SEASONAL VARIATIONS OF METABOLITE CONTENT IN PHLOEM SAP FROM *BROUSSONETIA PAPYRIFERA*

JIANG-TAO SHI[1*], HAI-CHONG LIU[1,2], JIA-YAN LUO[1] AND LIPING CAI [3,1]

[1]*College of Material Science and Engineering, Nanjing Forestry University, Nanjing 210037, P. R. China*
[2]*College of Economic and Management, Shandong Yingcai University, Jinan, 250104, P. R. China*
[3]*Department of Mechanical and Energy Engineering, University of North Texas, Denton TX 76203, USA*
*Corresponding author's email: shijt@njfu.edu.cn

Abstract

Phloem sap not only services important physiological function in tree growth but also can be selected for a potential raw material in chemical industrial products. As a tree species from Moraceae, Broussonetia papyrifera (*BP*) contains rich phloem sap.In order to investigate the phloem sap content and the variation during tree growh, Gas chromatography-mass spectrometry was employed to analyze the metabolites in phloem sap of *BP*, with a focus on seasonal variations in metabolite content. A total of 38 metabolites were detected in *BP* phloem exudates, with the highest content (44.59mg g^{-1}) of total metabolites observed in March. Moreover, large amounts of organic acids and sugars were detected in *BP* phloem exudates for all growing months, as well as lower amounts of fatty acids and alcohols. Metabolites associated with anti-cancer and anti-inflammation properties (e.g. PI3 kinase inhibitor, Chlorogenic acid, Chelerythrine and palmitic acid) were also detected. Quininic acid was the most abundant organic acid, representing up to 86.3% of all organic acids. In addition, D-fructose, D-glucose, and sucrose were the principle soluble sugars observed in phloem saps, with a maximum sugar content of 19.76mg g^{-1} in November. Seasonal changes in the metabolite content varied among individuals. Results of the metabolite analysis confirmed the role of *BP* phloem sap as an important resource for the synthesis of both pharmaceutical and human health products. All the results suggested that phloem sap content changes with the tree growth season.

Key words: Metabolites; Phloem sap; Sugars; Organic acid; Laticifers; GC-MS.

Introduction

Broussonetia papyrifera L. Vent. (*BP*) is widely planted in East Asia, the United States, and the Pacific Islands due to its fast growing and strong adaptability, germinating and regeneration capabilities (Fahrney *et al*., 1997; Saito *et al*., 2009). Similar to other Moraceae tree species, *BP* generates abundance of sap from its phloem tissue. Previous studies have reported the extraction of bioactive ingredients from the *BP* phloem sap which proved to have beneficial antioxidant (Xu *et al*., 2010), anti-inflammatory and anti-cancer properties (Wang *et al*., 2010; Guo *et al*., 2013; Park *et al*., 2016), and exhibited inhibitory activity against aromatase (Lee *et al*., 2001), tyrosinase (Zheng *et al*., 2008), α-Glucosidase (Ryu *et al*., 2010), and anticholinesterase (Ryu *et al*., 2012). Thus, *BP* phloem sap is a promising natural resource that can be potentially applied in the synthesis of new medicines and health products. Furthermore, *BP* phloem can also be effectively employed for the pulping and manufacturing of high quality paper (Hubbe & Bowden, 2009; Qu *et al*., 2014). Consequently, understanding the chemical composition in phloem sap is highly beneficial to the paper industrial.

Phloem sap is produced from the secondary metabolites of trees and some metabolites participate in the physiological pactivities of trees. Fresh phloem sap commonly consists of polychemical compositions produced from the metabolic activity of photosynthate. Such chemical ingredients are crucial for tree physiology, playing key roles in the resistance of pest infestation (Douglas, 2006; Kehr, 2006), the maintenance of osmotic pressure between conductivity cells (Turgeon & Wolf, 2009; Scartazza *et al.*, 2015), and the adjustment of the inorganic ion balance (Scartazza *et al*., 2015).

