Theor Appl Genet (1998) 96: 683–687

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Somatic hybridization in mint: identification and characterization of *Mentha piperita* (+) *M. spicata* hybrid plants

Received: 8 February 1997 / Accepted: 9 April 1997

Abstract Twenty eight somatic hybrid plants were identified following protoplast fusions between peppermint (*Mentha piperita* L. cv Black Mitcham), producing high-quality oil, and spearmint (*Mentha spicata* L. cv Native Spearmint), likewise producing high-quality oil and also possessing resistance to verticillium wilt. Prior to fusion, peppermint protoplasts were subjected to iodoacetic acid to inhibit cell division. Protoplasts of peppermint and spearmint were fused using polyethylene glycol plus DMSO. Fusion products were cultured according to an efficient protoplast-to-plant-cycle protocol developed for peppermint. Using this protocol, iodoacetic acid-treated peppermint protoplasts were not able to divide, whereas untreated spearmint protoplasts had the ability to produce callus but not shoots. Therefore, selection of somatic hybrid calli was based on the presumed capability of hybrid cells to form calli and shoots. Shoots in vitro were initially identified as hybrids using RAPD profiles. Subsequently, observations on morphology, chromosome counts, and Southern-hybridization patterns confirmed their hybrid status. The results of verticillium tests revealed that 18 somatic hybrids were more susceptible than Native Spearmint, while hybrid II-14 had a level of susceptibility intermediate between that of the fusion parents. Oil-analysis of hybrid plants indicated that they all have a GC-profile typical of spearmint oil.

Communicated by Y. Gleba

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Key words Essential oils · Mint · *Mentha piperita* · *M. spicata* · Somatic hybridization · Plant regeneration · RAPD · Southern hybridization

Introduction

The genus *Mentha* (*Laminaceae*) is composed of 19 geographically widespread species and 13 named hybrids (Chambers and Hummer 1994). Peppermint and spearmint are grown world-wide as perennial herbs, and produce different essential oils which are used as flavorings. Peppermint, *M.* × *piperita*, is native to Europe, and has become both cultivated and naturalized in North America. It is a sterile natural hybrid of *M. aquatica* × *M. spicata* (Tucker 1992) which is tetraploid (2n = 72) and produces the typical peppermint cyclic monoterpenes, menthol and menthone. Due to sterility it is not amenable to improvement by sexual crosses (Tucker 1992). Spearmint, *M. spicata*, was introduced from Europe to North America in the early 1800s. It is either tetraploid (2n = 48) or triploid (2n = 36) and produces the monoterpene carvone as the major oil component. The cultivar Native Spearmint which is commercially grown in The United States is a triploid plant (2n = 36). Both Black Mitcham and Native Spearmint are cultivars of high oil quality and productivity. However, peppermint is susceptible to the fungus *Verticillium dahliae* (Brandt et al. 1984), which in some years results in great losses to the mint industry. In contrast, Native Spearmint is resistant to verticillium (Lacy 1994, personal communication). To introduce verticillium resistance from spearmint into peppermint via interspecific hybridization is very difficult, because mints are sterile and seeds are rarely produced. Alternatively, biotechnologies such as somatic hybridization may be used to generate novel mint germplasms. Some biotechnological studies on mint, including micropropagation (Rech and Pires 1986; Repcakova et al. 1986; Hirata et al. 1990; Cellarova 1992; Buckley et al.

1995; Reed et al.1995), cell and tissue culture (Van Eck and Kitto 1992; Sato et al. 1993), somatic hybridization (Hisashi and Hiroshi 1994) and genetic transformation (Spencer et al. 1990, 1993), have already been published. Here, we report on the protoplast methodologies used to obtain symmetric somatic hybrids between peppermint and spearmint, and identify and confirm such plants by morphology, chromosome counts, RAPD profiles and Southern hybridization. Additionally, the results of characterizing the somatic hybrids for reaction to verticillium inoculation and oil-profile analysis are reported.

