticated statistical analyses and peak separation methods are commonly utilized to unravel the complexity of the data generated (Daskalchuk *et al*., 2006; Hall 2006).

Tolerance to abiotic stress is considered to be among the essential traits for maximizing biofuel production in poplar (Ragauskas *et al*. 2006), thus assessment of this attribute across the species range is of great importance. The objectives of this study were to: (1) test if there is a clinal trend in the metabolic profiles of *P. trichocarpa* trees sampled across the species geographic range within British Columbia and (2) evaluate the effect of main geographic and climate variables and date of bud set on levels of metabolite extracted from developing leaves.

In so doing, 106 genotypes originating from the species' range were sampled and metabolic profiles determined using GC-MS.

## 2 Materials and Methods

### 2.1 Sample collection

Sampling was conducted over two hours in the morning during the late summer of 2008 (September 3rd; 246 Julian days). The fifth down developing leaf was collected from *Populus trichocarpa* trees of completely randomized design where Julian days of bud set ranged between 213 and 351. The trees sampled as part of a common garden established at the Totem Research Field at the University of British Columbia, Vancouver, B.C. (49º 15′N, 123º 15′ W) and represent different populations from the species range spanning 44º 00′- 59º 19 N latitude and 121º 10′ - 133º 34′ W longitude (Fig. 2.1). Of the total 140 tree in the field, only 106 were sampled as the rest were infected. The collected leaves were immediately kept in an ice box covered with ice in the field and stored at - 80°C upon arriving at the laboratory to halt enzymatic activity.

### 2.2 Sample preparation and metabolite extraction

Frozen leaf tissue samples were ground to a fine powder under liquid nitrogen using a pre-cooled mortar and pestle (Fiehn 2002, Weckwerth *et al*., 2004). After grinding, samples were kept frozen at -80°C until further processing. To avoid any bias toward metabolites that are highly soluble in a specific solvent, a multi-solvent extraction method was used. Metabolite extraction was conducted using the liquid-liquid (water, methanol and chloroform were used) extraction method (Robinson *et al*., 2005). The organic extraction solvent used was methanol (with 3% distilled, deionised water, and an internal standard (0.25 mg/mL ribitol)). The extraction method contained two phases, the very polar water/methanol phase to extract polar (hydrophilic) metabolites, and the less polar chloroform phase to extract (lipophilic) metabolites (Fiehn, 2002, Robinson *et al*., 2007). For each sample, 1300 µL cold extraction solution was added to approximately 0.5 ml of frozen ground tissue in pre-weighted cold 2 ml lock-cap eppendorf tube. To enhance metabolite extraction, tubes were incubated at 70°C for 15 min with constant agitation at 1400 rpm and then centrifuged for 10 min at 14000 rpm. A 1000 µL aliquot of the supernatant was transferred to a new 2 ml tube and 200 µL was removed for GC-MS. The remaining liquid and pellets were dried overnight in the oven at 50°C to obtain the pellet (extracted tissue) dry weight based on the previously weighed empty tubes (approximately 50 mg).

For Gas chromatography mass spectrometry (GC-MS) analysis, 270 μL distilled, deionised water, and 130 μL chloroform were added to the extract with mild vortexing. Separation of the upper polar phase (methanol/water) and the lower less polar phase (methanol/chloroform) was made by centrifugation for 5 min at 14000 rpm. Part of the upper polar phase (320 μL) which preferentially partitions the more polar metabolites, was transferred to a fresh tube and dried overnight at 30°C in an eppendorf vacufuge. Samples were then derivatized as preparation for gas chromatography. The dried pellet was re-suspended by vortexing in 50 μL pyridine containing 20 mg/ml methoxyamine hydrochloride solution, and then incubated at 37°C for 2 h with orbital shaking at 1100 rpm. Methoxymation was used to protect the carbonyl moieties (Fiehn *et al*., 2000a and b). Tubes were briefly centrifuged to settle condensations and an N-alkane mixture (10 μL) (C12, C15, C19, C22, C28, C32, and C36) was added to determine retention time indices in gas chromatography analysis. Then, 70 μL of N-methyl-N-trimethylsilyltrifluoro acetamide (MSTFA) was added and incubated at 37°C for 30 min with shaking (1100 rpm) to eliminate acidic protons (Gullberg *et al*., 2004; Dettmer *et al*., 2006; O'Maille *et al*., 2008). Before filtration through compacted tissue paper, samples were left to stand at room temperature for 2 h for complete derivatization.

### 2.3 Metabolite extraction analysis

Gas chromatography mass spectrometry analysis was performed on a ThermoFinningan Trace GC-polarisQ ion trap system. This instrument was fit with an AS2000 auto-sampler and a split/splitless injector (Therm Electron Co., Waltham, MA, USA). For all analyses GC was equipped with low-bleed Restek Rtx-5MS column. The column was made from fused silica, 30 m, 0.25 mm ID, and a stationary phase diphenyl 5% dimethyl 95% polysiloxane. The GC parameters employed were: inlet temperature 250°C, helium carrier gas flow at constant 1 ml/min, injector split ratio 10:1, resting oven temperature 70°C, and the GC-MS transfer path temperature 300°C. From each sample, a 1 μL aliquot was injected at an oven temperature of 70°C, and beginning after 2 min the temperature was gradually increased to 325°C (8°C/min). The temperature was held at 325°C for 6 min then decreased rapidly to the initial resting temperature of 70°C in preparation for the subsequent run.

Mass spectrometry ions were formed by positive electron ionization (EI) where the fore-line was evacuated to approximately 40 mTorr, and with helium gas flow into the vacuum chamber at 0.3 ml/min. The initial temperature was held at 250°C, with an electron ionization potential of 70 eV. The detector signal was recorded from 3.35 min after sample injection until 35.5 min. Ions were scanned in the range of 50-650 mass units (mu) with a total scan time of 0.58s.

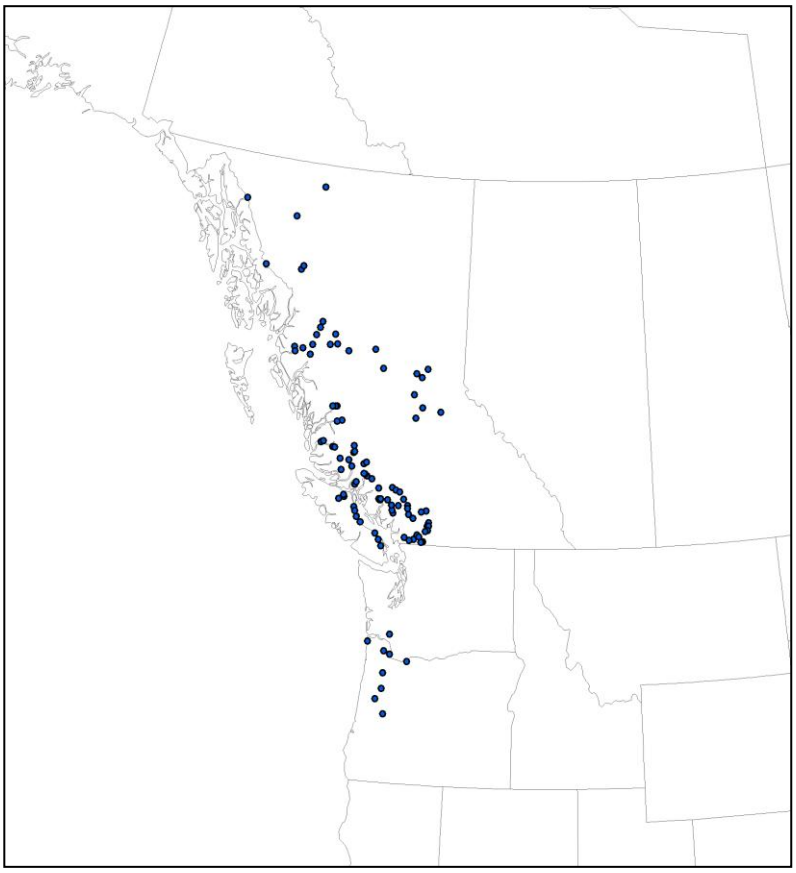

**Figure 2.1** Geographic origins of the *Populus trichocarpa* trees/populations sampled (N = 106).

## 2.4 Data compiling and processing

GC-MS data collection, peak determination and peak measurement were performed by ThermoFinningan 'Xcalibur' software (v1.3) associated with the GC-MS instrument. Peak identification, peak integration, and retention time correction was carried out by the R package XCMS (Smith *et al.*, 2006). Assuming that each metabolite detected by mass spectrometry is represented by at least two highly correlated m/z signals, integrated peaks in the XCMS output

were tested for integration. Only peaks with correlated intensities (m/z) (corr>0.95) and highly related retention times (RT) (difference in RT after XCMS RT correction <0.02 s) with at least one other m/z peak, were retained. Therefore, the peak with the highest intensity (m/z) was selected to represent the corresponding metabolite as the whole group contains the same metabolite signals. The NIST (National Institute of Standards and Technology) AMDIS Deconvolution algorithm was used to visually validate correctness of XCMS.

### 2.5 Metabolite identification

National Institute of Standards and Technology (NIST) MS-Search software provided with the NIST mass spectra, in addition to Gölm Metabolome Database (http://csbdb.mpimp-Golm.mpg.de/csbdb/gmd/gmd.html) (Kopka *et al.*, 2005; Daskalchuk *et al*., 2006), the Max Planck Institute Trimethylsilane (TMS) (http://www.mpimp-Golm.mpg.de/mms-library/index-e.html), and Dr. S. Mansfield's laboratory (Faculty of Forestry, UBC) TMS derivatized mass spectral libraries (including 513 known compounds) were jointly used to identify extracted GC-MS metabolites. The raw total ion chromatogram data was first standardized relative to the ribitol internal standard across all chromatograms, and then adjusted for the exact amount of dry tissue weight (mg) for each extracted sample. The final dataset consisted of 104 individual compound peaks across all samples.

### 2.6 Phenology data

Date of bud set, expressed as Julian day, for each individual tree was provided by Dr. R. Guy (Faculty of Forestry, UBC) and was used as a phenology indicator in the statistical analyses.

### 2.7 Geographic and climate variables

For each individual, geographic (latitude and elevation) and climate (mean annual temperature; MAT, °C and mean annual precipitation; MAP, mm was obtained from Wang *et al*. (2006)).

### 2.8 Statistical analysis

Metabolic variation in *Populus* ecotypes with respect to geographic, climate and phenology variables was examined using canonical correlation analysis (CCA) after reduction

of the metabolites via principle component analysis (PCA) under 'proc cancorr', 'proc princomp' and 'proc corr' (conducted to correlate the original variables to their PCs) procedures of the Statistical Analysis System (SAS v9.2) software, respectively. Two canonical correlations analyses were conducted, the first utilized the most significant original variables in the principle component matrices and the second used the principle components themselves. These two appropriate analyses were conducted to allow results comparison. Canonical correlation analysis studies the relationship between two groups of variables (X and Y) via transforming the data into canonical variables to maximize the variance between groups. In this study, CCA was performed to investigate the relationship between selected metabolites (response variables) produced by PCA and geographic, climate and bud set variables (predictor variables). Canonical variables are considered important based on the magnitude of the canonical correlation and significance at $\alpha$ level of 0.05.

## 3 Results

After compiling the GC-MS profiles of all samples, there were 104 detected metabolite peaks, of which 40 were identified (Appendix 1). Principle Component Analysis (PCA) was used as a preliminary data reduction step for the two analyses. The first ten PCAs accounted for 63% of the variation in the data and were selected for further analyses. Canonical Correlation Analysis (CCA) generated less than full rank correlation matrix between metabolite peaks and geo-climatic and phenology variables as a result of the correlations present among the metabolites. The PCA was then used to identify which metabolites contribute most to the variance of these variables; thus some variables were dropped (Jolliffe 2002). Therefore, PCA was mainly used for reduction of the original variables; i.e., the most significant metabolites were retained (Jolliffe 2002). The first CCA was conducted between the reduced set of metabolites while the second was based on the first ten principal components and geo-climatic and phenology variables, respectively.

The component matrices of PC-1 to PC-10 (Appendix 2) were screened for variables with high loadings (correlations between the original variables and their principal components) to retain. Based on the component loadings, only metabolites with absolute value of loadings >0.45 were retained (Table 3.1). PC-1 explained 15% of the metabolite variation and is represented mostly by flavonoids related to the shikimate pathway (quercetin and kaempferol), carbohydrates (glucose-6-phosphate, carbohydrate-1 and oligosaccharide-2) and metabolites related to raffinose biosynthesis (raffinose and galactinol) with loading as high as 0.80 (Table 3.1). Generally PC-2 explained 11% of the variation and is characterized by salicylates (salicin, salireposide, catechol and catechol glucoside), carbohydrates (glucose, fructose, carbohydrate-6, carbohydrate-8 and oligosaccharide-1) and malic acid with maximum loadings of 0.67 (Table 3.1). PC-3 accounted for 8% of the variation and is represented by organic acids related to the tricarboxylic acid (TCA) cycle (fumaric, citric and malic acids), an antioxidant (quinic acid) and gluconic acid with maximum loadings of 0.68 (Table 3.1). As PC 1-3, correlations reached 0.64, 0.55, 0.71, 0.56, 0.7 and 0.48 in PC-4, -5, -6, -7, -9 and -10, respectively. Collectively, the first ten principal components were represented by a broad range of metabolites.

