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Differences in the chemotype of two native spearmint clonal lines selected for rosmarinic acid accumulation in comparison to commercially grown native spearmint

B. Narasimhamoorthy*, L.Q. Zhao, X. Liu, W. Yang, J.A. Greaves

Specialty Crops Department, Kemin Industries, Inc., 2100 Maury Street, Des Moines, IA 50317, United States

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ABSTRACT

Rosmarinic acid (RA) is a water soluble antioxidant present in many Lamiaceae species at varying levels, including spearmint (*Mentha spicata* L). Two proprietary spearmint clonal lines, KI110 and KI42, with elevated levels of RA (designated Hi-RA) are currently being grown in Indiana for the commercial extraction of RA. The two Hi-RA clonal lines were selected from a native spearmint genetic background and are distinct in their phytochemical composition relative to native spearmint that is commercially grown for purposes of extraction of volatile oils for flavoring applications ("commercial spearmint"). The aim of this study was to describe the major compositional chemotypic differences between the proprietary Hi-RA native spearmint clonal lines relative to a commercial native clonal line of spearmint grown in Indiana. Leaf tissue samples of KI110, KI42 and the commercial spearmint clonal line were collected from field locations in Indiana during June 2013 for RA quantitation and chemical profiling of the essential oil fraction. RA levels in KI110 and KI42 were significantly higher than the commercial spearmint samples. GC–MS analysis revealed major compositional differences between the Hi-RA and commercial spearmint oils. Carvone was the dominant molecule in the commercial spearmint oil, constituting 54% of its oil content. In contrast carvone was below the detection levels in the oils of the two Hi-RA clonal lines, KI110 and KI42. Limonene, another notable constituent of commercial spearmint, was found in lower amounts in the Hi-RA mint clonal lines. Mint lactone and α-cubebene, the predominant compounds in the oil fractions of Hi-RA clonal lines KI110 and KI42, respectively, were either absent or present in insignificant amounts in the commercial spearmint. Differential amounts of myrcene and 1,8-cineole were observed in the three mint oil samples. The chemical fingerprints of the Hi-RA spearmint clonal lines clearly differentiate them from commercial spearmint and they should therefore be considered distinct, clonal lines of spearmint (*M. spicata*) with potential heath-promotive properties associated with KI110 and KI42 plant extracts that contain higher level of rosmarinic acid.

1. Introduction

Mint, belongs to the Lamiaceae family; genus *Mentha*, which comprises about 25–30 species. Of these, peppermints (*Mentha* × *piperita* L.) and two species of spearmints, 'Scotch' spearmint (*Mentha* × *gracilis* Sole) and 'Native' spearmint (*Mentha spicata* L.) are grown commercially in the U.S. (Zheljakov et al., 2010a,b) and many parts of the world (Lawrence, 2006). Peppermint and spearmints are important specialty crops valued for their essential oils used in many industries, including pharmaceuticals, cosmetics, food, and flavor (Mint Industry Research Council, 2014). Fresh and dried spearmint are also used in a variety of food and beverage applications to impart flavor. Rosmarinic acid (RA) is a potent phenolic antioxidant occurring at varying levels in the Mint family. Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenylacetic acid. It is also a secondary metabolite of various plant species including those of Lamiaceae. This molecule is also known to have unique properties, including antioxidant, antiviral, antibacterial, and anti-inflammatory (Alankar, 2009; Dhifi et al., 2013; Mazumder et al., 1997; Hooker et al., 2001) and neuroprotective activities (Braidy et al., 2014; Fallarini et al., 2009).

Spearmint is a perennial crop that can biosynthesize significant amounts of RA and other phenolic molecules. RA content in naturally existing spearmint (*M. spicata*) clonal lines was previously reported to range from 7.1 to 58.1 mg/g on a dry weight (DW)

* Corresponding author. Tel.: +1 515 559 5591; fax: +1 515 559 5232.
E-mail address: Brindha.Narasimhamoorthy@kemin.com
(B. Narasimhamoorthy).

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basis (Wang et al., 2004; Kosar et al., 2004; Shekarchi et al., 2012). However, *M. spicata* clones biochemically selected *in vitro* for elevated levels of phenolics ranged from 20 to 67 mg/g DW (Fletcher et al., 2005a,b). One particular *M. spicata* clone, selected *in vitro*, was found to produce 87–118 mg/g of RA on a DW basis (Kott and Fletcher, 2008). In addition, mint plants are easy to cultivate, can sustain several harvests annually, and exhibits a rapid re-growth after each harvest.

The specialty crop improvement (SCI) department at Kemin, independently developed two proprietary native spearmint (*M. spicata*) clonal lines, KI42 and KI110, capable of accumulating >100 mg/g RA from an open-pollinated spearmint population *via* conventional selection techniques. As a more polar molecule, RA is present only in the aqueous extract of spearmint in contrast to the steam distilled essential oil fraction where no RA accumulates. The composition of spearmint (*M. spicata*) essential oil has been well studied. Quantitative and qualitative differences have been reported in essential oil composition within different chemotypes of *M. spicata* collections from different geographical regions (Al-Marzouqi et al., 2007; Chauhan et al., 2009; Hussain et al., 2010; Joshi, 2013; Orav et al., 2013; Zheljakov et al., 2010a,b,c). The predominant compound in commercially available spearmint oil is carvone {R-(−)-carvone}, which gives spearmint its distinctive aroma (Zheljakov et al., 2010a,b; Dhifi et al., 2013). Spearmint also contains substantial amounts of limonene and 1,8-cineol (Hussain et al., 2010; Chauhan et al., 2009; Zheljakov et al., 2010a,b). In addition, morphological differences between different chemotypes are evidenced in plant height, leaf characteristics (including color, dimension and smoothness) and the presence of trichomes (Al-Marzouqi et al., 2007; Zheljakov et al., 2010a,b).