Materials and methods

Plant material

Stolons of Native Spearmint (NS) and Black Mitcham (BM) were received from Summit Plant Laboratories, Inc., Fort Collins, Colo., potted in 2-gallon plastic containers using Bacto Mix, and placed in a greenhouse at 18°C DT/16°C NT. Shoots were taken from greenhouse-grown plants, stripped of leaves, and washed for 5 min in 0.1% Sigmaclean soap. The shoots were rinsed with water and then placed in 70% ethanol for 1 min. The ethanol was subsequently removed and replaced with 10% Clorox plus 0.1% Tween 80. The shoots were surface-sterilized for 20 min followed by three sterile distilled water rinses. Two-centimeter nodal explants were inserted individually into 150 × 25 mm culture tubes each containing 15 ml of medium consisting of MS salts (Murashige and Skoog 1962), 10 mg/l thiamine, 1 mg/l nicotinic acid, 1 mg/l pyridoxine, 100 mg/l inositol, 20 g/l sucrose and 0.3% Phytagel, pH 5.8 (EM medium). The cultures were incubated at 26°C and with 25 μmol $m^{-2} s^{-1}$ of light, provided by Philips F96T12/CW cool white bulbs, on a 16-h photoperiod. Following growth and rooting, these donor plants were transferred and maintained in Magenta boxes, each containing 60 ml of EM medium, using the same environment.

Protoplast isolation and fusion

Donor plants grown and maintained in vitro were used for protoplast isolation. Stem protoplasts of BM, and leaf mesophyll protoplasts of NS, were isolated using the same protocol. First, 2–3 upper internode stem segments of BM were cut into 2-cm pieces and then cut again longitudinally. Two to three upper leaf pairs of NS (about 1 g) were sliced into 2-mm strips. Both tissues were incubated separately in 10 ml of an enzyme solution, consisting of CPW salts (Frearson et al. 1973), containing 0.5 M sucrose and 2.5% Cellulase "Onozuka" R10 (Yakult Honsha Co., Ltd., Japan) pH 5.6, for 16–20 h in the dark. Protoplasts were filtered through nylon mesh (61 μ) and placed in 10-ml glass tubes with 2 ml of KSm salt solution on the top (Krasnyanski and Menzcel 1993). Protoplasts that collected at the interface after centrifugation (80 g, 10 min) were removed and re-suspended in the KSm washing solution and purified twice by centrifugation (80 g, 2 min). Purified protoplasts were also washed twice in W5 solution (Menczel et al. 1981). Before fusion, BM protoplasts were inactivated using a 20-min treatment of 10 mM iodoacetic acid dissolved in W5, pH 5.6 (Krasnyanski and Menczel 1995), washed by centrifugation (80g, 2 min) in W5 and mixed (1 : 1) with purified NS protoplasts. Both species were at a density of 5.0×10^5/ml and were fused as described by Menczel et al. (1981) using a 25% PEG (6000) and 10% DMSO solution.

Protoplast culture and plant regeneration

Following PEG treatment, protoplasts were covered with 5 ml of V-KM medium (Krasnyanski et al. 1992) supplemented with naphthalene acetic acid (NAA), 0.1 mg/l, and 6-benzylaminopurine (BA), 2.0 mg/l, and cultured in 60 × 15-mm Petri dishes for 1 week in the dark at 25°C. Subsequently, protoplasts were collected and purified in KSm washing solution with 0.5 M sucrose by centrifugation (20g, 3 min). Protoplasts collected after purification were imbedded (4×10^4/ml) in 1 ml of 0.5% melted-agarose solidified (droplet) on the bottom of a 60 × 15-mm plastic Petri dish and covered with 10 ml of liquid VK-M4 medium (NAA 0.1 mg /l, BA 2.0 mg/l). One-week later dividing and non-dividing protoplasts were counted and plating efficiency was determined as the percentage of dividing protoplasts. Embedded protoplasts were cultured for 4 weeks in the dark at 26°C with 10 ml of fresh medium added at 10-day intervals. After 4 weeks, protoplast-derived colonies were released by gently pipetting the agarose droplet and the surrounding liquid medium with a Pasteur pipet and placing the mixture onto solid D4 medium, containing MS salts + B5 vitamins, sucrose 20 g/l, coconut water 25%, Phytagel 0.35%, BA 2 mg/l and N-phenyl-N′–1,2,3-thidiazol-5-ylurea (TDZ) 3 mg/l. After 5 weeks of culture in the dark, macrocalli were transferred onto 5T regeneration medium (MS salts + B5 vitamins, sucrose 20 g/l, coconut water 25%, Phytagel 0.35%, BA 1 mg/l, TDZ 2 mg/l) and incubated at 26°C and 25 μmol of light, provided by Philips F96T12/CW cool white bulbs, on a 16-h photoperiod. Calli that regenerated shoots were transferred to EM medium under the same light and temperature conditions, for shoot elongation and rooting.