Although all canonical correlations obtained between the original metabolites and geographic, climate and bud set variables were very strong in the first analysis, and moderate in the second analysis, they were not significant ($P > 0.05$). The amount of variance of metabolites explained by the canonical variables was small (from 0.0099 to 0.0181 in the first analysis and

between 0.02 and 0.0277 in the second analysis). While the statistical significance of these tests support their lack of significance, these tests do not represent the magnitude of the relationship, therefore the correlations between the original variables and their canonical variables are still interpretable and the main cause for the observed lack of "statistical significance" is the small sample size used in the experiment (Manly 1986; Wilkinson and APA Task Force on Statistical Inference 1999; Sherry and Henson 2005).

### 3.1 CCA performed using selected metabolites (the first analysis)

Five canonical variables were constructed based on genotypes' geo-climatic variables and bud set. Canonical correlations > 0.9 were used for interpretation. The first four canonical correlations all fit this criterion with values of 0.97, 0.94, 0.93 and 0.91, respectively.

In the first canonical correlation (r = 0.97, $P$ = 0.4), correlations between the original metabolites and their respective first canonical correlation CV1 variable (canonical loadings), indicated that latitude loaded highly and positively, while MAT and BS were negatively loaded (i.e., CV1 reached higher values with increasing latitude and decreasing mean annual temperature and early date of bud set) (Table 3.2). Quinic and phosphoric acids and taxifolin were negatively correlated with CV1 while shikimic and chlorogenic acids, kaempferol, salireposide, salicortin, catechol glucoside, pyroglutamic and ascorbic acids, galactinol, raffinose and steric acid methyl ester were positively correlated with CV1. Similar trends were observed for several unknowns with CV1 (Table 3.2). Metabolites related to shikimate, ascorbate, amino acids (glutamine) and raffinose metabolism and a group of unknowns were highly correlated to CV1. The strongest correlation has been detected between latitude (r = 0.94) and unknown-70 (r = 0.36) and unknown-85 and -94 (r = 0.26) (Table 3.2).

The second canonical correlation (r = 0.94, $P$ = 0.7) exhibited moderate and positive loading of MAP and BS on CV2, the second canonical variable (i.e., CV2 achieved higher values with increasing MAP and BS) (Table 3.3). In addition to the positive and negative correlations of unknown metabolites, pyroglutamic, phosphoric, fumaric, malic and citric acids and gluconic acid lactone and glutamine were negatively correlated with CV2 while quinic and chlorogenic acids, glucose, myo-inositol and fructose were positively correlated with CV2. A spread of metabolites associated with the TCA cycle (fumaric, malic and citric acids), the shikimate pathway (quinic and chlorogenic acids), amino acid metabolism (pyroglutamic acid and glutamine) and a cluster of unknowns were present. Significant correlations are apparent

for major soluble sugar pools (glucose and fructose). A strong correlation between MAP (r = 0.53) and phosphoric acid (r = - 0.33) and chlorogenic acid (r = 0.3) has been shown in the second canonical correlation (Table 3.3).

At the third canonical correlation (r = 0.93, *P* = 0.8), elevation was strongly and positively loaded on the third canonical variable, CV3 (i.e., CV3 approximated altitude level) (Table 3.4). Quinic and chlorogenic acids, taxifolin, kaempferol, catechin, salireposide, salicortin and ascorbic acid were negatively correlated with CV3, while shikimic acid, salicin, catechol, galactinol and raffinose were positively correlated with CV3. Similarly, positive and negative correlations between the unknown metabolites with CV3 were also observed. Significant correlations for metabolites related to shikimate (shikimic, quinic and chlorogenic acids, taxifolin, kaempferol, catechin, salicin, salireposide, salicortin, catechol), ascorbate (ascorbic acid), and raffinose metabolism (galactinol and raffinose) in addition to some unknowns with CV3 were present. The highest correlation detected was between elevation (r = 0.75) and raffinose (r = 0.26) and unknown-104 (r = - 0.24) (Table 3.4).

For the fourth canonical correlation (r = 0.91, *P* = 0.9), MAP was moderate and positively loaded on CV4, the fourth canonical variable (i.e., CV4 reached higher values with increasing MAP) (Table 3.5). The highest correlation occurred between MAP (r = 0.63) and an unknown-9 (r = - 0.31) (Table 3.5). Shikimic and quinic acids, taxifolin, kaempferol, ascorbic acid, galactinol and glucose-6-phosphate were negatively correlated with CV4, while salicortin, catechol glucoside, glutamine and fumaric, malic and citric acids and gluconic acid lactone were positively correlated with CV4. Shikimate (shikimic and quinic acids, taxifolin, kaempferol, salicortin and catechol glucoside), ascorbate (ascorbic acid), raffinose metabolism (galactinol), TCA cycle (glutamine, fumaric, malic and citric acids and gluconic acid lactone), amino acid (glutamine) related metabolites as well as a group of unknown compounds were highly correlated to CV4.

### 3.2 CCA performed using principal components (the second analysis)

Of the five constructed canonical variables based on the geo-climatic variables and bud set of the populations, two canonical correlations (> 0.45) were used for interpretation.

Correlations between the predictor variables and their respective first canonical correlation CV1 variable (canonical loadings) pointed to the positive and the negative loadings of the latitude and MAT and BS, respectively on CV1 in the first canonical correlation (r = 0.54,

*P* = 0.2) (i.e., CV1 achieved higher values with increasing latitude and decreasing mean annual temperature and early date of bud set) (Table 3.6). PC-5, -6 and -8 were positively loaded on CV1 while PC-9 and -10 were negatively loaded on CV1 (Table 3.6). The strongest correlation was detected between latitude (r = 0.47) and PC-6 (r = 0.33) (Table 3.6).

At the second canonical correlation (r = 0.45, *P* = 0.6), bud set was positively loaded on the second canonical variable, CV2 (i.e., CV2 reached higher values with late bud set) (Table 3.7). PC-1 was positively loaded on the second canonical variable, CV2 while PC-2, -3, -4 and -7 were negatively loaded on CV2 (Table 3.7). Bud set has a strong correlation (r = - 0.33) with PC-2 and -3 (r = - 0.2) (Table 3.7).

**Table 3.1** Metabolites loaded > 0.45 on the component matrices of the first ten principals. Metabolites represented by their peak number (sequence of elution in gas chromatography), loading (correlation between each metabolite and its principal component) and identity (otherwise metabolites are unknown).

| PC# | peak # | Loading | Identity |
|---|---|---|---|
| **PC-1** | 16 | 0.62 | unknown-16 |
| | 20 | 0.49 | unknown-20 |
| | 36 | 0.66 | unknown-36 carbohydrate-1 |
| | 47 | 0.49 | galacturonic acid |
| | 50 | 0.67 | unknown-50 |
| | 51 | 0.48 | galactonic acid |
| | 56 | 0.63 | unknown-56 |
| | 57 | 0.62 | steric acid methyl ester |
| | 59 | 0.80 | unknown-59 |
| | 61 | 0.66 | glucose-6-phosphate |
| | 62 | 0.80 | unknown-62 |
| | 63 | 0.80 | unknown-63 |
| | 68 | 0.80 | unknown-68 |
| | 72 | 0.57 | unknown-72 |
| | 75 | 0.56 | Monopalmitoyl-rac-glycerol |
| | 76 | 0.52 | unknown-76 oligosaccharide-2 |
| | 84 | 0.65 | unknown-84 |
| | 88 | 0.52 | galactinol |
| | 90 | 0.53 | kaempferol |
| | 94 | 0.62 | unknown-94 |
| | 95 | 0.63 | quercetin |
| | 102 | 0.51 | raffinose |
| | 104 | 0.63 | unknown-104 |
| **PC-2** | 3 | 0.53 | glutamine |
| | 5 | 0.63 | catechol |
| | 7 | 0.47 | fumaric acid |
| | 16 | 0.56 | unknown-16 |
| | 17 | 0.58 | unknown-17 |
| | 18 | 0.67 | malic acid |
| | 20 | 0.50 | unknown-20 |
| | 45 | 0.53 | fructose |
| | 46 | 0.61 | glucose |
| | 48 | 0.55 | ascorbic acid |
| | 54 | 0.51 | unknown-54 |
| | 64 | 0.60 | unknown-64 carbohydrate-6 |

| PC# | peak # | Loading | Identity |
|---|---|---|---|
| | 65 | 0.48 | unknown-65 carbohydrate-7 |
| | 66 | 0.61 | unknown-66 carbohydrate-8 |
| | 67 | 0.55 | unknown-67 oligosaccharide-1 |
| | 69 | 0.52 | catechol glucoside |
| | 71 | 0.48 | unknown-71 |
| | 74 | 0.55 | salicin |
| | 76 | 0.48 | unknown-76 oligosaccharide-2 |
| | 77 | 0.52 | unknown-77 |
| | 98 | 0.50 | salireposide |
| **PC-3** | 2 | 0.56 | Phosphoric acid |
| | 6 | 0.46 | glycolic acid |
| | 7 | 0.68 | fumaric acid |
| | 18 | 0.51 | malic acid |
| | 21 | 0.53 | pyroglutamic acid |
| | 22 | 0.54 | unknown-22 |
| | 26 | -0.52 | unknown-26 |
| | 30 | 0.47 | unknown-30 |
| | 41 | 0.67 | citric acid |
| | 44 | 0.55 | quinic acid |
| | 52 | 0.66 | gluconic acid lactone |
| | 54 | -0.48 | unknown-54 |
| | 97 | 0.49 | unknown-97 |
| **PC-4** | 5 | -0.53 | catechol |
| | 32 | 0.46 | unknown-32 |
| | 35 | -0.64 | unknown-35 |
| | 38 | 0.61 | unknown-38 organic acid-2 |
| | 60 | -0.56 | unknown-60 carbohydrate-5 |
| | 74 | -0.58 | salicin |
| **PC-5** | 6 | 0.51 | glycolic acid |
| | 40 | 0.55 | shikimic acid |
| | 72 | -0.51 | unknown-72 |
| **PC-6** | 39 | 0.70 | unknown-39 |
| | 49 | 0.71 | unknown-49 |
| **PC-7** | 9 | 0.46 | unknown-9 |
| | 31 | 0.50 | unknown-31 |

| PC# | peak # | Loading | Identity |
|---|---|---|---|
| | 99 | -0.56 | salicortin |
| **PC-8** | All metabolite loadings <0.45 | | |
| **PC-9** | 82 | 0.58 | catechin |
| | 93 | 0.70 | chlorogenic acid |
| **PC-10** | 14 | 0.48 | propanoic acid |
| | 15 | 0.46 | salicyle alcohol |

**Table 3.2** Canonical structure of correlations between latitude, mean annual temperature (MAT) and days to bud set (BS) variables and their first canonical variable CV1 and between metabolites and CV1. (All correlations are related to the first canonical correlation (r = 0.97, *P* = 0.4)).

| Variables | CV1 |
|---|---|
| Predictor variables | |
| Latitude | 0.94 |
| MAT | -0.68 |
| BS | -0.53 |
| | |
| Response variables | |
| Taxifolin | -0.18 |
| Steric acid methyl ester | 0.12 |
| Shikimic acid | 0.15 |
| Salireposide | 0.22 |
| Salicortin | 0.11 |
| Raffinose | 0.20 |
| Quinic acid | -0.18 |
| Pyroglutamic acid | 0.14 |
| Phosphoric acid | -0.10 |
| Unknown-76 Oligosaccharide-2 | 0.14 |
| Unknown-67 oligosaccharide-1 | 0.15 |
| Unknown-56 | -0.15 |
| Unknown-87 | 0.12 |
| Unknown-39 | 0.18 |
| Unknown-26 | 0.19 |
| Unknown-32 | -0.11 |
| Unknown-85 | 0.26 |
| Unknown-70 | 0.36 |
| Unknown-23 | -0.19 |
| Unknown-22 | 0.11 |
| Unknown-49 | 0.22 |
| Unknown-94 | 0.26 |
| Unknown-35 | -0.13 |
| Unknown-77 | 0.19 |
| Unknown-72 | -0.16 |
| Unknown-10 | 0.18 |
| Kaempferol | 0.10 |
| Galactinol | 0.14 |
| Chlorogenic acid | 0.14 |
| Catechol glucoside | 0.11 |

| Variables | CV1 |
|---|---|
| Predictor variables | |
| Latitude | 0.94 |
| MAT | -0.68 |
| BS | -0.53 |
| | |
| Response variables | |
| Unknown-64 carbohydrate-6 | 0.14 |
| Unknown-36 carbohydrate-1 | 0.14 |
| Ascorbic acid | 0.18 |

**Table 3.3** Canonical structure of correlations between mean annual precipitation (MAP) and days to bud set (BS) variables and their second canonical variable CV2 and between metabolites and CV2. (All correlations are related to the second canonical correlation (r = 0.94, *P* = 0.7)).

