Chapter 25

Spearmint Plantlet Culture System as a Means to Study Secondary Metabolism

Brent Tisserat, Mark Berhow, and Steven F. Vaughn

Summary

Spearmint has one major monoterpene, (-)-carvone, that constitutes up to 90% of all the monoterpenes present. Likewise, the major phenylpropanoid—rosmarinic acid—in spearmint accounts for up to 70% of the phenylpropanoids produced from the plant. These two compounds are each produced by separate distinct biosynthetic pathways which provide an excellent opportunity to study the influence of a wide number of environmental and chemical conditions on secondary metabolism and plant growth. The techniques presented in this chapter employ 1 g of fresh weight material for each secondary metabolite analyses. Analysis of single compounds obtained from the two distinct metabolic pathways simplifies the interpretation of the metabolic results allowing for direct correlations of culture factors on secondary metabolism.

Key words: Carvone, Rosmarinic acid, Plantlet culture, Natural plant products, Phenylpropanoids, Monoterpenoids

1. Introduction

Natural plant products can be generally divided into three major biosynthetic groups: the terpenoids (about 25,000), the alkaloids (about 12,000), and phenylpropanoids and allied phenolic compounds (about 8,000) (1). Spearmint plantlets grown in vitro constitutively accumulate a significant amount of two compounds, carvone (a monoterpene) (2), and rosmarinic acid (a substituted phenolic acid) (3). (-)-Carvone constitutes 90% of the monoterpenes produced in the cultured spearmint plants whereas rosmarinic acid constitutes up to 70% of the phenolics produced in the plant. Cultured spearmint plants also accumulate varying
levels of several flavones and flavonols (4, 5). The production of these two compounds from two distinctly different secondary pathways makes the spearmint plantlet culture a very useful experimental system. The accumulation of secondary metabolites in mint plantlets is very responsive to cultural factors (6, 7). The occurrence of dominate metabolites simplifies the interpretation of the metabolic results allowing for a direct correlation of the cultural factors on essential oil production.

Mint plantlets are miniature plants as they have the same morphologic features and chemical compositions as soil grown plants. Monoterpenes, being volatile essential oils, are manufactured exclusively in the green shoot foliage (especially in the leaves) and are stored in the oil glands. Monoterpenes are not found in roots or in callus, although some altered terpenes may occur within the callus. Rosmarinic acid, in contrast, is found in all plant organs (8), and has been reported to be particularly high in hairy roots (9), cell suspensions, and undifferentiated callus (10, 11). Within the sterile environment we can study the influence of various environmental and nutritional factors on the growth of plantlets (i.e., primary metabolism) and secondary metabolism. Various forms of chromatography have been used to separate extracts prepared from plants (12, 13). The distinctive light absorption characteristics were used to identify and quantify plant phenolics and terpenoids (13). Current qualitative and quantitative work is done using gas phase-driven chromatography (GC) for volatile compounds, such as the monoterpenoids, and high-performance liquid phase chromatography (HPLC) for nonvolatile compounds. Natural plant products are typically extracted in a suitable solvent or mixture of solvents and separated, identified, and quantified in one operation by coupling the HPLC or GC to a detecting system, such as a light absorbance detector (ultraviolet [UV], visible, or fluorescence), refractive index (RI), evaporative light scattering detector, a flame ionization detector (FID), or a spectrometer capable of detecting different mass ions (MS).

GC-FID is a standard and proven method for the detection and quantification of volatile monoterpenes such as carvone (13). HPLC reverse phase methodology using UV-VIS absorption detectors is the workhorse for phytochemical analysis with compounds that have good chromophores (i.e., conjugated double bond systems and/or phenolic ring structures). Reproducible HPLC methodology has been used for a variety of phenylpropanoid compounds such as the flavonoids (14, 15). This methodology can also be easily adapted for substituted phenolic acids such as rosmarinic acid. Low cost mass-spectrometry (MS) systems and their associated computer software packages now allow for the characterization of mass ion patterns produced by electron-impact ionization or soft ionization sources with quadrupole, ion trap, or time of flight MS system (16).
The problem of reproducible results from sample to sample and lab to lab still depends on the key steps of sample preparation and extraction. To maximize analyte extraction from the mark, the sample should be dry and ground to as fine a powder as possible. Depending on the time frame and the number of samples involved, extraction may be made exhaustive by either extracting the sample multiple times or by an increased solvent to solid ratio with time and provide enough heat to maximize the solubilization of the phytochemicals of interest. This is often a fine line between the amount of time and heat needed to maximize the efficiency of extraction or chemical alteration of the native state of the phytochemicals. This situation should be properly examined prior to the implementation of any new analytical procedure. We have found that most phenolic compounds are fairly stable when extracted for 1–2 d in methanol:dimethyl sulfoxide, with an extraction efficiency above 90% using a single step extraction method where the ratio of solvent to solid sample is greater than 10–1.