DNA isolation and RAPD analysis

Genomic DNA was isolated from young leaves of in vitro grown plants on EM medium using standard miniprep CTAB chloroform/isoamyl extraction according to Doyle and Doyle (1990). The random amplified polymorphic DNA (RAPD) procedure was carried out in a Perkin Elmer System 9600. Amplification parameters were 94°C for 4 min followed by three cycles with 94°C for 15 s, 35°C for 15 s, and 72°C for 1 min 15 s and then 34 cycles with 94°C for 15 s, 40°C for 15 s, and 72°C for 1 min 15 s. A 72°C incubation for 7 min as a final step was included. The 25 μl amplification mixture contained 25 ng of genomic DNA and 125 ng of OPP-7 (Promega) primer (5′-GTC-CAT-GCC-A-3′).

Southern-blot analysis

Total DNA extracted from 2–3 g of fresh leaf tissue, from greenhouse plants maintained in vegetative growth, was quantified using a DNA fluorometer (Hoefer Scientific Instruments, San Francisco) and 8–9 μg was digested with *Ste*I and electrophoresed in an 0.9% agarose gel in TAE buffer. DNA was transferred to a nylon membrane (Hybond N + , Amersham) using the capillary blotting technique. A 0.77-kb *Apa*I fragment from the clone pLC5.2 (Colby et al. 1993) carrying the limonene synthase (cyclase) gene was used as the probe. This probe was labeled with ^{32}P and used for hybridization. Blotting, labeling, hybridization and washing were carried out according to Sambrook et al. (1989).

Cytology

The roots of greenhouse-grown plants were taken, washed, and placed in water for 5–7 days. Root tips of young roots were collected and treated with colchicine (1%, 3 h) and stained with acetocarmine. Chromosomes were counted in 8–10 metaphase plates of each root tip.

Verticillium resistance test

Plants to provide cuttings were maintained vegetatively in a greenhouse, and verticillium stock cultures and the protocol for evaluating BM, NS and 19 somatic hybrids for resistance was carried out according to Lacy and Horner (1965) with some changes (Lacy, personal communication). Conidia were collected from PDA dishes and diluted with distilled water to 1×10^5 per ml. For each verticillium-evaluation experiment, 20 terminal shoot cuttings, 8–10 cm each, were collected from BM, NS and selected somatic hybrid plants and immediately placed in water. Simultaneously, for each genotype ten cuttings were placed in a 100-ml beaker containing 50 ml of water, and in another with conidia, for 15 min with stirring. The treated and untreated conidial sets of ten cuttings were inserted in blocks in sterilized galvanized flats containing moist sand in a greenhouse. Five-to-seven weeks after innoculation, when disease symptoms on BM were evident, cuttings were individually rated according to a visual scale of 1 = no symptoms, 2,3,4 = mild, moderate and severe, and 5 = dead or dying plants. Statistical comparisons were made within an experiment between somatic hybrids and the BM and NS parents treated at the same time. Each cutting was handled as a replication and the means of the ten verticillium-treated cuttings of hybrids were compared to those of BM and NS. Student's *t*-test was used to determine significance using $P \leq 05$.