| Variables | CV2 |
|---|---|
| Predictor variables | |
| MAP | 0.53 |
| BS | 0.50 |
| | |
| Response variables | |
| Chlorogenic acid | 0.30 |
| Unknown-87 | 0.28 |
| Unknown-50 | 0.27 |
| Unknown-16 | 0.26 |
| Unknown-63 | 0.24 |
| Glucose | 0.20 |
| Unknown-36 carbohydrate-1 | 0.19 |
| Unknown-94 | 0.17 |
| Unknown-68 | 0.15 |
| Fructose | 0.15 |
| Unknown-67 oligosaccharide-1 | 0.13 |
| Unknown-71 | 0.12 |
| Unknown-54 | 0.12 |
| Unknown-66 carbohydrate-8 | 0.11 |
| Quinic acid | 0.11 |
| Unknown-38 organicacid-2 | -0.16 |
| Pyroglutamic acid | -0.18 |
| Unknown-17 | -0.19 |
| Citric acid | -0.20 |
| Unknown-22 | -0.20 |
| Malic acid | -0.21 |
| Glutamine | -0.27 |
| Fumaric acid | -0.27 |
| Gluconic acid lactone | -0.27 |
| Unknown-62 | -0.27 |
| Myo-inositol | 0.10 |
| Phosphoric acid | -0.33 |

**Table 3.4** Canonical structure of correlations between elevation variable and their third canonical variable CV3 and between metabolites and CV3. (All correlations are related to the third canonical correlation (r = 0.93, *P* = 0.8)).

| Variables | CV3 |
|---|---|
| Predictor variables | |
| Elevation | 0.75 |
| | |
| Response variables | |
| Raffinose | 0.26 |
| Unknown-54 | 0.21 |
| Galactinol | 0.20 |
| Unknown-64 carbohydrate-6 | 0.19 |
| Unknown-10 | 0.17 |
| Salicin | 0.15 |
| Catechol | 0.15 |
| Shikimic acid | 0.14 |
| Unknown-60 carbohydrate-5 | 0.13 |
| Unknown-49 | 0.13 |
| Unknown-39 | 0.12 |
| Unknown-66 carbohydrate-8 | 0.11 |
| Unknown-35 | 0.10 |
| Kaempferol | -0.10 |
| Unknown-77 | -0.10 |
| Quinic acid | -0.13 |
| Ascorbic acid | -0.13 |
| Taxifolin | -0.14 |
| Salicortin | -0.14 |
| Salireposide | -0.15 |
| Catechin | -0.16 |
| Chlorogenic acid | -0.18 |
| Unknown-87 | -0.22 |
| Unknown-104 | -0.24 |

**Table 3.5** Canonical structure of correlations between mean annual precipitation (MAP) variable and their fourth canonical variable CV4 and between metabolites and CV4. (All correlations are related to the fourth canonical correlation (r = 0.91, *P* = 0.9)).

| Variables | CV4 |
|---|---|
| Predictor variables | |
| MAP | 0.63 |
| | |
| Response variables | |
| Malic acid | 0.21 |
| Unknown-67 oligosaccharide-1 | 0.19 |
| Salicortin | 0.18 |
| Gluconic acid lactone | 0.18 |
| Catechol glucoside | 0.17 |
| Citric acid | 0.17 |
| Glutamine | 0.16 |
| Unknown-66 Carbohydrate-8 | 0.14 |
| Fumaric acid | 0.13 |
| Taxifolin | -0.10 |
| Quinic acid | -0.11 |
| Unknown-56 | -0.11 |
| Ascorbic acid | -0.12 |
| Shikimic acid | -0.12 |
| Unknown-49 | -0.13 |
| Unknown-104 | -0.15 |
| Alanine | -0.15 |
| Unknown-16 | -0.15 |
| Glucose-6-phosphate | -0.15 |
| Galactinol | -0.16 |
| Kaempferol | -0.16 |
| Unknown-63 | -0.16 |
| Unknown-10 | -0.17 |
| Unknown-20 | -0.18 |
| Unknown-62 | -0.19 |
| Unknown-39 | -0.19 |
| Unknown-9 | -0.31 |

**Table 3.6** Canonical structure of correlations between latitude, mean annual temperature (MAT) and days to bud set (BS) variables and their first canonical variable CV1 and between metabolites and CV1. (All correlations are related to the first canonical correlation (r = 0.54, *P* = 0.2)).

| Variables | CV1 |
|---|---|
| Predictor variables | |
| Latitude | 0.47 |
| MAT | -0.43 |
| BS | -0.33 |
| | |
| Response variables | |
| PC-5 | 0.18 |
| PC-6 | 0.33 |
| PC-8 | 0.15 |
| PC-9 | -0.19 |
| PC-10 | -0.18 |

**Table 3.7** Canonical structure of correlations between days to bud set (BS) variable and its second canonical variable CV2 and between metabolites and CV2. (All correlations are related to the second canonical correlation (r = 0.45, *P* = 0.64)).

| Variables | CV2 |
|---|---|
| Predictor variables | |
| BS | 0.33 |
| | |
| Response variables | |
| PC-1 | 0.17 |
| PC-2 | -0.2 |
| PC-3 | -0.2 |
| PC-4 | -0.16 |
| PC-7 | -0.15 |

# 4 Discussion and Conclusion

As Populus trichocarpa covers a broad geographic area with physiological, morphological and developmental variability, climatic differences result in natural selection for local adaptation. In both analyses reported in this thesis, the metabolite profile of *P. trichocarpa* showed difference among ecotypes which can be linked to diverse of original environments (Zhen and Ungerer 2007).

In the first analysis, northern ecotypes showed an increase in different metabolites responsible for protection against cold (e.g., ascorbic acid and metabolites related to the raffinose biosynthesis pathway, Figure 4.2) that were accompanied by a reduction in metabolites involved in warmer climate (e.g., quinic acid). As well as, inhibition in different metabolites has been detected in northern genotypes as an indication of growth cessation and dormancy (phosphoric acid). Southern ecotypes exhibited continued growth and development activities as precursors of lignin, nucleic acids and proteins were higher and levels of Tricarboxylic Acid cycle (TCA) intermediates were lower (Figure 4.4).

The second analysis suggested an increase in photorespiration relative to photosynthesis of northern ecotypes during dormancy, as glycolic acid increase during slow growth rate. Also, compounds known to be consumed for energy during dormancy have been detected (e.g., organic acids). Increase in the shikimate pathway (Figure 4.5) and raffinose biosynthesis (Figure 4.6) intermediates were associated with southern ecotypes later bud set. Conversely, reductions in building blocks metabolites and metabolites responsible for protection against cold exposure damage have been observed. Decrease in TCA cycle intermediates with late bud set has been detected (Figure 4.4). While all these activities are taking place, the southern ecotypes did not approach dormancy yet.

Both analyses reflected strong genetic adaptation of bud set to photoperiod. *P. trichocarpa* ecotypes showed different growth stages when grown in the same environment because they have different geographical origins.

## 4.1 CCA performed using selected metabolites (the first analysis)

### 4.1.1 The first canonical correlation

The observed differences in the metabolite profiles among the studied 104 genotypes indicated the presence of gradient of physiological activities ranging from those who attained dormancy to those who were still in active growing phase. Northern genotypes were already

acclimating for winter when harvested (i.e., buds already set). For the first canonical correlation (Table 3.2), trends in metabolites related to the shikimate pathway (increasing and decreasing shikimic acid, chlorogenic acid, kaempferol, salireposide, salicortin and catechol glucoside and quinic acid and taxifolin, respectively) were associated with latitude of origin, low MAT and early bud set, suggesting that shikimate pathway intermediates are directed towards phenylpropanoid production under short photoperiod and low temperature climate (i.e., high latitude). Similar results were reported by Kaplan *et al*. (2004) in Arabidopsis where phenylpropanoid pathway intermediates have been detected under cold conditions (Figure 4.1). Phenylpropanoid metabolism, including the production of flavonoids and salicylates, plays an essential role in the growth and development of woody plants such as colouring of flowers and fruits (Bruneton 1999), pollination (Ylstra *et al*., 1992), phytohormone transport (Jacobs 1988), lignin biosynthesis (Hoffmann *et al*., 2004) and defense (Pietta 2000), in addition to their antioxidant effect (Dixon and Paiva 1995; Bandoniene and Murkovic 2002). In order to survive unfavorable conditions, changes at the genetic, physiologic and biochemical levels occur within plant system (Polesskaya 2006; Guy *et al*., 2008). Although Reactive Oxygen Species (ROS) are produced and assimilated normally through plant metabolism, an excess is produced when plants are exposed to environmental changes such as seasonal fluctuations or stress conditions (Vichnevetskaia and Roy 1999; Arora *et al*., 2002; Tausz *et al*., 2004). In order to protect different plant tissues from the damage caused by ROS, plants produce a variety of antioxidant compounds to scavenge the ROS (Vichnevetskaia and Roy 1999; Pietta 2000; Polesskaya 2006). It has also been reported that flavonoids have substantial antioxidant potential to contend with free radicals, thus functioning as a defense mechanism under different types of abiotic stresses (Solecka 1997; Sawa *et al*., 1999; Pietta 2000; Petersen *et al*., 2009). Similarly, salicylates have been shown to accumulate under chilling conditions in Arabidopsis (Scott *et al*., 2004) and poplar (Tsai *et al*., 2006; Morse *et al*., 2007), as they protect plants from low temperature damage. Phenylpropanoids seem to be produced and accumulate in young poplar leaves as well as other plant species as a defense response (Subramaniam *et al*., 1993; Solecka 1997; Grace and Logan 2000; Nugroho *et al*., 2002; Morreel *et al*., 2006). Therefore, genotypes originating from the north may have upregulated the phenylpropanoid pathway, including flavonoids and salicylates, in preparation for winter (Solecka 1997, Morreel *et al*., 2006).

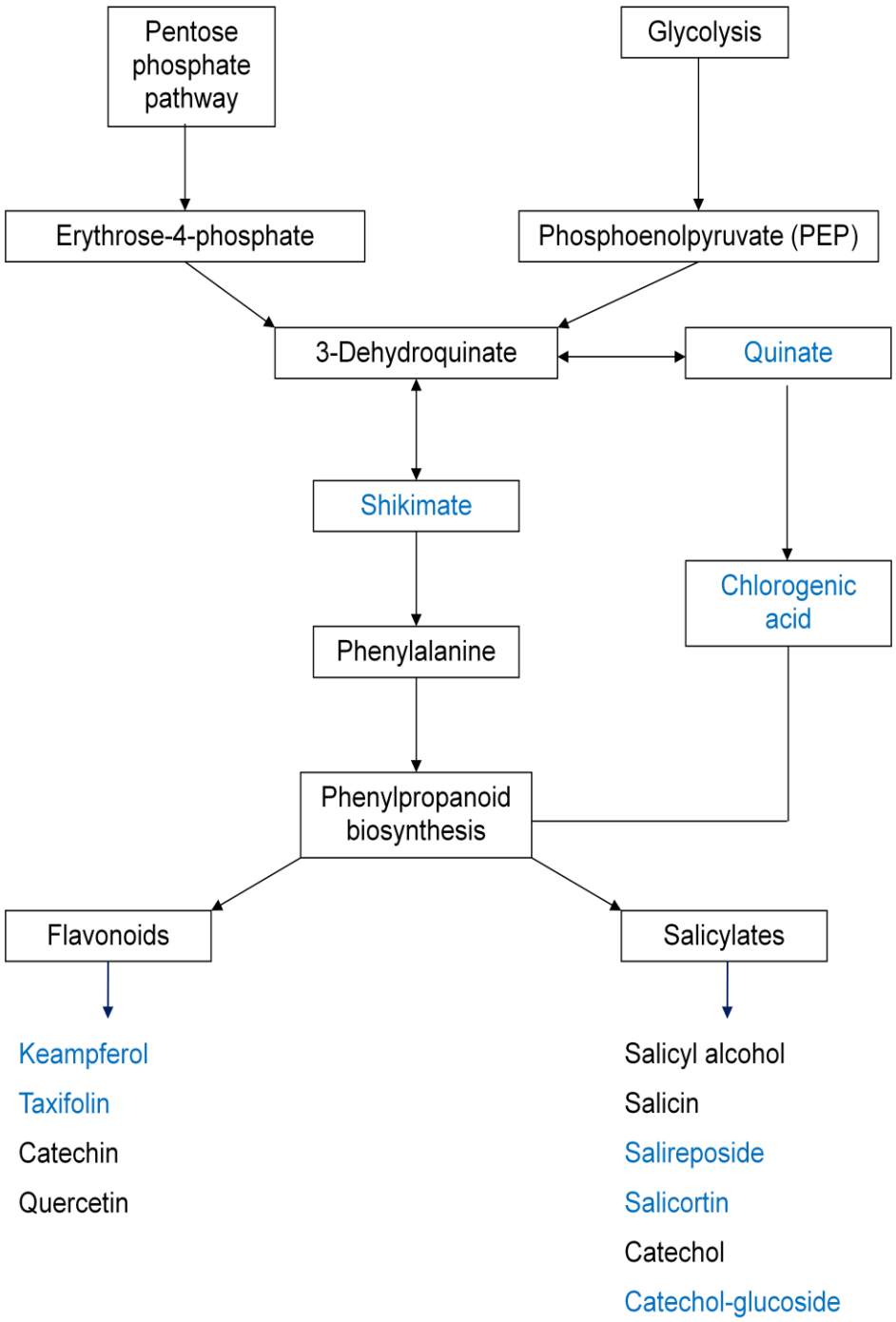


**Figure 4.1** Shikimic acid pathway (after Moorman *et al.*, 1992; Herrmann 1995; Vogt 2009). (Compounds in blue are associated with high latitude, low MAT and early bud set, i.e., those compounds are associated with early bud set of populations originating from the north).

