Reprinted from: Issues in new crops and new uses. 2007. J. Janick
and A. Whipkey (eds.). ASHS Press, Alexandria, VA.

In-Vitro Selection for Stress Tolerant Spearmint*

Hussein Al-Amier and Lyle E. Craker

Medicinal and aromatic plant species are important sources of secondary metabolites with therapeutic, cosmetic, and food processing applications. The quality of the metabolites, however, frequently varies due to inconsistencies in secondary metabolite composition of plant extracts, especially within the family Lamiaceae. A major contributing factor to these metabolite inconsistencies is undoubtedly due to plant heterogeneity, a characteristic of open-pollinated species. Thus, various genotypes occur within a plant population, differing in physical and metabolic traits that control plant growth, chemistry, and environmental susceptibility.

Spearmint (*Mentha spicata* L., Lamiaceae) is an essential oil crop with a number of culinary and aromatic uses (Yanishlieva et al. 2006; Szczerbanik et al. 2007). Similar to other Lamiaceae species, however, uniformity in essential oil content and composition and consistency in growth and development are especially susceptibility to environmental stress due to plant heterogeneity. Thus, crop yields and quality in major spearmint production regions of the US and in Egypt, areas that are frequently subject to dry periods, can fluctuate. The use of irrigation over the past several years to promote crop growth has increased the salt content of the soil, frequently forcing growers to apply 10% to 20% excess water to lower salt concentrations in the root zone (Takabayashi et al. 1994; Takabayashi and Dick 1996; Arndt et al. 2001; Mohamed et al. 2002).

Water stress in plants from a lack of moisture or from drought induced by salt stress is associated with increased proline synthesis and other metabolic changes, including the increased synthesis of phenolic acids and essential oil metabolites that are stimulated in response to stress (Matticci et al. 1995; Paré and Tuminson 1999; Mohamed et al. 2002). The exact nature and level of the response to stress is dependent upon the susceptibility of the plant genotype to stress (Rena and Splittstoesser 1975; Premachandra et al. 1992; Fatima et al. 2002). This variability among the assortment of genotypes within open-pollinated spearmint suggests that such variability could be exploited for development of drought tolerant types of spearmint (Rena and Splittstoesser 1975; Premachandra et al. 1992; Fatima et al. 2002) that over-express phenolic acids and other essential oil constituents (Mohamed et al. 2002).

METHODOLOGY

Shoot explants of spearmint developed from seeds (Geo. W. Park Seed Co., Inc, Greenwood, South Carolina) were used in this study. The seeds were surface sterilized by rinsing in 0.15% hypochlorite solution for 20 min, in 70% ethanol for 90 sec, and in distilled, deionized water three times for 5 min each. The seeds were then air dried in a laminar flow hood and subsequently sown on sterile (autoclaved at 120°C and 103 kilopascals for 20 min) MS medium (Murashige and Skoog 1962) containing 6-benzylaminopurine (MSBAP) (Table 1). Portions (25 mL) of the media were poured into sterile Petri plates (10 cm in diameter) and allowed to cool and solidify before sowing the seeds on the media surface (10 seeds/plate). The plates containing the seeds were wrapped with Parafilm® and placed in an incubator under a 16-hr light/8-hr dark cycle with illumination from cool white fluorescent lights (20 $\mu mol^{-1}cm^{-2}s^{-1}$) at 26°C for germination.

Shoot explants, each originating from a different seed and consisting of an apical bud and one adjacent leaf pair, were induced to produce multiple shoots via axillary shoot formation on the MSBAP medium. Of the initial 250 seeds aseptically germinated, 22 seedlings had the ability to form multiple shoots under the influence of BAP and were used to develop clonal populations for study of stress tolerance.

To determine stress tolerance, individual shoots (explants) of each clonal line were transferred from the MSBAP media to a stress media (Table 1), containing 0 (control) or 8 gL^{-1} NaCl, by immersing the cut end of the shoot into the stress media. The plates with the transferred shoot explants were incubated at 26°C under the same lighting conditions used for seed germination. After 30 days growth, all clonal lines were examined and selected for salt-tolerance by measurements of survival, biomass production, chlorophyll, proline, and phenolic acid levels. Each treatment had five replicates (Petri plates) and each replicate had seven explants.