Metabolic profiling is an effective technology for the investigation of plant tissue chemical composition, especially in response to condition changes (Andersson-Gunnerås *et al*., 2006; Lisec *et al*., 2006; Yeh *et al*., 2006; Shi & Li, 2012; Zrig *et al.*, 2018). However, research on metabolites and the corresponding seasonal variations of *BP* phloem sap is limited. It is commonly known that tree physiology is regulated by both endogenous and exogenous factors. In particular, the most important exogenous regulatory factors (temperature, illumination, and precipitation) vary according to the month. In addition, phloem sap is secreted from laticifer cells in the tree phloem tissue and the laticifer cell is divided yearly from cambium (Angyalossy *et al*., 2016). Numerous studies have demonstrated the seasonal dynamic changes of the anatomical structure during the formation of phloem (Evert & Murmanis, 1965; Tucker & Evert, 1969; Antonova & Stasova, 2006; Gričar & Čufar, 2008; Čufar *et al*., 2011; Jyske *et al*., 2015; Dong *et al*., 2016).

In the current study, we hypothesize the existence of seasonal changes on the chemical composition and concentration of *BP* phloem sap. The specific aims are as follows: i) To investigate the chemical compositions in *BP* phloem sap via Gas chromatography-mass spectrometry (GC-MS); and ii) to reveal the seasonal changes of these compounds in Nanjing city, China during the 2016 growing season.

Material and Methods

Phloem sap collection: Five healthy *Broussonetia papyrifera* trees were selected from the campus of Nanjing Forestry University (NJFU) (32º04'57.4'' N, 118º49'00.3'' E), Nanjing, China. Nanjing's climate is typical subtropical monsoon climate and the mean annual

temperture and precipitation is 15.4°C and 1062 mm, respectively. The average diameter at breast height of the trees was measured as 17.6 cm and the average tree age as 11.5 years. Phloem saps were collected between 10 a.m. to 12 p.m. on March 28, April 22, May 20, June 15, July 22, September 20, October 21, and November 15 in 2016. Sampling was straightforward as the phloem saps are generally stored in axial laticifer cells. Fig. 1 presents the cell types of the *BP* phloem. In general, the *BP* phloem consist of sieve tubes, laticifers, phloem rays, phloem fibres, and crystals. Even though in the region of nonconducting phloem featured with collapsed sieve tubes, latidifer cells still scatter intact (Esau & Cheadle, 1984; Evert, 2006; Angyalossy *et al.*, 2016). The sampling procedure is summarized as follows. Following the peeling of the outset bark (approximately 2 cm^2), the phloem was gashed with a disinfected scalpel at a 45° angle to the tree growth direction. The saps were carefully placed into prepared tubes, immediately frozen using liquid N_2 and stored at -20°C in refrigerator for future use. In order to avoid any interactions between the sap samples of different months, sampling was performed using a spiral position on the tree trunk at 10 cm intervals for all directions.

Sap pre-treatment: Phloem saps were extracted by applying a mixture of methanol, chloroform, and deionized water (Lisec *et al.*, 2006; Shi & Li, 2012). The saps were then finely grinded under liquid N_2 and 50 ± 3 mg of the sample was mixed with 1 mL methanol (-20°C pre-chilled) in a 1.5 mL centrifuge tube. Following this, 50 µl ribitol (CAS No.488-81-3, 2 mg mL^{-1} in ddH$_2$O), used as the internal standard for the quantitative analysis of the compounds, was added and the mixture was incubated for 20 min at 70°C and 950 rpm min^{-1}. Following 15 min of centrifuging at 10000 rpm min^{-1}, 500 µl supernatant was transferred to a new tube and mixed with equal volumes of chloroform (-20°C pre-chilled) and deionized water (4°C pre-chilled), and incubated for 20 min at 37°C and 950 rpm min^{-1}. Following 15 min of centrifuging at 4000 rpm min^{-1}, approximately 500 µl supernatant was transferred to a new tube and stored at -20°C for further testing. Ribitol and extraction reagents were purchased from Sigma-Aldrich (USA) and Nanjing chemical reagent Co., Ltd, China, respectively.