Of the flavonoids, only taxifolin showed a decreasing trend, perhaps because it acts as an absorbent antioxidant agent protecting plants from solar UV radiation injury associated with

higher temperatures (Pietta 2000; Warren *et al*., 2002; Morreel *et al*., 2006). UV irradiation has been shown to induce the accumulation of flavonoids in leaves of Arabidopsis (Li *et al*., 1993), tobacco (Nugroho *et al*. 2002), silver birch (Lavola *et al*., 2000), poplar (Schumaker *et al*., 1997; Warren *et al*., 2002) and Scots pine (Schnitzler, *et al*., 1997). The present study indicates that, generally, phenylpropanoids directed to increase flavonoids and salicylates are associate with the earlier bud set of genotypes that originated from more northern regions.

Shikimic acid and chlorogenic acid showed an increasing trend with latitude of origin, low MAT and early bud set, while quinic acid showed decreasing trend in northern genotypes. Similar observations were made by Passarinho *et al*. (2006) in *Quercus suber* who showed that quinic acid concentrations decreased with decreasing temperatures. In addition to acting as a precursor to lignin biosynthesis and an intermediate of aromatic amino acids and other secondary metabolites, shikimic acid behaves as an antioxidant in response to various stresses (Sawa *et al*., 1999; Passarinho *et al*., 2006). Chlorogenic acid has been shown to possess strong antioxidant potential which acts as a defense system in response to environmental stresses (Vichnevetskaia and Roy 1999; Grace and Logan 2000; Bandoniene and Murkovic 2002; Nugroho *et al*., 2002; Petersen *et al*., 2009).

Northern genotypes showed an increase in pyroglutamic acid, a glutamine precursor associated with early bud set (Table 3.2). Glutamine accumulates as one of the osmoprotectant amino acids associated with cold exposure. A general increase in the pools of amino acids derived from oxaloacetate and pyruvate during cold tolerance has been detected with inhibition in tricarboxylic acid (TCA) cycle intermediates (Kaplan *et al*., 2004; Baxter *et al*., 2007). This conclusion is similar to that reported for Arabidopsis (Kaplan *et al*., 2004, Baxter *et al*., 2007; Guy *et al*., 2008; Korn *et al*., 2009) and poplar (Renaut *et al*., 2004) where osmoprotectant amino acids accumulate under cold condition.

Ascorbic acid (vitamin C) showed an increasing trend with early bud set and high latitude (i.e., low MAT) (Table 3.2), suggesting that the accumulation of ascorbic acid is associated with early bud set of northern ecotypes. Vitamin C, the most important antioxidant compound in plants, has been detected in many tissues and acts as a protecting agent from oxidative damage during exposure to different stresses (Vichnevetskaia and Roy 1999; Pietta 2000; Arora *et al*., 2002; Smirnoff 2003; Kaplan *et al*., 2004). As an important naturally produced low-molecular weight vitamin, ascorbic acid utilization has gained great interest in metabolic engineering for improving crop stress resistance (Smirnoff 2003).

The increasing trend in ascorbic acid was also observed for raffinose and galactinol, which were correlated with the earlier bud set of northern genotypes (Table 3.2) suggesting that northern genotypes had started preparing for low temperatures. This might be an indication that raffinose and galactinol tend to accumulate in leaves as a cryoprotectant for protection against cold exposure, as suggested by Ögren (1996) and Renaut *et al*. (2004) (Figure 4.2). Accumulation of raffinose has also been detected in Arabidopsis leaves as a protective agent against freezing conditions (Taji *et al*., 2002; Stitt and Hurry 2002; Kaplan *et al*., 2004; Hannah *et al*., 2006; Guy *et al*., 2008; Korn *et al*., 2008; Nishizawa *et al*., 2008; Korn *et al*., 2009). Transcription of Raffinose Family Oligosaccharides (RFOs) genes, and especially galactinol synthase, is promoted under chilling conditions for membrane protection (Taji *et al*., 2002; Pennycooke *et al*., 2004; Nishizawa *et al*., 2008; Maruyama *et al*., 2009) and oxidative damage prevention in addition to their role in energy metabolism (Nishizawa *et al*., 2008; Maruyama *et al*., 2009; Sziderics 2010). It was also found that cold tolerance is associated with an increase in osmoprotectants, raffinose and its precursors (RFOs), in winter rye leaves (Antikainen and Pihakaski, 1994), Arabidopsis (Maruyama *et al*., 2009) and many plant species including woody plants (Patton *et al*., 2007; Yuanyuan *et al*., 2009). Increase of RFOs with low temperature during dormancy has also been found in *P. tremuloides* (Cox and Stushnoff 2001) and other woody plants (e.g., poplar) (Gómez *et al*., 2005). Raffinose level increased and diminished with low temperature and short photoperiod and high temperature and long photoperiod, respectively in poplar and ash (Cox and Stushnoff 2001; Jouve *et al*., 2007).

Photoperiod greatly controls plant growth and development; it is the main parameter known to affect bud set and dormancy. However, temperature parameters cannot be dismissed (Gómez *et al*., 2005; Ruttink *et al*., 2007; Lagercrantz, 2009). It should be stated that photoperiod, temperature and phenology are complex and separating one from the other is difficult. Studies that focused on bud phenology successfully identified the presence of three major genes with significant QTL effect indicating quantitative genetics additive gene action (see mapping experiments on *Populus* by Frewen *et al*. (2000) and Gómez *et al*. (2005)).

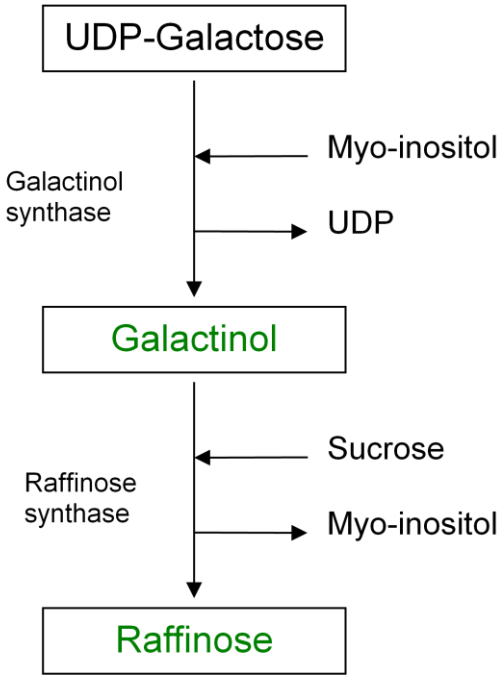


**Figure 4.2** The plant raffinose pathway (after Sprenger and Keller 2000; Taji *et al*., 2002; Amiard *et al*., 2003; Nishizawa *et al*., 2008). Compounds in green are positively associated with high latitude, low MAT and early bud set; i.e., those compounds are associated with early bud set of populations originating from further north.

For most native tree species, induction of growth cessation and bud set are controlled by shortening of the photoperiod in association with low temperature. In this case, latitude acts as a proxy, and variation in the length of growing season from plants originating from different latitudes often shows different photoperiodic responses between northern and southern genotypes. Trees from southern locations usually require shorter days to induce bud set than do northern trees (Gómez *et al*., 2005). Accordingly, in the northern hemisphere, *Populus* ecotypes originating from high latitudes and/or elevations set bud earlier than those from lower latitudes and elevations when grown in a common environment (Frewen *et al*. (2000), Lagercrantz (2009) and present study).

The observed positive association between steric acid methyl ester (methyl stearate) and early bud set of northern genotypes could be attributable to growth cessation and onset of dormancy (Table 3.2). This observation was also detected in other plant species and bacteria (Lightner *et al*., 1994; Terekhova *et al*., 2010). Fatty acid methyl esters, particularly methyl

stearate, are known to be naturally occurring under optimal growth and development conditions. Methyl stearate is thought to be involved in protective roles under osmotic and oxidative stress and has been detected in high concentrations of Arabidopsis young leaves (Lightner *et al*.; 1994, Terekhova *et al*., 2010).

The decrease in phosphoric acid (Table 3.2) might be a result of growth inhibition and dormancy of northern genotypes (Grace and Logan 2000). Under natural cold conditions soluble sugars are phosphorylated, reducing phosphate pools which results in activation of stress-related gene transcription including those involved in phenylpropanoid metabolism (Grace and Logan 2000).

Generally, genotypes originated from more northern locations start their acclimating for low temperatures earlier than those from southern sources, i.e., there is a clinal pattern of variation in cold acclimation associated with latitude of origin and climate.

### 4.1.2 The second canonical correlation

Under the second canonical correlation, quinic acid, chlorogenic acid, glucose and fructose were positively correlated with MAP and late bud set (Table 3.3). Genotypes originating from the south had not set bud yet, thus it is expected that they were still in active growth. Quinic and chlorogenic acid are known to be early intermediates for phenylpropanoids and lignin production (shikimate pathway, Figure 4.3). As one of the organic acids, quinic acid, is a key metabolite involved in lignifications and protein production and has been shown to increase during the late summer (September) in cork oak leaves (Passarinho *et al*., 2006).

Phosphoric acid plays an important role in plant metabolism; it is responsible for phosphorylation of sugars involved in DNA, RNA, and adenosine triphosphate (ATP) production. Accordingly, the observed decreasing trend of phosphoric acid in this study is likely associated with late bud set because of its consumption during active growth stages (Table 3.3).

The observed trend towards decreasing levels of the amino acid derivative, pyroglutamic acid, with MAP and late bud set (Table 3.3) might be attributable to an up-regulation in proteins synthesis as normal growth is still active in genotypes originated from the south. Pyroglutamic acid has been reported to act as the raw material for the production of the amino acid glutamine, a protein substrate (Ohkama-Ohtsu *et al*., 2009). Similarly, it could be speculated that the

amino acid glutamine has shown decreasing trend as a result of its consumption as a metabolic intermediate during active growth.

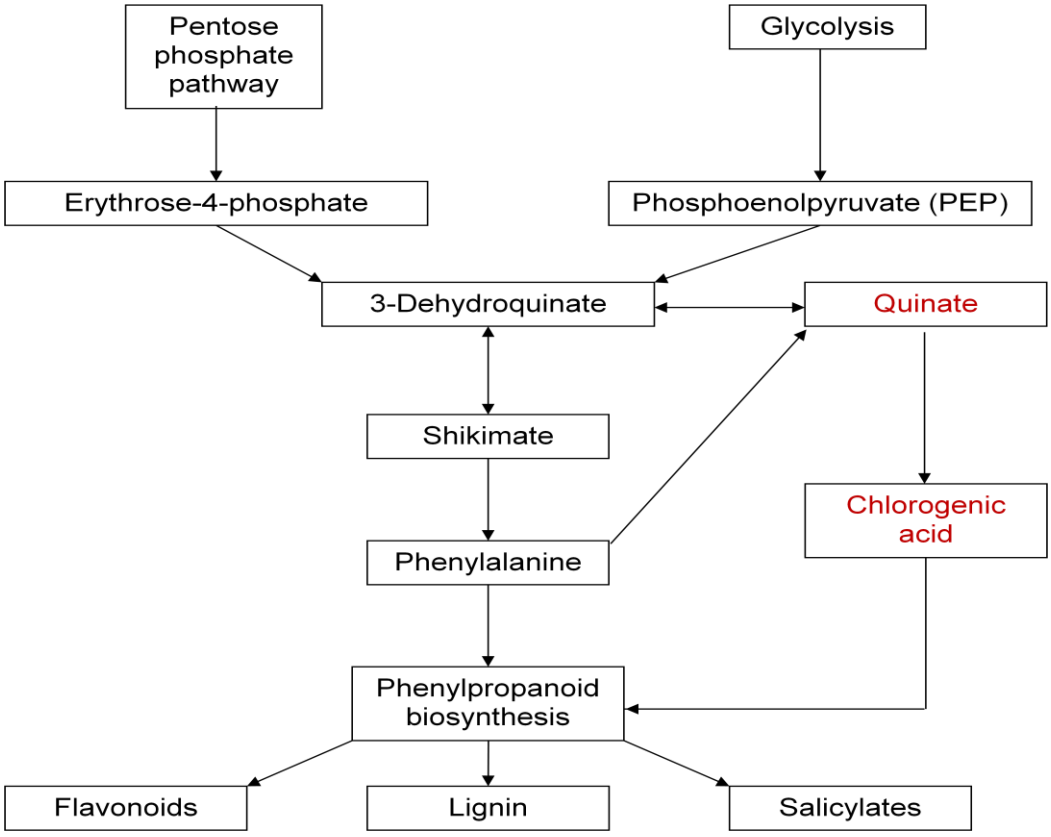


**Figure 4.3** Shikimic acid pathway (after Moorman *et al*., 1992; Herrmann 1995; Vogt 2009). (Compounds in red are associated with MAP and late bud set i.e., those compounds are associated with late bud set of populations originated from south).

Gluconic acid lactone decreased with late bud set and MAP (Table 3.3) which might be an indication of its involvement in normal metabolic activities of southern genotypes. Gluconic acid lactone showed increasing and decreasing trends in response to cold stress and deacclimation, respectively in Arabidopsis (Kaplan *et al*., 2004). This may be attributed to gluconic acid's antioxidant activity (Gheldof *et al*., 2002).