2. Materials

2.1. Plantlet Culture

1. Murashige and Skoog (Medium (MS) (Sigma; St. Louis, MO) supplemented with: 0.4 mg/L thiamine HCl, 100 mg/L myo-inositol and 30,000 mg/L sucrose. Adjust agar MS pH to 5.7 ± 0.1 with 0.1 N HCl or NaOH before adding 8 g/L agar (Difco Laboratories; Detroit, MI). Liquid MS pH was adjusted to 5.0 ± 0.1.

2. Magenta containers: GA-7 polycarbonate box, 77 mm × 77 mm × 97 mm H and GA-7-3 polycarbonate box, 77 mm × 77 mm × 77 mm H and polypropylene couplers to join boxes (Sigma).

3. 100-mL Glass baby food jar, 62 mm diam × 66 mm H (Sigma; St. Louis, MO).

4. Polypropylene translucent Magenta B-cap drilled with a 28-mm diam hole and Magenta 2-way cap (Sigma) drilled in the center top with a 5-mm diam hole.

5. Stainless steel breather/vent filter, 1/8” NPT, 11.11 mm H × 11.11 mm diam; 100 µm filtration (McMaster-Carr; Chicago, IL).

6. Polypropylene washers and miniature barbed fittings, 1/8” in. NPT threaded (McMaster-Carr).

2.2. Carvone Analysis

1. Dichloromethane.
2. Jars wrapped with aluminium foil seals.
3. 0.45-µm nylon 66 filters.
4. Rotoevaporation apparatus.
5. Gas chromatograph equipped with a flame ionization detector (GC-FID).
6. Gas chromatograph equipped with electron impact mass selective detector (GC-MS).
7. Fused silica HP-5MS capillary column (0.25 µm film thickness, 30 mL x 0.25 mm ID).
8. He carrier gas.

2.3. For Rosmarinic Acid Analysis

1. Mortar and pestle.
2. 30 mesh metal sieve.
3. 15 x 45-mm glass vial and locking cap.
4. 2 mL methanol:dimethyl sulfoxide (DMSO) (1:1).
5. Sonic water bath.
6. 0.45-µm nylon 66 filter.
7. A gradient-capable HPLC system with autoinjector, solvent mixer, column oven, and photodiode array detector.
8. 250 x 4.6 ODS-5, 5 micron reverse phase C-18 column).
9. Pure RA sample (Chromadex; Santa Ana, CA).
10. Electrospray ionization source, ion trap mass spectrometer with a gradient capable HPLC system (autoinjector, pump, degasser, and photodiode array or UV/VIS detector).
11. Flowmeter (Cole-Parmer, Chicago, IL)

3. Methods

3.1. Plantlet Culture Methodology

1. Prepare mint plantlet stocks by culturing a single 3-cm long shoot per 25 x 150 mm culture tube on agar medium for 2 wk in order to initiate roots (see Note 1).
2. Preparation of the plantlet culture system involves the interlocking of two magenta containers via a polypropylene coupler. A separate media container, baby food jar, is positioned within the bottom Magenta box container. Modify a B-cap closure with centrally positioned 28-mm diam drilled hole and fitted with a 25 mm diam translucent 2-way closure pre-drilled with a 5-mm hole (Fig. 1).
3. Transfer plantlets into Magenta containers containing an internal baby food jar with the root portion immersed in 47 mL of nutrient medium.

4. Physically separate media within the internal baby food jars from the plantlet foliage entirely. Any uptake of media occurs through the immersed roots. In addition, media is shielded from the atmospheric portion by polypropylene closure.

5. Treat atmospheric portion of the container with air applications administered through a stainless steel breather/filter fitted in the top magenta container (Fig. 1).

6. For demonstrative purposes of employing this system, give plantlets 0 and 16 times air applications for 4 wk. Air is generated by an aquarium pump, and monitor and maintain with a flowmeter and apply equally spaced intervals of 30 min at 100 mL/min (see Note 2).
3.2. Carvone Analysis

1. For analysis, excise the terminal 4-cm portion of 3–5 mint plantlets and mix together, and then sample by weighing out 1 g and immersing it in 15 mL dichloromethane for 72 h (see Note 3).

2. Filter samples with a 0.45 μm nylon 66 filter.

3. Analyze extracts on a GC-FID. An example GC-FID trace run is presented in Fig. 2.

4. Determine peak identities by running samples on a GC-MS system and compare the MS peaks with standards.

5. Follow GC-FID or GC-MS chromatography operation parameters: Splitless injection mode temperature programmed from 70 to 250°C at 10°C/min; He carrier gas flow rate 1.1 mL/min; and the injector temperature set at 250°C.