Derivatization and GC-MS: The frozen pure phloem sap samples were vacuum dried at -60°C and derivatized with *N*-methyl-*N*-trimethlsilyl-trifluoroacetamide (MSTFA) (CAS No.24589-78-4). Briefly, the dried samples were mixed with 50 µl Methoxyamine hydrochloride (CAS No.593-56-6, dissolved in pyridine, CAS No.110-86-1, 20 mg ml^{-1}) and incubated for 2 hours at 37°C and 950 rpm min^{-1}. A total of 100 µl MSTFA (containing 20 µl ml^{-1} alkanes) was then added and centrifuged at 37°C and 950 rpm min^{-1} for 30 min. The samples were then stored overnight at room temperature. All the derivatization reagents were purchased from Sigma-Aldrich (USA). A total of 0.4 µL of the derivatized sample was injected into the GC-MS (TRACE DSQ, USA) system fitted with a DB-5MS column (30 m × 0.32 mm × 0.25 µm). A flow rate of 1.0 mL min^{-1} was used for the He (99.999%) carrier gas. The parameters used for the gas chromatography testing are described as follows. The initial temperature was held at 50°C for 1 min, and subsequently increased to 300°C at a rate of 10°C min^{-1} for 5 min. Both the injector and the detector temperatures were set at 250°C. The EI ion and ionization voltages were pre-set at 70 eV with a scan range of 20-500 amu.

MS peak identification and data analysis: The gas chromatograms were viewed using MS Workstation (Microsoft Corporation, v. 6.9.3). In addition, both the NIST (National Institute of Standard and Technology, USA) mass spectral database and related literature were used for the identification and classification of metabolites (Yeh *et al.*, 2006; Shi & Li, 2012). The relative content of metabolites was determined using the following equation:

$$C_x=[(A_x/A_i)\times0.05\times2 \text{ mg mL}^{-1}]/m_0 \text{ (mg g}^{-1}),$$

where C_x is the relative content of the identified metabolite, A_x is the peak area of the identified metabolite, A_i is the peak area of the internal standard and m_0 is the dry weight of the phloem tissue (Andersson-Gunnerås *et al.*, 2006, Shi & Li, 2012).

Results and Discussion

Chemical composition of the BP phloem sap: Metabolites in the exudates of the *BP* phloem were analysed via GC-MS following their derivatization using MSTFA. A total of 38 metabolites were identified and their concentrations were determined based on their mass and the phloem tissue dry weight. Fig. 2 demonstrates the mean total concentrations of the metabolites and the percentage composition of the *BP* phloem sap. The total metabolite concentration varied according to the month, with the maximum (44.59 mg g^{-1}) observed in March and minimum in (6.78 mg g^{-1}) in May.