The observed decrease in fumaric, malic and citric acids with late bud set and MAP (Table 3.3) may be explained by a down regulation of the TCA cycle associated with late bud

set of southern genotypes (Figure 4.4). A similar relationship was found in Sitka spruce (S.D. Mansfield, Faculty of Forestry, UBC, personal communication (2009)). Increasing and decreasing trends in TCA cycle intermediates (including fumaric, malic and citric acids) during the cold acclimation and deacclimation process, respectively, have been reported in many plant species (Kalberer *et al*., 2006; Guy *et al*., 2008; Korn *et al*., 2009).

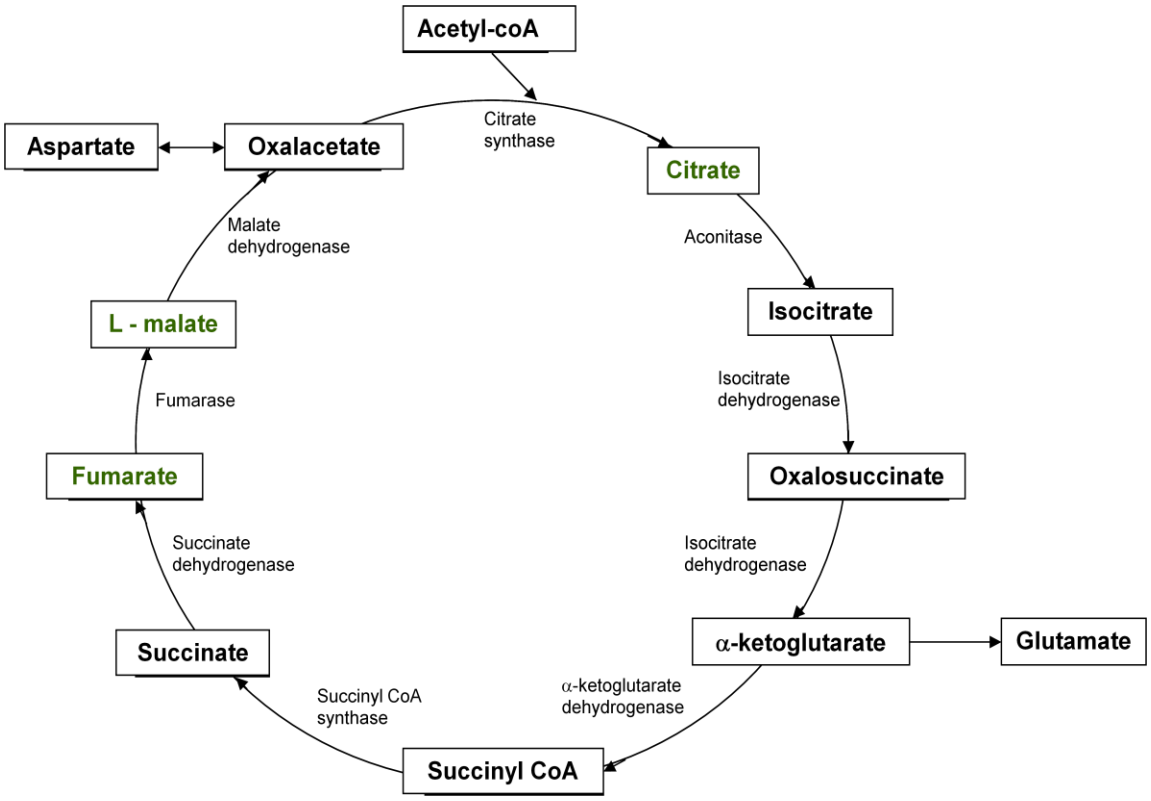


**Figure 4.4** Tricarboxylic acid (TCA) cycle diagram (Korn *et al*., 2009). (Compounds in green are negatively associated with late bud set and MAP).

### 4.1.3 The third canonical correlation

Results from the third canonical correlation indicated increasing (shikimic acid, salicin and catechol) and decreasing (quinic acid, chlorogenic acid, taxifolin, keampferol, catechin, salireposide and salicortin) trends with elevations (Table 3.4). These compounds are related to the shikimate pathway (Figure 4.1). Flavonoids showed decreasing trend with genotypes originated from high elevation, this might be related to its function as UV protectors (see section

4.1.1). It seems that the shikimate pathway directed the production of salicin and catechol in genotypes originated from higher elevations.

The observed decrease in ascorbic acid with high elevations (Table 3.4) might be related to genotypes originating from the north (see section 4.1.1 for its function).

Additionally, galactinol and raffinose showed increasing trend with higher elevation ecotypes (Table 3.4), which usually set bud earlier than those originating from lower elevations (discussed in section 4.1.1 above).

### 4.1.4 The fourth canonical correlation

Relative decrease in shikimic acid, quinic acid, taxifolin and kaempferol and increases in salicortin and catechol-glucoside with MAP (Table 3.5) might explain the direction of the shikimate pathway for producing salicylates (salicortin and catechol-glucoside) and not flavonoids (taxifolin and kaempferol) in association with increasing MAP. Quinic acid was found to accumulate under natural and applied drought conditions in cork oak and *Populus* spp., respectively (Gebre *et al*., 1994; Passarinho *et al*., 2006).

Decreases in ascorbic acid and galactinol with MAP (Table 3.5) might be related to genotypes originating from locations with sufficient precipitation. The antioxidant ascorbic acid and the osmoprotectant galactinol both showed an increasing trend under drought conditions (Arora *et al*., 2002; Taji *et al*., 2002; Guignard *et al*., 2005; Lei *et al*., 2007).

Increasing trends in glutamine, TCA cycle intermediates (fumaric, malic and citric acids) and gluconic acid lactone were also detected with MAP (Table 3.5). Under drought conditions an opposite trend was observed where inhibition in tricarboxylic acid cycle (TCA) intermediates was detected in many plants including Arabidopsis and cotton (Eaton 1949; Tausz *et al*., 2004; Huang *et al*., 2008).

Finally, it seems that the observed decrease in glucose-6-phosphate with MAP may be related to its consumption during metabolic activities associated with ecotypes that are still metabolically active.

## 4.2 CCA performed using principal components (the second analysis)

### 4.2.1 The first canonical correlation

For the first canonical correlation (Table 3.6), PC-5, PC-6 and PC-8 were positively while PC-9 and PC-10 were negatively associated with latitude of origin. Considering the

metabolites that loaded highly in the component matrix of each PC (Table 3.6), PC-5 associated positively with glycolic acid and shikimic acid and negatively with an unknown metabolite (Table 3.1) where the former and latter are increasing and decreasing with latitude of origin, low MAT and early bud set. These results indicate that glycolic acid concentration increased under early bud set and cold conditions as it protects plants under different stresses by behaving as ROS scavenger (Kinnersley 2002). Glycolic acid has shown a reversal role to stomatal closure induced by different stresses including low temperature (Zelitch and Walker 1964; Tausz *et al*., 2004; Wilkins *et al*., 2009). Glycolic acid has been detected in tomato leaves as an important intermediate in photorespiration during slow growth periods (i.e., dormancy) (Zelitch 1973; Jolivet *et al*., 1985). Shikimic acid showed increasing trends with northern genotypes (Figure 4.5) while an opposite trend was reported by Kaplan *et al*. (2004) in Arabidopsis with low temperatures. In addition to acting as a precursor to lignin biosynthesis and an intermediate of aromatic amino acids and other secondary metabolites, shikimic acid behaves as an antioxidant in response to various stresses (Sawa *et al*., 1999; Passarinho *et al*., 2006).

PC-6 showed positive associations with two unknown compounds (Table 3.1) that were related to the latitude of origin, low MAT and early bud set, while PC-8 showed weak association with all metabolites (loading < 0.45) (Table 3.1)

PC-9 indicated negative association with catechin and chlorogenic acid that showed decreasing trends with latitude of origin, low MAT and early bud set (Table 3.1). The observed chlorogenic acid decrease might be due to its consumption for phenylpropanoid biosynthesis which accumulates in plant young leaves, stems and apical buds as a defense response in winter (Subramaniam *et al*., 1993; Solecka 1997; Grace and Logan 2000; Nugroho *et al*., 2002; Morreel *et al*., 2006) (Figure 4.5). As one of the flavonoids, catechin is involved in plant protection against UV-light which is associated with high temperatures (Pietta 2000; Warren *et al*., 2002; Morreel *et al*., 2006).

PC-10 was associated with propanoic acid and salicyle alcohol which were negatively linked to the latitude of origin, low MAT and early bud set (Table 3.1). Propanoic acid decrease may hint towards its function in response to cold stress as suggested by Guy (1990) and Guy *et al*. (2008) who speculated on its role as an energy source during cold acclimation. Salicyl alcohol might be consumed for salicylates production; especially salicin as it increased with cold in Arabidopsis and poplar (Scott *et al*., 2004; Morse *et al*., 2007). All together, northern genotypes showed an increase in metabolites involved in cold acclimation as preparation for

winter supporting the presence of a clinal trend of those metabolites with bud set, latitude of origin and climate. The results from this analysis mirror those obtained from the previous (using different statistical analytical approach), thus providing credence and supporting the conclusions drawn.

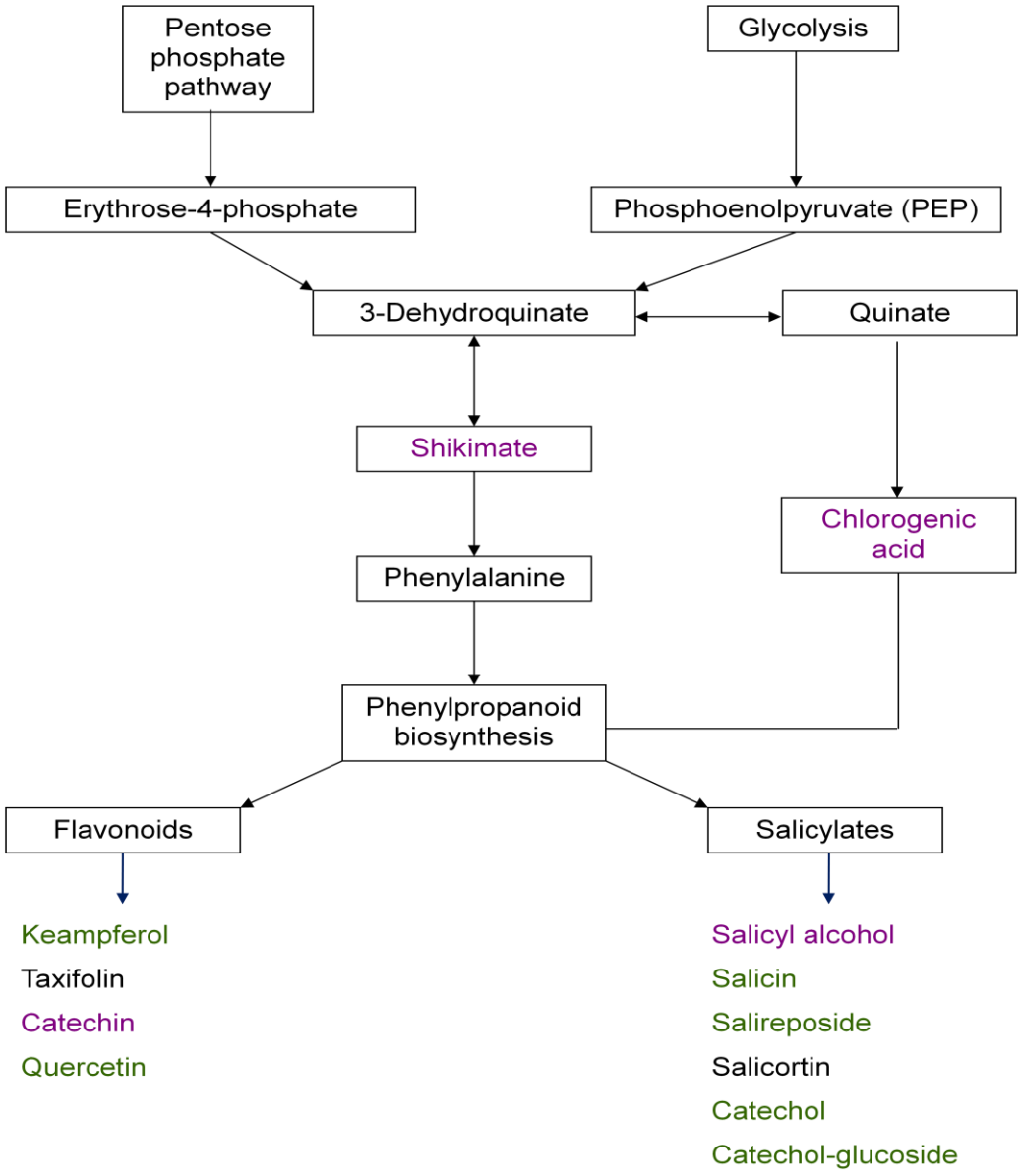


**Figure 4.5** Shikimic acid pathway (after Moorman *et al*., 1992; Herrmann 1995; Vogt 2009). (Trends of compounds in purple and green has been found in association with latitude, low MAT and early bud set and bud set, respectively).