*This work was supported in part by the Massachusetts Agricultural Experiment Station, University of Massachusetts, Amherst, MA 01003, paper number 3409, and by a US-Egypt Joint Grant, Foreign Agricultural Service/58-3148-7-045.

Survival was ascertained by the percentage of shoots that remained viable (explants with chlorosis in the shoot and/or leaf were scored as non-surviving). Biomass production was determined from shoot fresh weight and the number of shoots that survived the stress.

Chlorophyll levels were estimated spectrophotometrically, as described by Hipkins and Baker (1986), by measuring the absorbance at 650 nm and 665 nm of a 3 mL methanol extract of approximately 50 mg (fresh weight) of individual explants maintained in darkness for 2 hr at 23°C. Total chlorophyll (expressed as µg/ml methanol) = $25.8 \times A_{650} + 4.0 \times A_{665,}$ where A_{650} and A_{665} were the solution absorbance at 650 and 665 nm.

Free proline content was determined according to the method described by Bates et al. (1973) in which approximately 100 mg of spearmint tissue (containing both callus and developing shoots) was homogenized in 5 ml of 3% sulfosalicylic acid solution (Sigma-Aldrich Chemical Co., St. Louis, Missouri) and then filtered through Whatman #1 filter paper (Whatman, England) to remove plant residue. A 1 mL aliquot of the filtrate was subsequently mixed with an equal volume of glacial acetic acid-ninhydrin reagent (1.25 mg of ninhydrin, 30 mL of glacial acetic acid and 20 mL of 6 M H_3PO_4) and then incubated for 1 h at 100°C in boiling water bath. The reaction was terminated by placing the test tube containing the aliquot-reagent mixture in an ice bath and vigorously mixing the reaction mixture with 2 mL toluene. After warming the terminated reaction mixture to 25°C, the color intensity (an indication of proline level) was determined spectrophotometrically (Agilent 8453 spectrophotometer, Agilent Technologies, Santa Clara, California) at 520 nm (using toluene as blank and compared with standards made with proline in 3 % sulfosalicylic acid).

Phenolic levels in shoots were determined using a slight modification of the method of Chandler and Dodds (1983) that was originally based on the method of Singleton and Rossi (1965). Approximately 50 mg (fresh weight) of shoot tips were placed in 2.5 mL of 95% ethanol at 0°C for 48 h. Each sample was then homogenized with a Tissue Tearor (Biospec Product, Racine, Wisconsin) and centrifuged at 13,000 × g for 8 to 10 min. A 1 mL aliquot of the supernatant was transferred to a 16 × 100 mm test tube and mixed with 1 mL of 95% ethanol, 5 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent for 5 min followed by 1 mL of 5% Na_2CO_3 with thorough mixing. The mixture was allowed to stand for 60 min and the absorbance of the solution was read spectrophotometrically at 725 nm with an Agilent 8453 Spectrophotometer (Agilent Technologies, Santa Clara, California), using 95% ethanol as blank and solutions of gallic acid as standards). Absorbance values were converted to milligrams of phenolic acids per unit of fresh tissue. The yield of phenolic acids for each clone was calculated by multiplying the mean shoot survival by the mean fresh weight of each shoot by the mean phenolic acid level.

Table 1. Growth media used in culture of spearmint shoots.

Ingredient[z]	Proliferation media[y]	Salt stress media[y,x]
Basal salts	4.34 g/L	2.17 g/L
Murashige & Skoog vitamin solution	1 mL/L from 1000× commercial stock solution[w]	0.5 mL/L from 1000× commercial stock solution[w]
6-Benzylaminopurine (BAP)	1 mg/L	-
Sucrose	30 g/L	15 g/L
Salt (NaCl)	-	0 & 8 g/L
Deionized water	To make 1 L	To make 1 L
Phytogel®	3 g/L	3 g/L

[z]Except for the sucrose all media ingredients were sourced from Sigma-Aldrich Chemical Co., St. Louis, MO; the sucrose was sourced from Domino Sugar Co., New York.

[y]Both the proliferation and stress media were sterilized by autoclaving at 120°C and 103 kilopascals for 20 min. The pH was adjusted to pH 5.7 before autoclaving.