As demonstrated in Fig. 2, all identified metabolites can be classified into organic acids, sugars, fatty acids and alcohols. In particular, organic acids and sugars made up, on average, 86.6% of the overall metabolite concentration of the *BP* phloem sap over all growing months. Beside to the inter physiological caused of tress, we speculate that the differences in meatbolite content are also related to detection techniques. Previous research has highlighted the ability of the MSTFA derivatized method to detect a wide range of sugars and organic acids (Villas-Boas *et al.*, 2006). Fatty acids and alcohols were observed to be abundant in the *BP* phloem sap exudates, representing 1-7% of the total metabolites during all growing months. Other metabolites, such as the PI3-kinase inhibitor and silanamine, were also identified, compromising less than 1% of the total. Variations in relative metabolite abundances were observed to depended on the growing month. For example, the highest organic acid percentage was observed in May (66%) and the lowest in November (22%). In comparison, the lowest percentage of sugars was detected in May (17%) and the highest in November (72%). Furthermore, the maximum fatty acids and alcohols percentage was observed in September (7%).
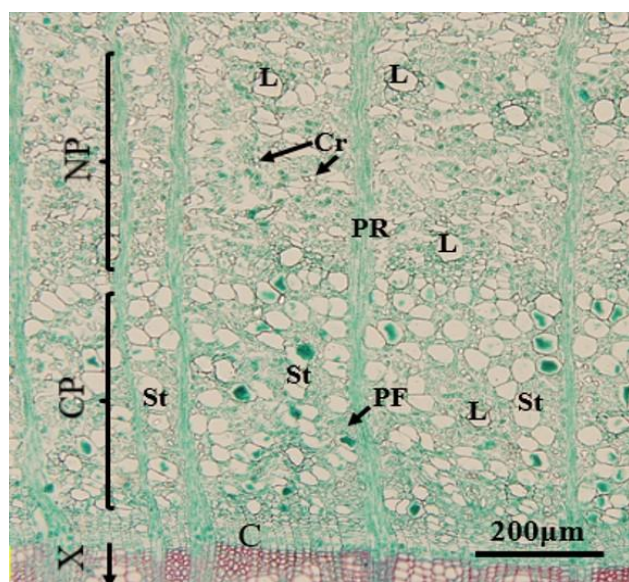
Fig. 1. Cross section of *BP* phloem collected in October, 2016. C: cambium; X: xylem; L: laticifers; CP: conducting phloem; NP: nonconducting phloem; Cr: crystals; PR: phloem rays; PF: phloem fibers; St: sieve tubes. Bar is 200μm.
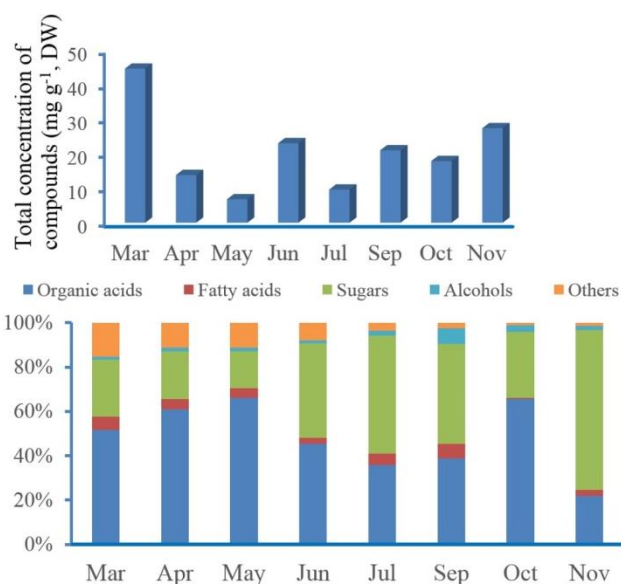

Fig. 2. Seasonal variation of total concentration of metabolites in *BP* phloem sap and the percentage of composition of the major groups. Mean value was used for drawing.

Seasonal variations in metabolites: Tables 1 and 2 report the chemical composition and content variations across the 2016 growing season, derived using the results from the qualitative and quantitative GC-MC analysis of all identified metabolites.

Organic acids: A total of 16 organic acids were detected in the *BP* phloem sap, with their compositions and contents varying with growing month (Table 1). Quininic acid was observed to be the most abundant organic acid, representing up to 86.3% of all organic acids for all growing months. More specifically, the maximum content of quininic acid (19.643 mg g^{-1}) was detected in March (Table 1), yet the highest percentage in November (Fig. 3). The content of the other metabolites exhibited a similar seasonal variation trend. Note that quininic acid, which is used as an astringent and starting material for the synthesis of new pharmaceuticals, can also be purified from *Eucalyptus globulus* (Santos *et al*., 2011), cinchona bark, and other plant products. Galactaric acid, a precursor for the synthesis of adipic acid (Li *et al*., 2014), was observed as the second most abundant organic acid in the *BP* phloem sap, with an average percentage of 3.6% of all organic acid contents. Both the maximum content (0.737 mg g^{-1}) and percentage (7.1%) of galactaric acid were detected in June. However, galactaric acid was not detected in September. Citric acid, an additional organic acid present in the *BP* phloem sap, exhibited peaks in the content and percentage (0.575 mg g^{-1} and 5.6%, respectively) in June, however, it was not detected in July, October and November (Table 1). Citric acid is an important industrial organic acid and is widely employed as an acidifier, flavouring and chelating agent, with more than a million tons manufactured each year. The seasonal variations in the citric acid content can be attributed to its role as an intermediate in the tricarboxylic acid cycle (TCA) (Fernie *et al*., 2004),

which itself is principally related to tree physiology at different growth stages. In addition, lactic acid was detected for all growing months, with a peak in content (0.559 mg g^{-1}) observed in March, and maximum percentage in October. Lactic acid is generally employed in food additives, pharmaceuticals and cosmetics. Additional organic acids (e.g. oxalic acid, glycolic acid, and propanedioic acid) were detected at percentages less of than 2% of total organic acids, with several present only in specific growing months (Table 1).