Commercial spearmint and peppermint is currently limited to north of the 41st parallel in the U.S. because of longer days (>15 h) and grown solely for the purposes of oil extraction (Johnson, 2001). Spearmint and peppermint plants initiate flowers during long days when the plants produce higher amounts of essential oils (Langston and Leopold, 1954; Burbott and Loomis, 1967). Kemin's proprietary Hi-RA mint clonal lines, KI110 and KI42 are currently grown in north central Indiana where commercial spearmint is grown for oil extraction. It is important to differentiate the proprietary Hi-RA native spearmint clonal lines KI110 and KI42, from other commercial native spearmint grown in the same geographical area. Chemotypic profiling and molecular fingerprinting of the proprietary Hi-RA clonal lines will be an effective tool in the authentication of the Hi-RA spearmint clonal lines. In addition, chemotypic profiles will be used to differentiate extracts derived from Kemin's proprietary Hi-RA clonal lines from other commercially grown native spearmint extracts. The present study was conducted to compare and contrast the two proprietary, elite Hi-RA native spearmint clonal lines, KI110 and KI42, against typical, native commercial spearmint grown in Indiana on the basis of chemical fingerprinting of the essential oil fraction and measurement of RA accumulation in the aqueous fraction.

2. Materials and methods

2.1. Plant material

Hi-RA native spearmint clonal lines KI110, KI42 and commercial native spearmint were grown at a field location in north central Indiana, USA. The native commercial spearmint was a clonal line that had been grown in Indiana since the early 1950s. Leaf biomass from each clonal line was cut at ground level during June 2013 and taken to the laboratory to extract essential oil. Three smaller sub-samples of leaf and stem tissue from each of the clonal lines were dried using a patent-pending microwave drying method (Ruden et al., 2011) for RA quantitation. For each sample, RA was quantitated from a random sub-sample, expressed on dry weight basis and replicated three times. The remaining biomass from each clonal line was wilted for 24 h before essential oil extraction *via* steam distillation.

2.2. Essential oil extraction

Partially wilted stem and leaf tissue (500 g) samples were steam distilled for approximately 3 h using a 5-L Clevenger type steam distillation unit. Replicate oil samples (three replicates) were obtained from the commercial spearmint and KI110 while a single oil sample was obtained from KI42. Steam distillation was allowed to continue until oil production from each sample ceased which was typically after 1.5 h. Oil samples from each replicate of each clonal line were recovered, weighed and sent to the lab for chemotypic profiling. Spent biomass from each replicate of each clonal line was recovered and dried to obtain dry weight measurements. The essential oil yields were calculated as % of dry weight as an average of three extractions.

2.3. RA quantitation

Dried leaf and stem tissue was ground using an electrical mill (IKA A11 basic, Guangzhou, China). Accurately weighed 20 mg of freshly ground spearmint leaf and stem tissue was placed into a 2.0 mL centrifugal tube. 1.8 mL of the extraction solvent [20 mM KH_2PO_4 (adjust pH to 2.5 with phosphoric acid):ethanol (1:1)] was transferred to each tube and vortexed each for 1 min. Tubes were placed into an ultrasonic bath (KQ5200DE, Ultrasonic Instrument Company, Kunshan, China) for 10 min followed vortexing for 1 min. The tubes were then placed in a microcentrifuge (Eppendorf model 5418, Germany) and pelleted for 10 min at 10,000 rpm (at 11,200 rcf). The supernatant was filtered through 0.45 μm PTFE syringe filter before being poured into autosampler vials for HPLC analysis.

Agilent 1200 series HPLC modules were used with diode array detector, quaternary pump, autosampler, column heater, and online degasser. The column was a Merck Purospher STAR LP RP-18 endcapped HPLC column (250 × 4.6 mm, 5 μm), maintained at 35 °C. The mobile phase consisted of 0.1% o-phosphoric acid in water (Channel A) and 0.1% o-phosphoric acid in acetonitrile (Channel B). The gradient program was as follows; 0–7 min, linear gradient from 28% to 40.6% B; 7–7.2 min, linear gradient from 40.6% to 100% B; hold at 100% B until 8.2 min; 8.2–8.3 min, linear gradient from 100% to 28% B; hold at 28% B until 10 min. The flow rate was 1.0 mL/min; detection wavelength was 330 nm; and injection volume was 5 μL. Standard curve for rosmarinic acid was generated using externally sourced standard (Sigma–Aldrich) and injected into the HPLC. Peak area response for rosmarinic acid in the target samples were compared to the standard curve for relative quantitation purposes. RA content was expressed in mg/g of dried tissue from each sample. The mean RA content and standard deviation ($P < 0.05$) were computed using Microsoft EXCEL.

2.4. Gas chromatography-flame ionization detector (GC-FID) analysis

Essential oil samples from KI42, KI110 and commercial native spearmint were analyzed using a Agilent 6890N apparatus (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector (FID) and an HP-5 capillary column (30 m × 0.25 mm ID × 0.25 μm; Agilent Technologies, Hewlett-Packard, Palo Alto, CA). The flow of the carrier gas was nitrogen at 1 mL/min. The oven was programmed as follows: initial temperature 50 °C, increased to 150 °C at a rate of 3 °C/min; and then ramped to 250 °C at a rate of 15 °C/min. Both injector and detector temperatures were held at 250 °C. The injection volume was 1 μL of 10 mg/mL solution in