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Perilla frutescens (L.) Britton: honeybee forage and preliminary results on the metabolic profiling by NMR spectroscopy

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Abstarct: Nuclear magnetic resonance (NMR) spectroscopy has emerged as a technology for metabolite characterisation of both foods and plants. NMR technique allows to analyse metabolite content in a single experiment, in a non-destructive way and with a very simple sample preparation. This study characterises the metabolites of *Perilla frutescens* var. *crispa* leaf and flower for the first time by NMR. Our results showed higher metabolite content in leaves compared to flowers, highlighting the presence of amino acids, organic acids, saccharides and large amounts of aromatic compounds, mainly in the form of rosmarinic acid. Moreover, we cultivated Perilla, an important medicinal plant native to Asia, in a low mountain environment in Italy, to continue its evaluation as a honeybee attractive species. Interestingly, even in this type of environment, Perilla has been confirmed to be a good bee plant for both nectar and pollen.

INTRODUCTION

In recent years, several studies on metabolite characterisation of foods and plants have been carried out (Schripsema, 2010). Among different analytical approaches, nuclear magnetic resonance (NMR) emerged as an elective and reliable technique for monitoring the metabolic profiles of plant tissues. The NMR approach is an advantageous analytic procedure for complex mixtures as it determines the metabolite content with a single experiment in a non-destructive way. Moreover, it requires a simple or no sample preparation, which makes the analysis of low demand in terms of experimental acquisition time. Several studies in the literature describe the metabolic profiling of plant extracts by NMR coupled with multivariate statistical data analysis. This approach allows the comparison of metabolite content among genotypes, between biotic/abiotic stress conditions or between different pedoclimatic growth conditions (Consonni, Cagliani, Stocchero, & Porretta, 2009, 2010; Fumagalli et al., 2009; Gavaghan et al., 2011; Genga et al., 2011; Marina et al., 2010; Mattana et al., 2005). In this context, NMR represents a very suitable technique for carrying out the analysis of complex mixtures with the simultaneous detection of several chemical compounds (flavonoids, amino acids, organic acids, alkaloids and so forth). The application of this technique is spreading to medicinal plants, which are of increasing interest in a world searching for alternatives to chemicals as a route to well-being and human health.

In order to use plant bioactive molecules appropriately, it is necessary to deepen our knowledge on the properties of these plants. *Perilla frutescens* (L.) Britton, an edible species native to eastern Asia, is increasingly important for its antioxidant (Duelung, Amiot, Fillon, & Mouritsen, 2012; Meng, Lozano, Bombarda, Gaydou, & Li, 2006; Meng, Lozano, Gaydou, & Li, 2009; Saita et al., 2012), anti-cancer (Asif, 2011; Osakabe, Yasuda, Natsume, & Yoshikawa, 2004; Ueda, Yamazaki, & Yamazaki, 2003) and anti-allergic properties (Makino et al., 2003; Osakabe, Takano, et al., 2004; Ueda, Yamazaki, & Yamazaki, 2002; Zekonis, Sadzeviciene, Simoniene, & Kevelaitis, 2008). These effects derive mainly from its high content of polyphenols, anthocyanin, flavonoids, luteolin, rosmarinic acid, omega-3,6,9 fatty acids, limonene and perillaldehyde. On the basis of these beneficial properties, this plant is now cultivated in many regions of the world and recently also in Italy. In China and India, its native regions, Perilla is found at altitudes as high as 1200 m asl (Roecklein & Leung, 1987). It has also been cultivated successfully in Finland using black plastic mulch (Tanaka, Kim, & Yu, 1997). Studies carried out in the Piemonte region (Italy) focused on the beekeeping implications of this plant, which resulted in a new source for *Apis mellifera* L. (Barbieri & Ferrazzi, 2011). The goals of this research were to learn more about the bee value of this species in a low mountain zone of northwestern Italy, to analyse the metabolic content of *P. frutescens* (L.) Britton var. *crispa* (Bentham) Deane ex Bailey forma *purpurea* Makino leaf and flower using NMR spectroscopy and to find its therapeutic potential.

Given the wide use of Perilla leaves and flower heads in Far East populations (Yu, 1997) and the growing demand for plant foods with health benefits from the European population, *P. frutescens* might be used as a new food source in Europe.

RESULTS AND DISCUSSION

Pests and pollinators

P. frutescens plants did not suffer pest attacks, except for limited damage to leaves caused by Lepidoptera: Geometridae larvae. Since the start of flowering, in the first week of September, *P. frutescens* was intensively visited by *A. mellifera*, which gathered nectar and less frequently the whitish pollen. The frequency of foraging was high, up to 15 honeybees/m^2/5 min.

On the basis of these data, *P. frutescens* was confirmed to be a good bee plant for both nectar and pollen in this low mountain environment, as found in plain areas (Barbieri & Ferrazzi, 2011), but any other insect pollinator was not observed.