[x]The media contained salt at indicated concentrations, the non-stress control media contained no salt.

[w]Final concentration = 2 µg glycine, 100 µg myo-inositol, 0.5 µg nicotinic acid, 0.5 g pyridoxine hydrochloride, & 0.1 µg thiamine hydrochloride for the propagation media and ½ those amounts for the salt stress media.

RESULTS

Of the 22 clonal lines developed, only 12 survived a salt stress of 8 gL^{-1} NaCl (Table 2). Growth of the 12 surviving clones, however, was at a reduced rate as evidenced by a reduction in survival, shoot numbers, and, except for clone 12, fresh weight in the salt stressed as compared with the unstressed controls. No consistent pattern was noted in chlorophyll synthesis of the clones under salt stress.

In the surviving clonal lines, the salt stress increased the synthesis of proline as compared with controls (Fig. 1). All 12 of the clones that survived the salt stress increased synthesis of proline, as compared with unstressed controls, when transferred to media containing salt. No relationship between the synthesis of proline in the unstressed control and the salt stressed shoots was observed.

Except for clonal line 5, the phenol acid concentration increased in the surviving clonal lines stressed with salt as compared with unstressed controls (Fig. 2). In clonal line 5, the salt stress resulted in an observed, but not significant, decrease in phenol acid concentration. The small increase in phenol acid concentration in salt stressed clonal lines 8, 9, and 12 as compared with the unstressed controls was not significant.

Table 2. Characteristics of 12 spearmint lines surviving salt stress.

Clone	Survival (% control)	Shoots (% control)	Fresh wt. (% control)	Chlorophyll (% control)
1	61.9	63.7	15.4	92.6
2	66.7	32.9	47.5	149.6
3	85.7	39.0	23.9	114.0
4	81.0	56.6	52.9	25.8
5	66.7	42.7	61.5	29.1
6	81.0	57.1	84.4	66.8
7	47.6	51.3	17.0	28.4
8	90.5	45.8	54.8	49.0
9	95.2	30.3	20.5	29.8
10	76.2	35.5	47.5	13.0
11	85.7	22.2	26.5	116.6
12	66.7	36.5	122.5	57.0

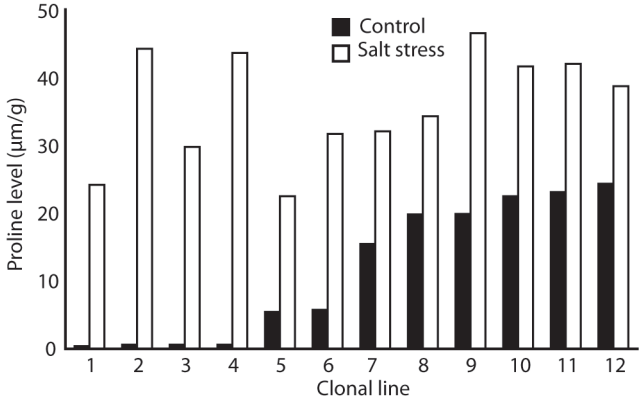

Fig. 1. Proline accumulation in plants subjected to salt stress. For salt stress, shoot explants were exposed to 8 gL^{-1} NaCl. All shoot explants were harvested after 30 days growth.

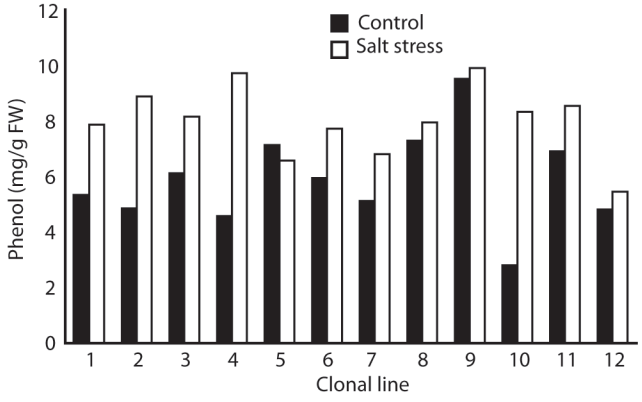

Fig. 2. Phenol concentration in plants subjected to salt stress. For salt stress, shoot explants were exposed to 8 gL^{-1} NaCl. All shoot explants were harvested after 30 days growth.