Essential-oil analysis

Between 50 and 100 g of fresh leaf tissue were collected from greenhouse-grown plants that were approximately 10% in flowering. The leaves were inserted into a 500-ml round bottom flask containing several boiling chips. Distilled water was added to just cover the leaves, and the flask was enclosed in a heating mantle. A Clevenger trap was placed on the flask and the trap was filled with distilled water. A reflux condenser was then fitted above the trap and the jacket of the condenser was cooled with circulating ice water. After 60 mins of boiling, the apparatus was cooled for 10 mins. The oil was collected in 100-µl aliquots. Such oil samples were analyzed by A. M. Todd Company, Kalamazoo, Mich., and I. P. Callison and Sons, Chehalis, Wash.

Results and discussion

Pre-requisites for successful somatic hybridization include an efficient protoplast-culture scheme and similarly a plant-regeneration scheme for at least one fusion parent. In preliminary studies, both BM and NS protoplasts were successfully isolated from different tissues. However, for stem- and leaf-isolated protoplasts of both BM and NS, cultured in V-KM4 medium, plating efficiencies and colony formation frequencies varied (data not shown). Stem protoplasts of BM were selected for fusion because they exhibited a high capability to form colonies, p-calli, and to regenerate shoots on D4 medium. In contrast, BM leaf-mesophyll protoplasts cultured in V-KM4 medium had only 40–60% of the p-calli on D4 medium form shoots. Stems of in vitro grown plants of NS were more rigid in structure and yielded fewer protoplasts of lower quality. In contrast, NS leaf-mesophyll protoplasts were higher in quality and were consistently and readily isolated. The regeneration system developed for BM stem protoplast-derived calli gave 100% plant regeneration. It is important to mention that the provision of coconut water in both the V-KM4 protoplast culture and the D4 plant regeneration medium was essential. Neither colony formation nor p-calli growth were observed without coconut water in these media (4% and 25% in V-KM4 and D4, respectively). Another critical factor was the use of a combination of thidiazuron (TDZ) at 0.1–4 mg/l, and BA at 1–10 mg/l. Both TDZ and BA alone at various concentrations, or in a combination with other hormones, failed to give sufficient shoot regeneration. It is known that TDZ is a very efficient cytokinin for plant regeneration in woody plants where it is used at rather low concentrations (Huetteman and Preece 1993). We found that TDZ (3 mg/l) and BA (2 mg/l) was the most-efficient hormone combination for shoot regeneration; giving 100% frequency for BM. When the same conditions developed for plant regeneration for BM were applied to NS no shoot regeneration from p-calli was observed. The strategy, therefore, to obtain somatic hybrids of BM and NS was to culture the fused protoplasts, and later protoplast-derived colonies, according to the BM protocol. In order to eliminate possible cell division and subsequent plant regeneration from non-fused BM protoplasts, they were treated with iodoacetic acid immediately prior to fusion.

After protoplast fusion, cultures yielded many calli with shoot primordia, which were initially placed in the dark and later under light. Only calli that formed shoots were selected and further subcultured. Other calli without shoot regeneration were presumed to be non-fused NS. Altogether about 100 calli were intially selected. Thirty of these randomly selected shoots were rooted and only after RAPD confirmation as hybrids were the in vitro maintained plants transferred to the greenhouse. When parental DNAs were amplified by the OOP-7 primer the RAPD patterns were distinctly different (Fig. 1). The presence of distinct parental bands in the individual patterns of all 30 plants, each of separate callus origin, confirmed their hybrid status. Although it is possible that multiple shoots from a