### 4.2.2 The second canonical correlation

Positive (PC-1) and negative (PC-2-4 and PC-7) associations between late bud set and the second canonical correlations scores were observed (Table 3.7). In view of each PC component matrix (Table 3.1), PC-1 showed an increasing trend of metabolites related to the raffinose pathway (galacturonic acid, galactonic acid, galactinol and raffinose) with late bud set. This suggested that during the active growth, the raffinose pathway of southern ecotypes works in two directions producing galacturonic acid and galactonic acid on the one hand, and galactinol and raffinose on the other hand (Figure 4.6). Although accumulation of the Raffinose Family Oligosaccharides (RFOs) has been detected in Arabidopsis leaves as a protective agent against freezing conditions, it was also detected under high temperature climate (Taji *et al*., 2002; Stitt and Hurry 2002; Kaplan *et al*., 2004; Hannah *et al*., 2006; Guy *et al*., 2008; Korn *et al*. 2008; Nishizawa *et al*., 2008). It was also reported that RFOs function as oxidative damage prevention in response to different environmental stresses (Taji *et al*., 2002; Kaplan *et al*., 2004; Nishizawa *et al*., 2008; Maruyama *et al*., 2009). Additionally, Kaplan *et al*. (2004) has detected an increase in galactonic acid in response to both heat and cold stress.

PC-1 showed an increasing trend of steric acid methyl ester (methyl stearate) accumulation with late bud set of southern genotypes, an indication of normal growth activities associated with late bud set (Table 3.1). Methyl stearate has been detected under optimal growth and development conditions in young leaves of Arabidopsis (Lightner *et al*., 1994).

The phosphate sugar, glucose-6-phosphate is very common in plant cells as a result of glucose phosphorylation; an increase in glucose-6-phosphate during active metabolism has been found in association with late bud set of southern ecotypes as shown in PC-1 (Table 3.1). Similarly, monopalmitoyl-rac-glycerol has a positive association with late bud set in PC-1 which might be produced and stored during growth stage to be used as a source of energy during dormancy (Table 3.1).

As flavonoids, kaempferol and quercetin exhibited an increasing trend with late bud set (Table 3.1), which might accumulate as a protecting agent against UV light. Previous studies have reported that flavonoids act as absorbent antioxidants protecting plants from solar UV radiation injury associated with high temperature (Pietta 2000; Warren *et al*., 2002; Morreel *et al*., 2006). Accumulation of flavonoids has been detected in leaves of different plants including poplar as a response to UV irradiation associated with high temperatures (Li *et al*., 1993; Schnitzler, *et al*., 1997; Schumaker *et al*., 1997; Lavola *et al*., 2000; Nugroho *et al*. 2002;

Warren *et al*., 2002). Additionally, PC-1 showed increasing trend of 14 unknown metabolites with late bud set (Table 3.1).

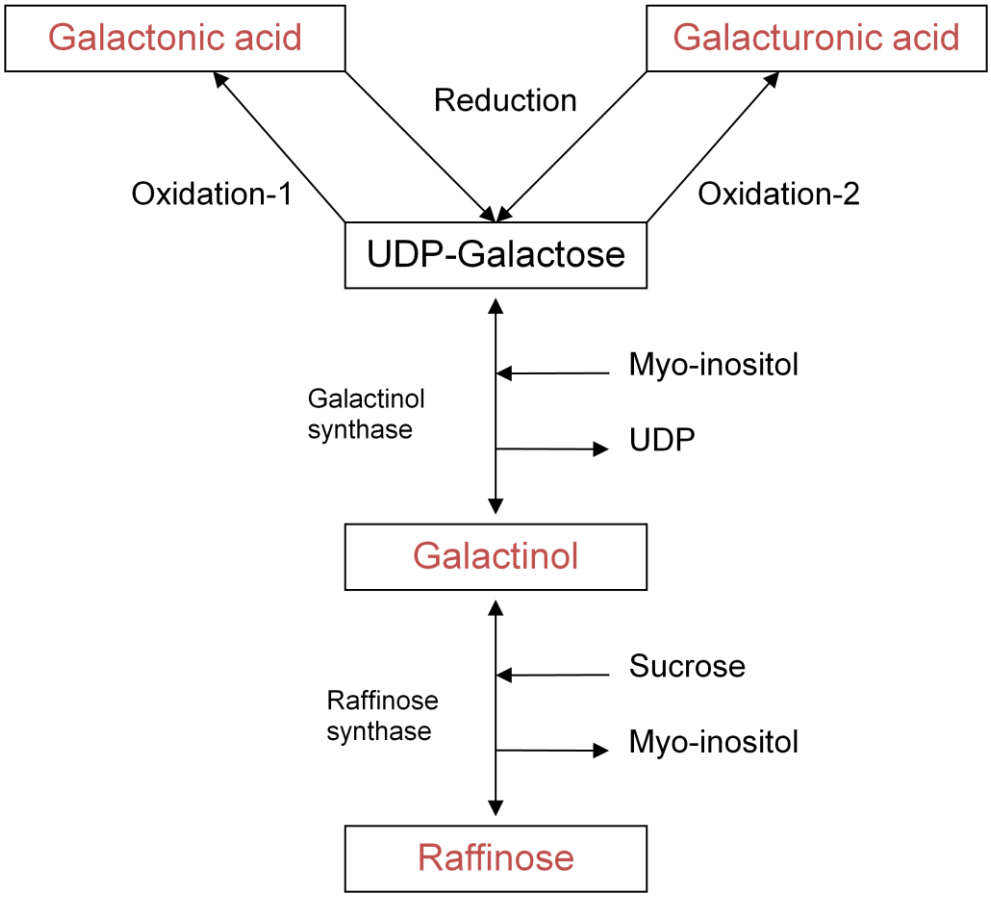


**Figure 4.6** Raffinose pathway in plants (after Sprenger and Keller 2000; Taji *et al*., 2002; Amiard *et al*., 2003; Nishizawa *et al*., 2008). (Metabolites in red are increasing with late bud set)

PC-2 showed a decreasing trend of glutamine, monosaccharides (fructose and glucose), ascorbic acid (vitamin C), and metabolites related to shikimate pathway (catechol, catechol glucoside, salicin and salireposide) and to TCA cycle (fumaric and malic acids) and with late bud set (Table 3.1). Decreased trends in: 1) glutamine suggests its consumption during growth and development of southern genotypes (e.g., protein synthesis), 2) fructose and glucose indicative of their consumption in different metabolic pathways, 3) ascorbic acid as an enzyme cofactor in synthesis of many compounds such as hormones (Isherwood and Mapson 1962; Benzakour *et al.*, 2000; Smirnoff 2003), 4) the observed shikimate pathway metabolites (Figure

4.5) as salicylates (salts and esters of salicylic acid) all are involved in *Populus* sp. protection mechanisms (Zhang *et al*., 2006; Morse *et al*., 2007) and 5) metabolites related to TCA cycle (fumaric and malic acids (Figure 4.4)) suggesting an inhibition in TCA cycle in association with late bud set of southern ecotypes (discussed in section 4.1.2). Furthermore, 11 unknowns are negatively associated with late bud set as explained in PC-2 (Table 3.1).

In PC-3 a group of organic (glycolic, fumaric, malic, pyroglutamic, citric, quinic acids and gluconic acid lactone) and an inorganic acid (phosphoric) in addition to five unknown metabolites decreased with late bud set (Table 3.1). As normal growth of southern ecotypes continues, a reduction in phosphoric acid is expected as it is consumed in the biosynthesis of many compounds (e.g., nucleic acids; Sherman 2009). Also reduction in glycolic acid has been detected in actively growing southern ecotypes. A drop in TCA cycle intermediates (fumaric, malic and citric acids) has been reported in association with late bud set (discussed in section 4.1.2). The observed decrease of pyroglutamic and quinic acids and gluconic acid lactone with late bud set has been discussed in section 4.2.

The observed increase in catechol and salicin in PC-4 and salicortin in PC-7 (Table 3.1) with late bud set might reflect the role salicylates have during active metabolism of southern ecotypes. Salicylates have been reported to have a protective role against UV-light associated with temperature (Lavola 1998). Furthermore, trends of unknowns were loaded in PC-4 and PC-7 (Table 3.1).

Generally, southern ecotypes were still growing normally and developing (i.e., are exhibiting active metabolic status) as they did not set buds yet.

## 4.3 Conclusion

The ability of *Populus* spp. to grow and develop under different environmental conditions is of great importance to thier survival and the production of considerable biomass in a short period of time. This attribute is essential for this ecologically dominant and economically important genus (Ragauskas *et al*., 2006; Lei *et al*., 2007; Wilkins *et al*., 2009).

When grown in a common location, in the late summer, southern genotypes are still actively growing, while their northern counterparts have already set bud, i.e., growth cessation and dormancy has occurred. Northern genotypes, with their low MAT, set bud early and show increasing concentrations of antioxidants and metabolites that are known to be associated with cold exposure. These include shikimic acid, chlorogenic acid, flavonoids (kaempferol),

salicylates (salireposide, salicortin and catechol glucoside). Conversely, they show a decrease in quinic acid and taxifolin; metabolites related to the shikimate pathway (Figure 4.1). Similarly an increase in cryoprotectant RFOs (galactinol and raffinose) has been detected (Figure 4.2), as well as an increase in the osmoprotectant amino acid glutamine and its precursor pyroglutamic acid, the antioxidant ascorbic acid, and protective steric acid methyl ester and a decrease in phosphoric acid.

Growth activities and metabolism of southern ecotypes can be explained with increases in quinic acid, chlorogenic acid, glucose and fructose and decreases in osmoprotectant such as amino acid glutamine and its precursor pyroglutamic acid, phosphoric acid, antioxidant gluconic acid lactone and TCA cycle intermediates (fumaric acid, malic acid and citric acid) (Figure 4.4).

Generally, considerable physiological and biochemical changes occur at the genetical level of *P. trichocarpa* along its natural geographic range, reflecting local adaptation when growing in a common environment. Therefore, bud set in *Populus* is expected to show clinal variation with latitude (i.e., photoperiod) controlled by genetics and environmental components (e.g., temperature), as well as their interactions (Frewen *et al*., 2000; Ingvarsson *et al*., 2006). Thus, the observations made from this common garden study reflect the within-species genetic variability.

Finally, metabolite profiling in conjunction with data-mining tools has proven to be an effective approach in investigating the individual and/or collective role of both genetics and environmental adaptation in cold-hardy woody plants (Cox and Stushnoff 2001; Roessner *et al*., 2001a).

## 4.4 Limitations and recommendation for further research

The present study has shown strong relationships between metabolites and geo-climatic and phenology variables of *Populus trichocarpa* genotypes reflecting species local adaptation. For further research, a larger sample representing multiple genotypes within population and multiple populations representing the entire species' range, including extreme populations, is recommended. Sampling from reciprocal transplant experiments where the same genotypes are planted over multiple environments will also assist in better understanding of genotypes reaction to various environmental conditions and the better matching between genetics and environment (i.e., better deployment of planting stocks). Sampling over the growing season may allow better understanding of *P. trichocarpa* population's adaptation. Additionally, increased efforts towards the identification of unknown/unidentified metabolites are required for expanding the metabolite libraries.

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## APPENDIX 1 Metabolites list in GC-MS chromatogram

List of all 104 metabolites determined by GC-MS chromatogram.

| Peak number | RT (min) | Identity |
|---|---|---|
| GC_001 | 10.18 | Unidentified GC_001 |
| GC_002 | 10.75 | Phosphoric acid |
| GC_003 | 10.98 | Glutamine |
| GC_004 | 11.35 | Succinic acid |
| GC_005 | 11.47 | Catechol |
| GC_006 | 11.73 | Glycolic acid |
| GC_007 | 11.90 | Fumaric acid |
| GC_008 | 12.18 | Alanine |
| GC_009 | 12.47 | Unidentified GC_009 |
| GC_010 | 12.53 | Unidentified GC_010 |
| GC_011 | 13.07 | Unidentified GC_011 |
| GC_012 | 13.20 | Unidentified GC_012 |
| GC_013 | 13.25 | Hydriodic acid |
| GC_014 | 13.40 | Propanoic acid |
| GC_015 | 13.40 | Salicyl alcohol |
| GC_016 | 14.05 | Unidentified GC_016 |
| GC_017 | 14.27 | Unidentified GC_017 |
| GC_018 | 14.28 | Malic acid |
| GC_019 | 14.37 | Unidentified GC_019 |
| GC_020 | 14.55 | Unidentified GC_020 |
| GC_021 | 14.80 | Pyroglutamic acid |
| GC_022 | 15.10 | Unidentified GC_022 |
| GC_023 | 15.12 | Unidentified GC_023 |
| GC_024 | 15.47 | Threonic acid |
| GC_025 | 15.60 | Alpha-ketoglutaric acid |
| GC_026 | 15.80 | Unidentified GC_026 |
| GC_027 | 15.80 | Unidentified GC_027 |
| GC_028 | 15.97 | Unidentified GC_028 |
| GC_029 | 16.07 | 2,3-Dihydroxybutanedioic acid |
| GC_030 | 16.22 | Unidentified GC_030 |
| GC_031 | 16.77 | Unidentified GC_031 |
| GC_032 | 16.85 | Unidentified GC_032 |
| GC_033 | 17.20 | Unidentified GC_033 |
| GC_034 | 17.45 | Unidentified GC_034; Sugar alcohol |
| GC_035 | 17.68 | Unidentified GC_035 |
| GC_036 | 18.07 | Unidentified GC_036; Carbohydrate-1 |
| GC_037 | 18.23 | Unidentified GC_037; Organic acid-1 |
| GC_038 | 18.40 | Unidentified GC_038; Organic acid-2 |
| GC_039 | 18.63 | Unidentified GC_039 |
| GC_040 | 18.85 | Shikimic acid |
| GC_041 | 19.03 | Citric acid |
| GC_042 | 19.43 | Unidentified GC_042; Carbohydrate-2 |
| GC_043 | 19.48 | Unidentified GC_043; Carbohydrate-3 |
| GC_044 | 19.65 | Quinic acid |
| GC_045 | 19.82 | Fructose |
| GC_046 | 20.38 | Glucose |