With the exception of clonal line 5, the calculated yield of phenolic acids for each clonal line was lower in the salt stressed plants than in the unstressed control plants (Fig. 3). In the case of clonal line 5 and clonal lines 10 and 12, the yield of phenolic acids was very similar in both the salt stressed and the unstressed control plants. The highest yield of phenolic acids was in the non-stressed clonal line 9.

DISCUSSION

The relatively high variability in survival, shoot numbers, fresh weight, and chlorophyll levels among the clonal lines of spearmint in response to salt stress reflects genetic heterogeneity among the spearmint clonal lines. Similarly, the lack of any relationship between the synthesis of proline level in the unstressed control clonal lines and the stressed clonal lines indicates that shifts in metabolism were a factor of genetic variability within each clonal line. Such variability in growth and metabolism is common in open-pollinated plants and is thought to enable plant species to adapt to different environments (Whitham and Slobodchikoff 1981). In the cultivation of medicinal and aromatic plants, however, such variability, arising from the plant genome or the growth environment, is undesirable, leading to harvests that frequently vary in the quantity of plant material and in the array and level of bioactive constituents (Al-Amier et al. 2005).

Medicinal and aromatic plants are primarily used in two ways, either as the plant material *per se* or as a source of plant metabolites. When plant material or an extract of the plant material is directly consumed, plant tissues with the same concentration of bioactive material under non-stress and stress environments would help insure the same dose of bioactives whether the plant material was sourced from a non-stressed or stressed field. In instances in which the plant material is used for the production of a plant metabolite, then the clonal lines that gave the highest metabolite yield under a non-stress and under a stress environment, respectively, would provide the highest metabolite yield for each stress condition.

In our study, the similar phenolic acid concentrations in the paired unstressed control and salt stressed spearmint clonal lines 5, 8, 9, and 12 suggest these lines would meet requirements for plant material from both non-stressed and stressed plants to be consumed directly. The anticipated effects from consuming the same quantity of plant material from either the non-stressed or environmental stressed plants would be same because the amount of bioactive consumed would be essentially equal. In contrast, spearmint clonal line 9 would provide the highest yield of extractable metabolite (a factor of plant growth and metabolite concentration) in the non-stressed environment and clonal line 5 would provide the highest yield in the stressed environment as compared with any of the other clonal lines.

The development and use of spearmint clonal lines demonstrated the feasibility of rapid selection of plant material suitable for production of secondary metabolites growing in unstressed and stressed environments. The differences in response among the clonal lines to a stress environment suggest the possibility of selection for stability. In our test, the desired spearmint clonal selections needed to be able to survive and grow in both non-stressed and salt stressed environments to produce uniform concentrations or relatively high yields of phenolic acids. Such selections will ensure that spearmint growers in salt-stressed areas of Egypt and in variable environments of the US can produce the type of spearmint desired in the marketplace.

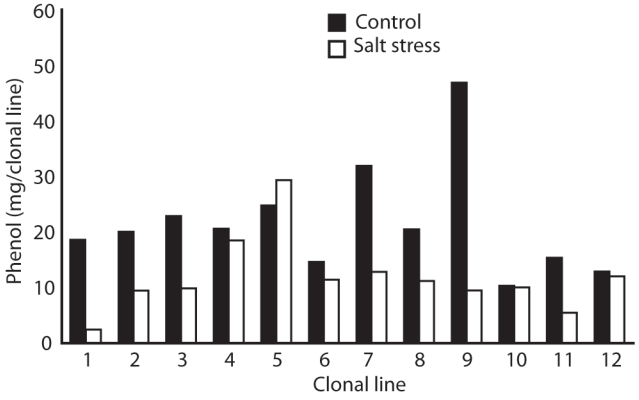

Fig. 3. Phenol yield in plants subjected to salt stress. For salt stress, shoot explants were exposed to 8 gL^{-1} NaCl. All shoots were harvested after 30 days growth. Yield was calculated as [(% surviving shoots/clonal set) × (g F.Wt/plant) × (mg phenol/g F.Wt.)]. A clonal set consisted of the seven shoot explants placed on the stress media at initiation of the salt stress.

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