Sugars and other metabolites: Natural sugars are fundamental for the photosynthetic process in trees, and are precursors for tree physiological traits and the biosynthesis of other organic compounds. Sucrose, D-fructose, D-glucose, D-psicose, and D-lactulose were all detected in the *BP* phloem sap. The maximum total sugar content (19.761 mg g^{-1}) was observed in November and the minimum (1.123 mg g^{-1}) in May (Table 2). Similar to the organic acids, the content and percentage of individual sugars varied with the growing month. D-fructose was the most abundant sugar, representing 40% of the soluble sugars in March, July, October, and November (Fig. 4). Sucrose was the most abundant sugar in the remaining 4 growing months. D-fructose content exhibited a maximum (7.614 mg g^{-1}) and minimum (0.134 mg g^{-1}) in November and May, respectively. Similarly, the D-glucose content peaked in November, with the minimum value reached in May. The phloem sap collected from September exhibited the greatest sucrose content at 7.344 mg g^{-1}, almost 80% of all sugars for this month (Table 2 and Fig. 4). Previous research has demonstrated the ability of sucrose synthase to convert sucrose into glucose and fructose (Babb & Haigler, 2001). Moreover, the abundance of sugars in phloem is linked to the presence of phloem sap-sucking insects (Douglas, 2006; Kehr, 2006).

Table 1. Seasonal variations in the contents of organic acids detected in *BP* phloem sap (n=5). Unit: mg g⁻¹.

Compounds	March	April	May	June	July	Sept.	Oct.	Nov.
Lactic acid	0.559±0.062	0.128±0.016	0.068±0.008	0.052±0.005	0.031±0.007	0.230±0.039	0.447±0.053	0.078±0.012
Oxalic acid	0.157±0.032	---	0.049±0.006	---	0.025±0.002	0.120±0.039	0.079±0.018	0.049±0.013
Glycolic acid	0.144±0.029	0.054±0.011	0.041±0.004	0.057±0.011	---	0.016±0.001	0.034±0.010	---
Propanedioic acid	0.079±0.018	0.078±0.025	0.050±0.003	0.191±0.042	---	0.024±0.003	---	---
3-Hydroxyisovaleric acid	0.392±0.063	0.159±0.047	0.095±0.009	0.130±0.033	---	---	---	---
Butanedioic acid	0.192±0.025	---	0.126±0.023	0.052±0.007	---	---	---	---
Benzoic acid	---	---	0.018±0.000	---	---	0.030±0.002	0.037±0.009	---
2-Butenedioic acid	---	---	0.215±0.048	0.085±0.016	---	---	---	---
Malic acid	0.486±0.071	0.070±0.002	0.072±0.012	0.105±0.027	0.019±0.000	---	0.041±0.016	---
Citric acid	0.235±0.039	0.353±0.039	0.121±0.036	0.575±0.055	---	0.110±0.035	---	---
Ribonic acid	---	0.190±0.022	0.048±0.009	0.239±0.038	0.050±0.004	---	---	---
Shikimic acid	0.156±0.018	---	0.217±0.059	---	---	---	---	---
Quininic acid	19.634±2.336	6.915±0.319	3.106±0.116	7.575±0.407	3.148±0.085	7.576±0.526	10.842±1.338	5.662±0.527
L-Threonic acid	0.094±0.015	0.069±0.025	0.038±0.004	0.219±0.036	---	---	---	---
5-O-Feruloylquinic acid	0.317±0.068	---	0.117±0.020	0.447±0.052	---	---	---	0.035±0.005
Galactaric acid	0.534±0.092	0.398±0.051	0.095±0.018	0.737±0.049	0.153±0.028	---	0.206±0.045	0.152±0.033
Total (mean value)	22.980	8.416	4.478	10.467	3.426	8.107	11.687	5.977

Values calculated by dividing metabolite concentration phloem tissue dry weight. Values given as mean ± SD (n=5). "---" denotes not detected.