List of all 104 metabolites determined by GC-MS chromatogram - continued

| Peak number | RT (min) | Identity |
|---|---|---|
| GC_047 | 20.67 | Galacturonic acid |
| GC_048 | 20.70 | Ascorbic acid |
| GC_049 | 21.15 | Unidentified GC_049 |
| GC_050 | 21.30 | Unidentified GC_050 |
| GC_051 | 21.38 | Galactonic acid |
| GC_052 | 21.60 | Gluconic acid |
| GC_053 | 22.45 | Myo-inositol |
| GC_054 | 23.52 | Unidentified GC_054 |
| GC_055 | 23.58 | Unidentified GC_055 |
| GC_056 | 23.58 | Unidentified GC_056 |
| GC_057 | 23.78 | Steric acid methyl ester |
| GC_058 | 24.02 | Unidentified GC_058; Carbohydrate-4 |
| GC_059 | 24.58 | Unidentified GC_059 |
| GC_060 | 24.92 | Unidentified GC_060; Carbohydrate-5 |
| GC_061 | 25.05 | Glucose-6-phosphate |
| GC_062 | 25.32 | Unidentified GC_062 |
| GC_063 | 25.40 | Unidentified GC_063 |
| GC_064 | 25.48 | Unidentified GC_064; Carbohydrate-6 |
| GC_065 | 25.63 | Unidentified GC_065; Carbohydrate-7 |
| GC_066 | 25.68 | Unidentified GC_066; Carbohydrate-8 |
| GC_067 | 25.85 | Unidentified GC_067; Oligosaccharide-1 |
| GC_068 | 25.97 | Unidentified GC_068 |
| GC_069 | 26.13 | Catechol glucoside |
| GC_070 | 26.23 | Unidentified GC_070 |
| GC_071 | 26.38 | Unidentified GC_071 |
| GC_072 | 26.57 | Unidentified GC_072 |
| GC_073 | 26.85 | Unidentified GC_073 |
| GC_074 | 26.95 | Salicin |
| GC_075 | 27.30 | 1-Monopalmitoyl-rac-glycerol |
| GC_076 | 27.33 | Unidentified GC_076; Oligosaccharide -2 |
| GC_077 | 27.35 | Unidentified GC_077 |
| GC_078 | 27.63 | Unidentified GC_078 |
| GC_079 | 27.87 | Unidentified GC_079; Oligosaccharide-3 |
| GC_080 | 28.20 | Sucrose |
| GC_081 | 29.65 | Unidentified GC_081 |
| GC_082 | 30.12 | Catechin |
| GC_083 | 30.47 | Unidentified GC_083 |
| GC_084 | 30.67 | Unidentified GC_084 |
| GC_085 | 30.77 | Unidentified GC_085 |
| GC_086 | 30.82 | Taxifolin |
| GC_087 | 31.13 | Unidentified GC_087 |
| GC_088 | 31.25 | Galactinol |
| GC_089 | 31.67 | Unidentified GC_089 |
| GC_090 | 31.70 | Kaempferol |

List of all 104 metabolites determined by GC-MS chromatogram - continued

| Peak number | RT (min) | Identity |
|---|---|---|
| GC_091 | 31.83 | Unidentified GC_091 |
| GC_092 | 32.00 | Unidentified GC_092 |
| GC_093 | 32.08 | Chlorogenic acid |
| GC_094 | 32.28 | Unidentified GC_094 |
| GC_095 | 32.57 | Quercetin |
| GC_096 | 32.67 | Unidentified GC_096 |
| GC_097 | 32.95 | Unidentified GC_097 |
| GC_098 | 33.23 | Salireposide |
| GC_099 | 33.25 | Salicortin |
| GC_100 | 34.17 | Unidentified GC_100 |
| GC_101 | 34.28 | Unidentified GC_101 |
| GC_102 | 34.40 | Raffinose |
| GC_103 | 34.70 | Unidentified GC_103 |
| GC_104 | 35.35 | Unidentified GC_104 |

## APPENDIX 2 Significant metabolites in principal component matrices

All metabolites significantly loaded on principal component matrices. Metabolites represented by their peak number (sequence of elution in gas chromatography), loading (correlation between each metabolite and its principal component), and identity (otherwise metabolites are unknown).

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| **PC-1** | 4 | 0.43 | succinic acid |
| | 5 | 0.2 | catechol |
| | 8 | 0.24 | alanine |
| | 9 | 0.34 | unknown-9 |
| | 13 | 0.41 | hydriodic acid |
| | 16 | 0.62 | unknown-16 |
| | 17 | 0.33 | unknown-17 |
| | 20 | 0.49 | unknown-20 |
| | 21 | 0.24 | pyroglutamic acid |
| | 22 | 0.33 | unknown-22 |
| | 25 | 0.45 | unknown-25 |
| | 28 | 0.45 | unknown-28 |
| | 29 | 0.46 | dihydroxybutanedioic acid |
| | 30 | -0.25 | unknown-30 |
| | 32 | -0.27 | unknown-32 |
| | 33 | 0.41 | unknown-33 |
| | 34 | 0.4 | sugar alcohol |
| | 36 | 0.66 | unknown-36 carbohydrate-1 |
| | 37 | 0.24 | organic acid |
| | 38 | 0.35 | unknown-38 organic acid-2 |
| | 42 | 0.23 | unknown-42 carbohydrate-2 |
| | 44 | 0.43 | quinic acid |
| | 45 | 0.34 | fructose |
| | 46 | 0.4 | glucose |
| | 47 | 0.49 | galacturonic acid |
| | 48 | 0.41 | ascorbic acid |
| | 51 | 0.48 | galactonic acid |
| | 55 | 0.4 | unknown-55 |
| | 56 | 0.63 | unknown-56 |
| | 57 | 0.62 | steric acid methyl ester |
| | 58 | 0.42 | unknown-58 carbohydrate-4 |
| | 59 | 0.8 | unknown-59 |
| | 60 | 0.44 | unknown-60 carbohydrate-5 |
| | 61 | 0.66 | glucose-6-phosphate |
| | 62 | 0.8 | unknown-62 |
| | 63 | 0.8 | unknown-63 |
| | 65 | 0.28 | unknown-65 carbohydrate-7 |
| | 68 | 0.8 | unknown-68 |

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| | 69 | 0.27 | catechol glucoside |
| | 70 | 0.3 | unknown-70 |
| | 71 | 0.22 | unknown-71 |
| | 72 | 0.57 | unknown-72 |
| | 73 | 0.24 | unknown-73 |
| | 74 | 0.55 | Salicin |
| | 75 | 0.56 | Monopalmitoyl-rac-glycerol |
| | 76 | 0.52 | unknown-76 oligosaccharide-2 |
| | 79 | 0.45 | unknown-79 oligosaccharide-3 |
| | 80 | 0.29 | Sucrose |
| | 83 | 0.37 | unknown-83 |
| | 84 | 0.65 | unknown-84 |
| | 86 | 0.42 | Taxifolin |
| | 87 | 0.27 | unknown-87 |
| | 88 | 0.52 | galactinol |
| | 89 | 0.43 | unknown-89 |
| | 90 | 0.53 | kaempferol |
| | 91 | 0.42 | unknown-91 |
| | 92 | 0.43 | unknown-92 |
| | 93 | 0.24 | chlorogenic acid |
| | 94 | 0.62 | unknown-94 |
| | 95 | 0.63 | quercetin |
| | 96 | 0.35 | unknown-96 |
| | 97 | 0.44 | unknown-97 |
| | 100 | 0.38 | unknown-100 |
| | 101 | 0.3 | unknown-101 |
| | 102 | 0.51 | raffinose |
| | 104 | 0.63 | unknown-104 |
| **PC-2** | 2 | 0.39 | phosphoric acid |
| | 3 | 0.53 | glutamine |
| | 4 | -0.24 | succinic acid |
| | 5 | 0.63 | Catechol |
| | 6 | -0.26 | glycolic acid |
| | 7 | 0.47 | fumaric acid |
| | 8 | 0.21 | Alanine |
| | 14 | 0.36 | propanoic acid |
| | 15 | 0.38 | salicyle alcohol |
| | 16 | 0.56 | unknown-16 |
| | 17 | 0.58 | unknown-17 |
| | 18 | 0.67 | malic acid |
| | 19 | -0.37 | unknown-19 |
| | 20 | -0.5 | unknown-20 |

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| | 21 | 0.22 | pyroglutamic acid |
| | 22 | 0.4 | unknown-22 |
| | 23 | 0.41 | unknown-23 |
| | 24 | -0.3 | threonic acid |
| | 25 | -0.44 | alpha-ketoglutaric acid |
| | 27 | 0.43 | unknown-27 |
| | 28 | -0.25 | unknown-28 |
| | 30 | 0.29 | unknown-30 |
| | 31 | -0.34 | unknown-31 |
| | 32 | -0.37 | unknown-32 |
| | 33 | -0.38 | unknown-33 |
| | 35 | 0.36 | unknown-35 |
| | 40 | -0.22 | shikimic acid |
| | 41 | 0.37 | citric acid |
| | 42 | 0.32 | unknown-42 carbohydrate-2 |
| | 44 | -0.23 | quinic acid |
| | 45 | 0.53 | Fructose |
| | 46 | 0.61 | Glucose |
| | 47 | -0.42 | galacturonic acid |
| | 48 | 0.55 | ascorbic acid |
| | 50 | -0.28 | unknown-50 |
| | 52 | 0.32 | gluconic acid |
| | 54 | 0.51 | unknown-54 |
| | 55 | -0.24 | unknown-55 |
| | 57 | 0.35 | steric acid methyl ester |
| | 58 | 0.25 | unknown-58 carbohydrate-4 |
| | 59 | 0.19 | unknown-59 |
| | 60 | 0.45 | unknown-60 carbohydrate-5 |
| | 61 | -0.2 | glucose-6-phosphate |
| | 62 | -0.22 | unknown-62 |
| | 64 | 0.6 | unknown-64 carbohydrate-6 |
| | 65 | 0.48 | unknown-65 carbohydrate-7 |
| | 66 | 0.61 | unknown-66 carbohydrate-8 |
| | 67 | 0.55 | unknown-67 oligosaccharide-1 |
| | 69 | 0.52 | catechol glucoside |
| | 70 | 0.26 | unknown-70 |
| | 71 | 0.48 | unknown-71 |
| | 74 | 0.55 | Salicin |
| | 75 | 0.35 | Monopalmitoyl-rac-glycerol |
| | 76 | 0.48 | unknown-76 oligosaccharide-2 |
| | 77 | 0.52 | unknown-77 |
| | 78 | 0.3 | unknown-78 |
| | 79 | 0.43 | unknown-79 oligosaccharide-3 |

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| | 80 | -0.34 | Sucrose |
| | 81 | 0.41 | unknown-81 |
| | 82 | -0.25 | Catechin |
| | 83 | -0.37 | unknown-83 |
| | 84 | 0.36 | unknown-84 |
| | 86 | -0.22 | Taxifolin |
| | 88 | -0.4 | galactinol |
| | 89 | 0.25 | unknown-89 |
| | 98 | 0.5 | salireposide |
| | 101 | 0.19 | unknown-101 |
| | 104 | -0.27 | unknown-104 |
| **PC-3** | 1 | -0.19 | unknown-1 |
| | 2 | 0.56 | Phosphoric acid |
| | 4 | 0.36 | succinic acid |
| | 5 | 0.22 | Catechol |
| | 6 | 0.46 | glycolic acid |
| | 7 | 0.68 | fumaric acid |
| | 8 | 0.28 | Alanine |
| | 17 | 0.34 | unknown-17 |
| | 18 | 0.51 | malic acid |
| | 19 | 0.22 | unknown-19 |
| | 20 | 0.35 | unknown-20 |
| | 21 | 0.53 | pyroglutamic acid |
| | 22 | 0.54 | unknown-22 |
| | 23 | 0.2 | unknown-23 |
| | 24 | 0.4 | threonic acid |
| | 25 | 0.38 | alpha-ketoglutaric acid |
| | 26 | -0.52 | unknown-26 |
| | 29 | 0.2 | dihydroxybutanedioic acid |
| | 30 | 0.47 | unknown-30 |
| | 31 | -0.16 | unknown-31 |
| | 34 | 0.27 | sugar alcohol |
| | 36 | -0.44 | unknown-36 carbohydrate-1 |
| | 38 | 0.19 | unknown-38 organic acid-2 |
| | 40 | 0.28 | shikimic acid |
| | 41 | 0.67 | citric acid |
| | 43 | -0.33 | unknown-43 carbohydrate-3 |
| | 44 | 0.55 | quinic acid |
| | 48 | -0.25 | ascorbic acid |
| | 49 | 0.29 | unknown-49 |
| | 52 | 0.66 | gluconic acid lactone |
| | 53 | 0.26 | myo-inositol |