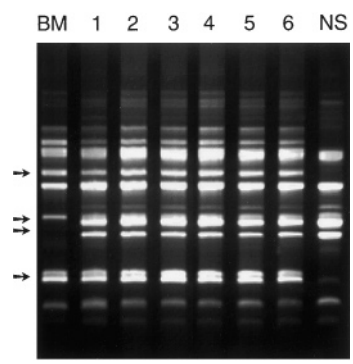

Fig. 1 RAPD analysis of somatic hybrids plants (1–6) and parental species *M. piperita* cv Black Mitcham (BM) and *M. spicata* cv Native Spearmint (NS)

selected callus may have had different origins, only a single shoot/callus was removed, maintained, and analyzed individually. Leaf morphology was intermediate between BM and NS (Fig. 2). By the leaf color (dark green of BM in contrast to light-green of NS) and leaf size, somatic hybrid plants resembled BM; whereas by the leaf margin they resembled NS. Chromosome counts also revealed that all somatic hybrid plants have a chromosome number ranging from 98 to 110 which is close to the expected 108 chromosomes, i.e. *M. piperita* 2n = 72 plus *M. spicata* 2n = 36. It was very difficult to be precise in counting the mint chromosomes because of the high numbers involved. Additional verification for somatic hybrid status was provided by Southern- blot hybridization. Species-specific banding patterns were generated by the hybridization of *Spe*I-digested genomic DNA from BM and NS when probed with a 0.77-kb *Apa*I fragment from the limonene synthase gene. At least two bands, 6.1-kb specific for BM and 3.9-kb for NS, were both present in the six somatic hybrids tested (Fig. 3). The presence of hybridization patterns of both BM and NS in the profile of somatic hybrids confirmed that they contain DNAs from both parents.

The verticillium reactions of BM as susceptible, 3.2–4.7 disease ratings, and NS as resistant, 1.7–2.6 ratings, were confirmed (Brandt et al. 1984; Lacy 1994, personnal communication and Table 1). Nineteen somatic hybrids were evaluated for verticillium resistance at least once, and 6 of the 19 hybrids 2–3 times, in seven independent experiments (Table 1). Twelve of the hybrids were as verticillium-susceptible, or more so, than BM. Ten plants, I-4, II-21, II-31, II-33, II-5, II-51, III-14, III-16, III-19 and III-41, had mean disease ratings which were significantly higher than NS, but not significantly different from BM. Hybrid II-12 had a mean disease rating of 4.8, significantly higher than NS or BM. Hybrid II-13 was highly susceptible to the extent that all ten cuttings were dead at the time of symptom rating (5.0). Although the mean disease ratings were higher than either parent for II-13, no *t*-test was performed due to a zero variance within the group. Hybrid II-14 had a rating of 3.2 which was significantly different from, and intermediate between, that of BM and NS. One hybrid, I-31, had a mean disease rating of 3.0, intermediate with respect to the two parents, but did not differ significantly from either parent. Five hybrids, II-3, II-4, III-11, III-12 and III-13, that were included in more than one experiment, had disease ratings which were higher than NS in at least one experiment, but were not different, or else had a zero variance, in the other experiment(s). In no case did any of these five hybrids have a disease rating significantly lower than BM.

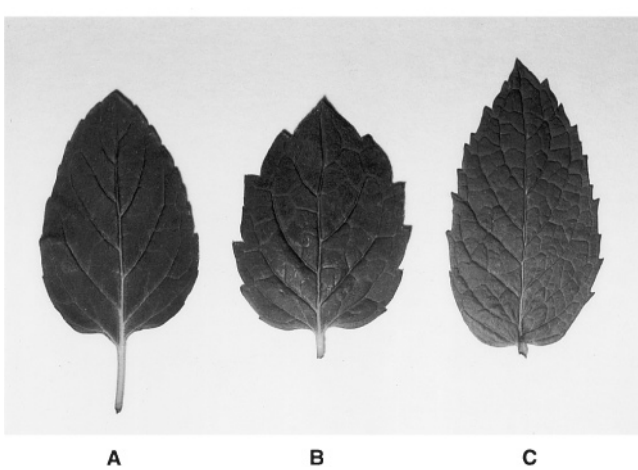

Fig. 2A–C Leaf morphology of a somatic hybrid plant and parental species. **A** *M. piperita* (cv Black Mitcham); **B** somatic hybrid; **C** *M. spicata* (cv Native Spearmint)