The late flowering period allowed no honey production; however, Perilla did provide winter provisions to assist in honeybee survival. Abundant supplies of honey are essential and conspicuous pollen stocks provide honeybee colonies with a better wintering (Mattila & Otis, 2007).

NMR analyses

^{1}H NMR spectra of leaves and flowers are presented in Figure 1(A)–(C). Spectra were scaled to external common standard to be properly compared. Resonance assignments of chemical compounds reported on spectra were achieved by bi-dimensional homo-correlated total correlation spectroscopy (TOCSY), hetero-correlated experiments like heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) in comparison to reference compounds. The aliphatic region of leaf and flower samples (Figure 1(A)) showed the presence of several amino acids, alanine (Ala), valine (Val), threonine (Thr) and asparagine (Asn), as well as some organic acids like succinate (Succ), malate (Mal), citrate (Cit) and gamma amino butyric acid (GABA). In this region, fatty acids (mostly linolenic and stearic) were also observed (Figure 1(A)). In this part of the ^{1}H NMR spectrum, no particular differences have been detected between leaf and flower samples. In the anomeric region of the spectra reported in Figure 1(B), sucrose (Suc), α- and β-glucose (α-Glu and β-Glu) and fructose (Fru) signals were detected. In this part of ^{1}H NMR spectrum, small differences have been observed between leaf and flower extracts. In particular, leaf samples showed signals close to the anomeric proton of sucrose resonating at 5.30 ppm, defined as S1, and close to α-glucose resonating at 5.09 ppm and defined as S2 (Figure 1(B-b)). These two additional resonances were likely due to saccharide derivatives.

In the aromatic region of both plant tissue extracts (Figure 1(C)), we detected mainly rosmarinic acid (Ros), which is reported to be one of the major bioactive compounds in Perilla leaves (Makino et al., 2003). Formic acid (For) was also detected. Additionally, resonance peak (Ald) at 9.25 ppm due to aliphatic aldehyde was revealed. Two not-yet-identified aromatic compounds, indicated as U1 and U2, were observed only in leaf extracts. These signals might account for phenolic derivatives. Although flavonoids are usually observed in fresh tissues (Gu, Wu, & Wang, 2009), we

did not detect apigenin, luteolin or chrysoeriol, most likely as a consequence of the sample treatment.

Our preliminary results suggested that different classes of chemical compounds can be detected simultaneously in a single experiment. This could also be performed quantitatively, allowing evaluation of the relative content of different compounds, with particular attention to those responsible for antioxidant or beneficial effects. The assessment of the entire metabolic profiling of *P. frutescens* will highlight its nutraceutical value and its therapeutic range.