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| | 54 | -0.48 | unknown-54 |
| | 56 | 0.42 | unknown-56 |
| | 57 | 0.23 | steric acid methyl ester |
| | 61 | 0.45 | glucose-6-phosphate |
| | 62 | -0.27 | unknown-62 |
| | 63 | -0.31 | unknown-63 |
| | 65 | -0.46 | unknown-65 carbohydrate-7 |
| | 67 | -0.31 | unknown-67 oligosaccharide-1 |
| | 70 | -0.34 | unknown-70 |
| | 73 | 0.28 | unknown-73 |
| | 76 | -0.22 | unknown-76 oligosaccharide-2 |
| | 77 | -0.2 | unknown-77 |
| | 78 | -0.21 | unknown-78 |
| | 79 | -0.2 | unknown-79 oligosaccharide-3 |
| | 81 | -0.29 | unknown-81 |
| | 83 | -0.2 | unknown-83 |
| | 85 | -0.45 | unknown-85 |
| | 87 | -0.34 | unknown-87 |
| | 90 | -0.31 | kaempferol |
| | 91 | -0.38 | unknown-91 |
| | 92 | -0.32 | unknown-92 |
| | 93 | -0.27 | chlorogenic acid |
| | 94 | -0.49 | unknown-94 |
| | 95 | -0.33 | Quercetin |
| | 97 | 0.49 | unknown-97 |
| | 100 | -0.41 | unknown-100 |
| | 101 | -0.4 | unknown-101 |
| | 102 | -0.31 | Raffinose |
| | 103 | -0.2 | unknown-103 |
| **PC-4** | 1 | -0.21 | unknown-1 |
| | 2 | 0.32 | Phosphoric acid |
| | 3 | -0.2 | glutamine |
| | 5 | -0.53 | Catechol |
| | 9 | -0.26 | unknown-9 |
| | 13 | 0.31 | hydriodic acid |
| | 14 | -0.23 | propanoic acid |
| | 15 | -0.23 | salicyle alcohol |
| | 16 | -0.28 | unknown-16 |
| | 17 | 0.41 | unknown-17 |
| | 18 | 0.23 | malic acid |
| | 21 | 0.28 | pyroglutamic acid |
| | 23 | -0.45 | unknown-23 |

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| | 24 | -0.23 | threonic acid |
| | 28 | -0.38 | unknown-28 |
| | 31 | 0.41 | unknown-31 |
| | 32 | 0.46 | unknown-32 |
| | 33 | 0.33 | unknown-33 |
| | 34 | 0.22 | sugar alcohol |
| | 35 | -0.64 | unknown-35 |
| | 38 | 0.61 | unknown-38 organic acid-2 |
| | 41 | 0.37 | citric acid |
| | 42 | 0.29 | unknown-42 carbohydrate-2 |
| | 43 | 0.38 | unknown-43 carbohydrate-3 |
| | 50 | -0.39 | unknown-50 |
| | 52 | 0.38 | gluconic acid lactone |
| | 53 | -0.26 | myo-inositol |
| | 54 | 0.27 | unknown-54 |
| | 59 | 0.36 | unknown-59 |
| | 60 | -0.56 | unknown-60 carbohydrate-5 |
| | 68 | 0.25 | unknown-68 |
| | 70 | 0.2 | unknown-70 |
| | 72 | 0.25 | unknown-72 |
| | 73 | -0.2 | unknown-73 |
| | 74 | -0.58 | Salicin |
| | 78 | 0.21 | unknown-78 |
| | 82 | 0.24 | Catechin |
| | 85 | 0.24 | unknown-85 |
| | 87 | 0.2 | unknown-87 |
| | 89 | -0.39 | unknown-89 |
| | 93 | 0.16 | chlorogenic acid |
| | 97 | 0.32 | unknown-97 |
| **PC-5** | 2 | -0.44 | Phosphoric acid |
| | 6 | 0.51 | glycolic acid |
| | 12 | 0.23 | unknown-12 |
| | 20 | 0.2 | unknown-20 |
| | 21 | 0.2 | pyroglutamic acid |
| | 24 | 0.35 | threonic acid |
| | 26 | -0.32 | unknown-26 |
| | 27 | 0.24 | unknown-27 |
| | 29 | 0.4 | dihydroxybutanedioic acid |
| | 30 | -0.32 | unknown-30 |
| | 31 | 0.26 | unknown-31 |
| | 32 | 0.22 | unknown-32 |
| | 36 | -0.28 | unknown-36 carbohydrate-1 |

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| | 38 | 0.3 | unknown-38 organic acid-2 |
| | 40 | 0.55 | shikimic acid |
| | 41 | -0.25 | citric acid |
| | 42 | 0.3 | unknown-42 carbohydrate-2 |
| | 45 | 0.34 | Fructose |
| | 46 | 0.21 | Glucose |
| | 47 | 0.19 | galacturonic acid |
| | 49 | 0.19 | unknown-49 |
| | 51 | 0.26 | galactonic acid |
| | 52 | -0.26 | gluconic acid lactone |
| | 53 | 0.33 | myo-inositol |
| | 54 | 0.29 | unknown-54 |
| | 64 | 0.36 | unknown-64 carbohydrate-6 |
| | 66 | 0.3 | unknown-66 carbohydrate-8 |
| | 67 | 0.37 | unknown-67 oligosaccharide-1 |
| | 69 | 0.41 | catechol glucoside |
| | 72 | -0.51 | unknown-72 |
| | 77 | 0.3 | unknown-77 |
| | 79 | 0.28 | unknown-79 oligosaccharide-3 |
| | 80 | 0.31 | Sucrose |
| | 83 | -0.3 | unknown-83 |
| | 88 | 0.24 | galactinol |
| | 90 | -0.26 | kaempferol |
| | 91 | -0.23 | unknown-91 |
| | 95 | -0.33 | Quercetin |
| | 98 | 0.21 | salireposide |
| | 102 | 0.21 | Raffinose |
| | 104 | -0.35 | unknown-104 |
| **PC-6** | 3 | -0.32 | glutamine |
| | 10 | 0.3 | unknown-10 |
| | 12 | -0.2 | unknown-12 |
| | 19 | 0.24 | unknown-19 |
| | 23 | -0.2 | unknown-23 |
| | 27 | -0.26 | unknown-27 |
| | 29 | -0.19 | dihydroxybutanedioic acid |
| | 31 | -0.37 | unknown-31 |
| | 32 | -0.45 | unknown-32 |
| | 33 | -0.32 | unknown-33 |
| | 34 | -0.39 | sugar alcohol |
| | 35 | -0.21 | unknown-35 |
| | 38 | -0.28 | unknown-38 organic acid-2 |
| | 39 | 0.7 | unknown-39 |

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| | 40 | 0.25 | shikimic acid |
| | 49 | 0.71 | unknown-49 |
| | 51 | -0.34 | galactonic acid |
| | 53 | 0.42 | myo-inositol |
| | 56 | -0.24 | unknown-56 |
| | 57 | 0.27 | steric acid methyl ester |
| | 70 | 0.4 | unknown-70 |
| | 75 | 0.4 | Monopalmitoyl-rac-glycerol |
| | 77 | 0.41 | unknown-77 |
| | 80 | 0.33 | Sucrose |
| | 85 | 0.43 | unknown-85 |
| | 87 | 0.21 | unknown-87 |
| | 88 | 0.28 | galactinol |
| | 89 | -0.26 | unknown-89 |
| | 93 | 0.28 | chlorogenic acid |
| | 98 | 0.2 | salireposide |
| | 99 | -0.22 | Salicortin |
| | 100 | -0.23 | unknown-100 |
| | 102 | 0.2 | Raffinose |
| **PC-7** | 1 | 0.25 | unknown-1 |
| | 3 | 0.31 | glutamine |
| | 4 | 0.32 | succinic acid |
| | 7 | 0.22 | fumaric acid |
| | 9 | 0.46 | unknown-9 |
| | 10 | 0.43 | unknown-10 |
| | 13 | 0.26 | hydriodic acid |
| | 14 | 0.37 | propanoic acid |
| | 15 | 0.36 | salicyle alcohol |
| | 18 | -0.21 | malic acid |
| | 21 | 0.33 | pyroglutamic acid |
| | 22 | 0.23 | unknown-22 |
| | 26 | 0.41 | unknown-26 |
| | 31 | 0.5 | unknown-31 |
| | 32 | 0.28 | unknown-32 |
| | 38 | 0.36 | unknown-38 organic acid-2 |
| | 44 | -0.35 | quinic acid |
| | 49 | 0.2 | unknown-49 |
| | 56 | -0.24 | unknown-56 |
| | 60 | 0.28 | unknown-60 carbohydrate-5 |
| | 64 | -0.2 | unknown-64 carbohydrate-6 |
| | 65 | -0.2 | unknown-65 carbohydrate-7 |
| | 66 | -0.29 | unknown-66 carbohydrate-8 |

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| | 70 | 0.25 | unknown-70 |
| | 82 | -0.25 | Catechin |
| | 84 | -0.22 | unknown-84 |
| | 85 | 0.22 | unknown-85 |
| | 86 | -0.24 | Taxifolin |
| | 88 | 0.24 | galactinol |
| | 89 | 0.39 | unknown-89 |
| | 96 | -0.31 | unknown-96 |
| | 99 | -0.56 | Salicortin |
| **PC-8** | 1 | 0.26 | unknown-1 |
| | 3 | -0.24 | glutamine |
| | 8 | 0.44 | Alanine |
| | 19 | 0.27 | unknown-19 |
| | 21 | 0.37 | pyroglutamic acid |
| | 22 | 0.34 | unknown-22 |
| | 23 | -0.39 | unknown-23 |
| | 24 | -0.33 | threonic acid |
| | 25 | -0.2 | alpha-ketoglutaric acid |
| | 29 | -0.33 | dihydroxybutanedioic acid |
| | 30 | 0.27 | unknown-30 |
| | 35 | 0.25 | unknown-35 |
| | 37 | -0.33 | unknown-37 organic acid-1 |
| | 40 | -0.2 | shikimic acid |
| | 46 | 0.32 | Glucose |
| | 48 | 0.27 | ascorbic acid |
| | 51 | -0.3 | galactonic acid |
| | 53 | 0.31 | myo-inositol |
| | 55 | 0.26 | unknown-55 |
| | 59 | -0.2 | unknown-59 |
| | 64 | 0.31 | unknown-64 carbohydrate-6 |
| | 66 | 0.3 | unknown-66 carbohydrate-8 |
| | 68 | -0.25 | unknown-68 |
| | 69 | 0.32 | catechol glucoside |
| | 71 | -0.49 | unknown-71 |
| | 73 | 0.33 | unknown-73 |
| | 78 | -0.3 | unknown-78 |
| | 80 | 0.37 | Sucrose |
| | 82 | 0.32 | Catechin |
| | 89 | 0.24 | unknown-89 |
| | 91 | -0.2 | unknown-91 |
| | 99 | 0.33 | Salicortin |
| **PC-9** | 9 | 0.25 | unknown-9 |

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| | 19 | -0.25 | unknown-19 |
| | 20 | -0.22 | unknown-20 |
| | 23 | 0.2 | unknown-23 |
| | 27 | 0.26 | unknown-27 |
| | 28 | -0.19 | unknown-28 |
| | 43 | 0.21 | unknown-43 carbohydrate-3 |
| | 47 | 0.22 | galacturonic acid |
| | 48 | -0.21 | ascorbic acid |
| | 55 | 0.36 | unknown-55 |
| | 64 | -0.26 | unknown-64 carbohydrate-6 |
| | 66 | -0.25 | unknown-66 carbohydrate-8 |
| | 70 | -0.29 | unknown-70 |
| | 76 | -0.23 | unknown-76 oligosaccharide-2 |
| | 81 | 0.3 | unknown-81 |
| | 82 | 0.58 | Catechin |
| | 86 | 0.6 | Taxifolin |
| | 87 | 0.58 | unknown-87 |
| | 93 | 0.7 | chlorogenic acid |
| | 99 | -0.32 | Salicortin |
| | 101 | -0.23 | unknown-101 |
| **PC-10** | 3 | 0.22 | glutamine |
| | 11 | -0.26 | unknown-11 |
| | 12 | -0.29 | unknown-12 |
| | 14 | 0.48 | propanoic acid |
| | 15 | 0.46 | salicyle alcohol |
| | 19 | 0.34 | unknown-19 |
| | 21 | -0.24 | pyroglutamic acid |
| | 22 | -0.27 | unknown-22 |
| | 27 | 0.21 | unknown-27 |
| | 37 | -0.28 | unknown-37 organic acid-1 |
| | 39 | 0.24 | unknown-39 |
| | 42 | -0.38 | unknown-42 carbohydrate-2 |
| | 43 | -0.32 | unknown-43 carbohydrate-3 |
| | 49 | 0.23 | unknown-49 |
| | 54 | -0.2 | unknown-54 |
| | 56 | 0.2 | unknown-56 |
| | 69 | 0.32 | catechol glucoside |
| | 73 | -0.28 | unknown-73 |
| | 77 | 0.33 | unknown-77 |
| | 79 | 0.28 | unknown-79 oligosaccharide-3 |
| | 83 | 0.27 | unknown-83 |
| | 95 | 0.2 | Quercetin |

| PC# | Peak # | Loading | Identity |
|---|---|---|---|
| | 98 | 0.22 | salireposide |
| | 100 | -0.2 | unknown-100 |
| | 102 | -0.32 | raffinose |
| | 104 | 0.33 | unknown-104 |