Table 2. Seasonal variations in the sugars and other metabolites detected in *BP* phloem sap (n=5). Unit: mg g⁻¹.

Compounds	March	April	May	June	July	Sept.	Oct.	Nov.
D-Fructose	6.524±0.368	0.464±0.076	0.134±0.062	1.289±0.094	2.256±0.117	0.491±0.094	2.198±0.118	7.614±1.039
D-Glucose	4.002±0.207	0.653±0.085	0.139±0.055	1.354±0.083	1.915±0.105	0.599±0.069	1.392±0.301	7.147±1.146
Sucrose	0.997±0.093	1.166±0.117	0.648±0.073	3.533±0.136	0.932±0.075	7.344±1.225	1.191±0.057	4.406±0.317
D-Psicose	---	0.231±0.045	---	2.161±0.226	---	---	---	---
D-Lactulose	---	0.448±0.063	0.210±0.054	1.512±0.110	---	1.011±0.095	0.499±0.082	0.593±0.062
Total Sugars (mean value)	11.754	2.962	1.123	9.851	5.103	9.445	5.282	19.761
Palmitic acid	1.322±0.115	0.262±0.059	0.164±0.038	0.195±0.042	0.159±0.038	0.338±0.073	0.087±0.022	0.194±0.056
Stearic acid	0.829±0.064	0.154±0.044	0.079±0.021	0.116±0.019	0.061±0.026	0.083±0.020	0.031±0.006	0.069±0.014
1-Monopalmitin	0.292±0.037	0.124±0.038	0.033±0.006	0.112±0.031	---	---	---	---
Octadecenoic acid	---	---	---	---	0.283±0.047	0.960±0.091	---	0.455±0.072
Glycerol monostearate	0.229±0.055	0.095±0.012	0.032±0.000	0.151±0.028	---	---	---	---
D-Pinitol	0.123±0.021	---	---	0.132±0.021	---	0.049±0.022	---	---
Myo-Inositol	0.315±0.048	0.128±0.047	0.045±0.008	0.519±0.099	0.148±0.039	0.261±0.034	0.034±0.016	0.169±0.048
PI3-Kinase Inhibitor	0.121±0.036	0.059±0.005	0.040±0.002	0.069±0.017	---	0.115±0.056	---	---
Chlorogenic acid	3.427±0.229	0.111±0.031	---	0.390±0.059	---	---	---	0.058±0.013
Chelerythrine	---	---	---	---	0.142±0.044	---	---	0.032±0.002
Pentasiloxane	0.089±0.021	---	0.030±0.001	0.057±0.014	0.014±0.000	0.062±0.027	0.024±0.009	0.019±0.000
Silanol, trimethyl-, phosphate (3:1)	2.736±0.205	1.248±0.115	0.622±0.072	0.742±0.092	---	---	---	---
silanamine,	0.121±0.062	---	0.026±0.004	0.034±0.008	0.039±0.003	0.094±0.033	0.081±0.025	0.046±0.007
3-Acetamid-ocoumarin	---	---	---	---	---	---	0.043±0.016	0.064±0.011
1-Hexanol	0.099±0.037	---	0.019±0.001	---	0.033±0.001	---	0.067±0.021	0.048±0.005
Linalool	---	---	---	---	0.183±0.029	1.175±0.085	0.050±0.006	0.411±0.063
Glycerol	---	---	---	---	---	0.275±0.062	0.456±0.069	0.061±0.017

Values calculated by dividing metabolite concentration by phloem tissue dry weight. Values given as mean ± SD (n=5). "---"denotes not detected.

Two fatty acids, palmitic acid and stearic acid, were detected in the *BP* phloem sap over all growing months (Table 1), with peaks in both acids observed in March. Pascual *et al*., (2017) reported that palmitic acid is able to strongly boost the metastatic potential of CD36+ in mouse models of human oral cancer cells (Pascual *et al*., 2017). Stearic acid is a chemical industrial resource and is wildly used in the production of cosmetics and lubricants. In addition, glycerol monostearate, 1-monopalmitin, and octadecenoic acid were detected in the *BP* phloem sap for several months. In particular, values of glycerol monostearate, a food additive and control release agent in pharmaceuticals, reached 0.229 mg g⁻¹ and 0.151 mg g⁻¹ in March and June, respectively.