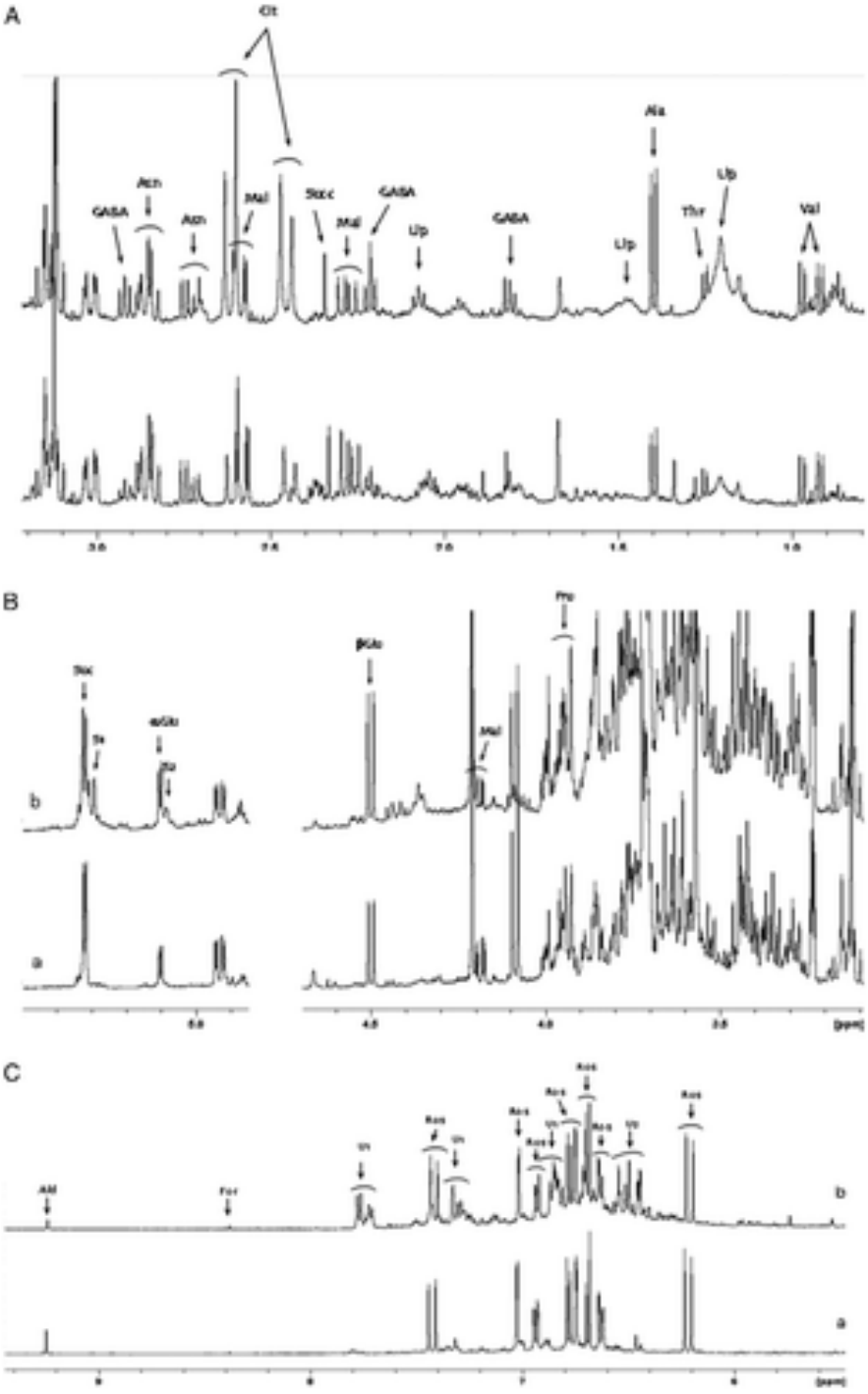

Figure 1 Expansion of the aliphatic (A), anomeric (B) and aromatic (C) region of ^{1}H NMR spectra of alcoholic extracts of Perilla: (a) flowers and (b) leaves.

EXPERIMENTAL

Plant material

Seeds of *P. frutescens* (L.) Britton var. *crispa* (Bentham) Deane ex Bailey forma *purpurea* Makino provided by a Piemonte farmer were sown in the spring of 2011; the seedlings were then transplanted at an in-row distance of 30 cm from each other in several small plots at Mattie, 730 m asl, located in the alpine Susa Valley near Torino (Piemonte, Italy). Some plants were harvested to dry them for NMR analysis. The drying process took place in darkness at room temperature (22 ± 2°C).

Perilla was collected during two different vegetative phases of the plants, during flowering (sample 1) and during fruiting (sample 2). The harvesting was made from Mattie (Susa Valley, northwestern Alps, Italy). Voucher specimens were deposited at the Herbarium of the Department of Life Sciences and Systems Biology of Torino University (viale Mattioli 25, Torino, Italy; voucher no. 2654, TO-HG section Herbarium generale).

Pests and pollinators' investigations

The plants were monitored during their growth to detect the presence of pests and, in the flowering period, to detect pollinator species and their behaviour. The count of pollinators (number of individuals/m^2 of flowering canopy surface/5 min) visiting flowers and the assessment of their foraging activity (products collected: nectar and pollen) were carried out according to the protocol developed by Ferrazzi (1974).

Plant extraction

The extraction procedure was performed according to Kim, Choi, and Verpoorte (2010) with slight modifications. Air-dried *P. frutescens* leaves and flowers were ground under liquid nitrogen and freeze-dried for 1 day. Dried materials of 70 mg were extracted with buffered (KH_2PO_4) aqueous methanol (D_2O/MeOH d_4) in a 1:1 ratio. Samples were vortexed for 1 min at room temperature and centrifuged for 10 min (17,000g). Supernatant of 600 µL were transferred into the 5-mm NMR tube.

NMR analysis

Spectra were recorded at 300 K with both 500 and 600 MHz Bruker Avance spectrometers (Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany). ^{1}H 1D spectra were acquired with pulse sequences incorporating a solvent suppression scheme based on low-power radiofrequency pulse saturation. 2D homo- and hetero-nuclear spectra (TOCSY and HSQC, HMBC) were also acquired for resonance assignment of chemical compounds. All spectra were recorded using TOPSPIN software (Bruker Biospin).

CONCLUSIONS

In conclusion, leaf and flower samples did show the presence of amino acids, organic acids, saccharides and aromatic compounds (mainly Ros). In particular, leaves displayed higher content levels of each of these components compared to flowers. Additionally, two not-yet-identified aromatic compounds were observed only in leaf. The qualitative comparison between NMR spectra of leaf and flower indicated that leaf is the most interesting tissue in terms of metabolite content, especially in aromatic compounds. Moreover, this research brings new evidence about the honeybee importance of this medicinal species recently introduced in Italy. Perilla is both a nectariferous and a polliniferous plant.

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