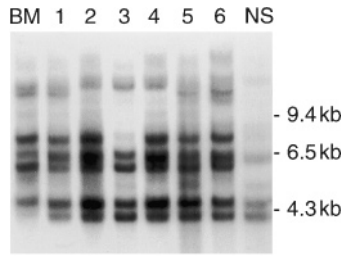

Fig. 3 Southern-blot hybridization of *Spe*I digests of total DNA of *M. piperita* cv Black Mitcham (*BM*), *M. spicata* cv Native Spearmint (*NS*) and somatic hybrid plants (1–6) probed with the 0.77-kb *Apa*I fragment from clone pLC5.2 carrying the limonene synthase gene

Table 1 Visual rating response of NS, BM and 19 somatic hybrid plants to verticillium inoculation

Genotype	Experiment						
	1	2	3	4	5	6	7
BM	3.9[a]	3.2[a]	3.5[a]	3.6[a]	4.1[a]	4.7[a]	3.9[a]
I-31	3.0						
I-4						4.3[a]	
II-12					4.8[a,b]		
II-13				5.0[c]			5.0[c]
II-14						3.2[a,b]	
II-21	4.4[a]						
II-3					3.9[a]		5.0[c]
II-31			4.4[a]				
II-33						4.7[a]	
II-4	3.6				4.7[a,b]		
II-5		2.9[a]	3.9[a]	4.0[a]			
II-51	4.5[a]						
III-11			3.8[a]				5.0[c]
III-12		4.6[a,b]	3.0	3.9[a]			
III-13		3.8[a]		5.0[c]			
III-14							4.3[a]
III-16		3.4[a]					
III-19						3.7[a]	
III-41					4.1[a]		
NS	2.6[b]	1.7[b]	2.6[b]	1.9[b]	1.4[b]	1.7[b]	1.9[b]

[a] Significantly different from N.S. at $P = 0.05$
[b] Significantly different from B.M. at $P = 0.05$
[c] Not tested for significant difference due to unequal variance between groups

Table 2 Range in percent oils of Black Mitcham, Native Spearmint and seven somatic hybrid plants

Mint species	Menthone	Menthol	Carvone
Black Mitcham	23.0–47.7	16.0–40.8	–
Native Spearmint	0–0.1	–	60.8–66.0
Somatic hybrids[a]	1.3–2.5	–	50.4–64.4

[a] Oil profiles combined for: SHII-4, SHII-13, SHII-31, SHIII-9, SHIII-11, SHIII-14, and SHIII-19

Two other experiments were conducted in which, for unknown reasons, verticillium disease symptoms failed to progress. The results of these experiments, in which the mean disease ratings for BM and NS were not significantly different, are not included in those given in Table 2.

The results of the GC analysis of essential oils indicated that BM had a typical range in the concentrations of menthone and menthol, but that carvone was absent (Table 2). In contrast, NS had predominantly carvone, a trace of menthone and no menthol. The oil type in 29 of the somatic hybrids was initially assessed by olfactory means by four evaluators. All 29 hybrid plants were found comparable to NS. The range of oils found in seven somatic hybrids selected for GC analysis indicated they had a profile similar to NS. They had predominantly carvone, and just slightly more (1.3– 2.5%) menthone than NS (0–0.1%).

In conclusion, the fact that NS is a triploid while BM is a tetraploid interspecific hybrid, and that the genetics of verticillium resistance has not been reported in *Mentha*, renders the interpretation of the verticillium responses of the somatic hybrids as problematic. In a few cases, possibly due to genomic rearrangements, a gain/loss of chromosomes or even somaclonal variation, some somatic hybrids were even more susceptible to verticillium than BM. Conversely, hybrid II-14 was lower in verticillium susceptibility than BM and this reaction was presumably derived from NS. Thus, some disease resistance was introduced from NS into susceptible peppermint, but the level of resistance was not comparable to that of the NS parent. This level of verticillium response, coupled with the undesired NS oil-type profile being predominant in the somatic hybrids, indicates that other biotechnologies may warrant exploration for the improvement of cultivated mints.

Acknowledgements This study was supported in part by a grant from Mint Industry Research Council. The authors thank R. May and A. Loskutov for their assistance and the A. M. Todd Company for the oil profile analyses.

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