Alcohols are also a key substrate and intermediate product in the metabolism cycle. Myo-inositol, an important intermediate in plants, has proven to have positive physiological effects on humans (McLaurin *et al*., 2000; Sanz *et al*., 2004). In the current study, myo-inositol content peaked (0.519 mg g⁻¹) in June, and varied across other growing months. Moreover, minimal amounts of D-pinitol were detected in March, June, and September. Numerous studies have reported D-pinitol as a bioactive ingredient in biological and medical activities (Bianchi *et al*., 2015; Chen *et al*., 2014).

Interestingly, the PI3 kinase inhibitor, an anti-cancer and anti-inflammation drug (Neri *et al*., 2003; Crabbe 2007; Wu *et al*., 2009; Kurtz & Ray-Coquard, 2012), was detected in the *BP* phloem sap. In particular, maximum PI3 kinase inhibitor amounts were observed in March and September (0.121 mg g⁻¹ and 0.115 mg g⁻¹, respectively). Chlorogenic acid, used to reduce inflammation and blood pressure (Onakpoya *et al*., 2014, Tajik *et al*., 2017), was also detected in the *BP* phloem sap, peaking in March (3.427 mg g⁻¹) (Table 2). In addition, Linalool content, with a proven anti-fungal ability (Vila *et al*., 2002), was at its maximum (0.411 mg g⁻¹) in November. Lastly, the concentration of Chelerythrine, a benzophenanthridine alkaloid with potent anti-inflammatory effects in vivo (Lenfeld *et al*., 1981), was observed as 0.142 mg g⁻¹ in July.
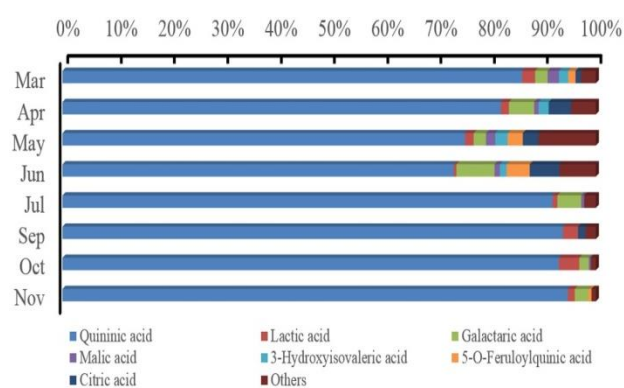
Fig. 3. Percentage composition of organic acids in the *BP* phloem saps and their season changes.

Fig. 4. Percentage composition of sugars in the *BP* phloem saps and their season changes.

Conclusions

The chemical composition of the exudates from *BP* phloem were analyzed via GC-MS following their derivatization using MSTFA. A total of 38 metabolites were detected, with total concentrations varying according to growing month. The highest total metabolites concentration was observed as 44.59 mg g^{-1} in March and the lowest as 6.78mg g^{-1} in May. Organic acids and sugars were the most abundant metabolites in the *BP* phloem sap for all growing months. In addition, small amounts of fatty acids and alcohols were also detected in the *BP* phloem exudates. Seasonal variations in the content of metabolites were distinct amongst individuals. More specifically, organic acid content peaked (22.98mg g^{-1}) in March, with quininic acid observed as the most abundant organic acid (86.3%). In November, the maximum sugar content reached 19.76 mg g^{-1}, with D-fructose, D-glucose, and sucrose as the major soluble sugars. Interestingly, some metabolites, such as the PI3 kinase inhibitor, Chlorogenic acid, Chelerythrine, and palmitic acid, which exhibit anti-cancer and anti-inflammation bioactivity, were also detected and varied across growing months. The metabolite analysis confirmed that *BP* phloem sap can be employed as an important resource for the synthesis of pharmaceutical and human health products.

Acknowledgements

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