6. Prepare standard curves monthly by weighing pure (-)-carvone into a vial and diluting with dichloromethane to 1 mg/mL. Three serial dilutions are prepared and injected on the GC. The GC calibration software is used to prepare a standard curve and report the unknown carvone peak data as mg/mL.

7. Essential oil analysis is reported as carvone/ treatment (mg carvone /g FW).

3.3. Rosmarinic Acid Analysis

1. Excise the terminal 4-cm portion of 3–5 mint plantlets, mix together, and then weigh 1 g sample.

Fig. 2. GC-FID trace of a spearmint extract injection.
2. Plant materials are then freeze-dried for at least 24 h (see Note 4).
3. Grind samples into a fine powder with a mortar and pestle, pass through a number 30 mesh sieve to remove stem materials.
4. For analysis, samples typically 0.1 g are placed in a glass vial with 2 mL DMSO:methanol (1:1). Cap vials and wrap with a sealing tape.
5. Samples are sonicated at 42 Hz for 15 min at room temperature.
6. Allow samples to stand overnight at room temperature.
7. Remove aliquot samples from the vial and then filter through a 0.45-μm nylon 66 filter.
8. Samples are run on a dual pump HPLC system with an autoinjector, and with the column oven set to 40°C.
9. Monitor peaks using a photodiode array detector running under a chromatography software control program.
10. Initial solvent conditions are 20% methanol and 80% 0.01 M phosphoric acid in water.
11. Effluent is monitored at 285 nm; after injections of 15 μL, the column is held at the initial conditions for two min, then developed to 100% methanol in a linear gradient over 55 min.
12. Standard curves are prepared from a series of dilutions of freshly prepared pure RA dissolved in methanol, 1 mg/mL, 2.78 nM/μL, and a series of serial dilutions are made. After running the standards at the same injection volume as the unknowns, the extinction coefficient based on peak absorbance area versus concentration in nM is determined. Alternatively, use the calibration program in the chromatography software only if reliable RA retention times can be obtained over time.
13. The spectra and retention time of the RA in the mint extracts is identical to that of the standard. An example trace HPLC injection is presented in Fig. 3.
14. After identification, peaks are integrated to obtain the area under the peak in mAbs units.
15. The presence of RA is confirmed by LC-MS by comparison of retention time and mass spectra.
16. Samples are run on ThermoFinnigan LCQ DECA XP Plus LC-MS system with a Surveyor HPLC system-autoinjector, pump, degasser, and PDA detector and a nitrogen generator all running under the Xcaliber 1.3 software system.
17. The MS is run with the ESI probe in the negative mode, with the source temperature set at 300°C and the needle gas set at 80 arbitrary units and the sheath gas set at 20 arbitrary units.

18. The MS is optimized for the detection of RA by using the autotune feature of the software while infusing a solution of RA standard into with the effluent of the column and tuning on 359 $m/z$ [M–H]$^–$.

19. The initial HPLC conditions are 20% methanol and 0.25% acetic acid in water, at the flow rate 0.3 mL/min. The column is then developed to 100% methanol and 0.25% acetic acid over 50 min.

20. RA in selected samples is confirmed by comparison of retention and MS spectra of the sample RA peak to that of a standard (see Note 5).

4. Notes

1. These protocols can be adapted for many other plant materials and species.
2. For illustrative purposes, spearmint plant growth and secondary responses employing the plantlet culture systems are shown in Figs. 4 and 5. Note that both RA and Carvone quantities can be dramatically altered depending on the treatment given to plantlets, even through the plantlet growth rate remains relatively unaffected (Fig. 5).

3. Carvone levels can be deceivingly unreliable to determine unless the investigator consistently employs the same plant material types and its location of growth. Please note in this investigation we have used terminal 4-cm shoot sections only.

4. RA analysis likewise can be difficult to determine accurately. RA is susceptible to chemical degradation and care must be taken to avoid exposure to excessive heat and acidic conditions. The quicker and efficient removal of water from the samples, HPLC will determine more accurately RA values. In general freeze-drying seems to yield the most reproducible results.

5. Because of the time required for chromatographic analysis, a large number of samples generated during the course of a plant culture study can be overwhelming. We have used near infrared spectroscopic (NIRS) analysis for rapidly determination of relative concentrations of RA in dried mint samples.
Fig. 5. Influence of ventilation treatments on growth and secondary metabolism of spearmint plantlets. Ventilation treatments consisted of a single 30 min exposure to 100 mL/min air equally spaced apart for a day. Data were averaged for five replications per treatments. Note that higher air treatments promoted more secondary metabolites to be produced while growth was relatively unaffected.
Once a set of samples has been analyzed by HPLC, use them to prepare a calibration curve in the NIRS instrument. Powdered samples can be quickly read on the NIRS instrument for rapid determination of RA levels.

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References
