

In Vitro Storage Conditions for Mint Germplasm

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Abstract. Four tissue-cultured mints, *Mentha arvensis* L., *M. spicata* L., *M. suaveolens* Ehrh. hybrid, and *M. suaveolens* cv. *Variegata*, were evaluated for survival during storage in media containing three concentrations of N and in four light and temperature regimes. Shoots were placed in plastic, five-chamber, tissue-culture bags on Murashige and Skoog medium (1962) containing 25%, 50%, and 100% of the normal N concentration (MS-N) and stored at 4 °C and -1 °C in darkness, at 4 °C with a 12-hour photoperiod, and at 25 °C with a 16-hour photoperiod. Shoots of all four genotypes stored at 25 °C were in excellent condition after 6 months but required subculture after 18 months. Condition ratings of stored shoots varied with genotype and N concentration. Cultures survived longest at 4 °C with a 12-hour photoperiod on 50% MS-N. Under this regime, all four genotypes were rated in good condition at 30 months but declined to poor condition by 36 months. Based on these data, I recommend that mint cultures be stored on MS medium with 50% MS-N at 4 °C with a 12-hour photoperiod. This regime should provide a minimum of 24 to 36 months of storage before subculture is required. Cold-sensitive genotypes could be stored for 18 months at 25 °C on 50% MS-N medium.

Breeders use collections of valuable plant germplasm to develop cultivars with improved crop yield and disease and insect resistance. Exotic germplasm, even that without currently identified economically important traits, requires safe storage. Obscure genes from these plants may provide solutions to new disease, insect, environmental, or crop production problems (Westwood, 1989). The United States produces \$112 million of peppermint and spearmint oil each year and new oil flavor components are under investigation (Chambers and Hummer, 1992). Mint germplasm must be preserved as clonal field-grown or potted plants because many cultivars are sterile. The two commercial mint types, peppermint and spearmint, are sterile hybrids and maintenance of specific genotypes is highly important to the mint oil industry. Mints can be readily cultured in vitro, although they may have associated internal bacterial contaminants (Buckley et al., 1995; Gunning and Lagerstedt, 1985; Reed et al., 1995).

Some clonal crops are kept in slow-growth storage as in vitro cultures for germplasm conservation (Ashmore, 1997; Engelmann, 1991; Withers, 1991; Withers et al., 1990). Previously, mint cultures held at the National Clonal Germplasm Repository (NCGR) were

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stored at 4 °C in darkness in 13 × 100 mm glass tubes on MS (Murashige and Skoog, 1962) medium. Under these storage conditions, 50% of the cultures were lost to fungal or bacterial contamination (Reed, unpublished data). These losses were reduced by changing storage vessels from glass tubes to polyethylene bags (Reed, 1991). The objective of this study was to contrast three MS medium N concentrations, four light and temperature conditions, and four genotypes in an effort to prolong in vitro storage of mint germplasm.

Materials and Methods

Four in vitro-grown mints [*Mentha arvensis* (NCGR local number 185.001), *M. spicata* (75.001), *M. suaveolens* hybrid (202.001), and *M. suaveolens* cv. *Variegata* (587.001)] were multiplied in Magenta GA7 boxes on 40 mL MS medium containing 2.2 (M^N-benzyladenine (BA) and 0.5 μM indole-3-butyric acid (IBA), 0.3% Bitek agar (Difco, Detroit), and 0.125% Gelrite (Schweitzer-Hall, South Plainfield, N.J.) at pH 5.7. Shoots were harvested from 3-week-old cultures. Shoot tips and internodal sections (2.5–3.0 cm) were placed in five-cell Starpac bags (Garner Enterprises, Willis, Texas) on three formulations of MS medium (10 mL/cell) containing 0.3% agar and 0.15% Gelrite, but no growth regulators. Shoots stored at 25 °C in light were placed in bags with 20 mL medium per cell, as moisture loss is greater at the higher temperature (Reed, 1993). The medium contained 100%, 50%, or 25% of the standard MS N concentrations (100%, 50%, and 25% MS-N).

Shoots were sealed in tissue culture bags and grown in the growth room for 1 week [25 °C, 16-h photoperiod, 25 μmol·m⁻²·s⁻¹ photo-

synthetic photon flux (PAR)]. Shoots destined for 4 °C and -1 °C storage were then cold acclimatized (8 h, 22 °C day/16 h, -1 °C night) for 1 week before storage. One bag of each treatment with five cells (replicates) was placed in each storage environment. Shoots were stored in four environments: 4 °C in darkness or 4 °C with a 12-h photoperiod (25 μmol·m⁻²·s⁻¹ PAR from cool white fluorescent bulbs), -1 °C in darkness, or 25 °C with a 16-h photoperiod (10 μmol·m⁻²·s⁻¹).

After 6, 18, 24, 30, 36, 48, and 54 months of storage in the four environments, shoots were rated on a vigor scale of 0 to 5, based on plant appearance: 5 = dark green leaves and stems, no etiolation; 4 = green leaves and stems, little etiolation; 3 = shoot tips and upper leaves green, some etiolation, 2 = shoot tips green, leaves and stems mostly brown, base may be dark brown, should be removed for subculture; 1 = plant mostly brown, only extreme shoot tip green, much of base dark brown; 0 = plant totally brown, no visible green on shoot tip, dead.

A completely randomized design was used with genotype, N concentration, and storage environment (light/temperature) as factors for the cold storage experiments. Warm storage data were analyzed separately with genotype and N concentration as factors. Data were analyzed for ANOVA using the factor program on MSTATC (1988).

Results and Discussion

For mint plants stored at 25 °C in the light, the MS-N concentration was the most important factor and the interaction of genotype and MS-N concentration was also significant (Table 1). Each of the genotypes survived growth room storage at 25 °C for up to 18 months. Their mean vigor rating was 3.8 at 6 months, but declined to below 2 by 18 months, at which time they required subculture (Fig. 1a). At 25 °C, ratings varied with genotype and MS-N: *M. spicata* and 'Variegata' on 50% MS-N remained in good condition (ratings of 3.4 and 2.8) at 18 months, but were dead by 24 months; *M. arvensis* and *M. suaveolens* hybrid shoots on 50% MS-N were rated poor (mean 1.2) at 18 months; and most shoots on treatments other than 50% MS-N were dead at 18 months (data not shown).

The three-way interaction (genotype × environment × MS-N concentration) was significant for cold-stored shoots after 24 months

Table 1. Analysis of variance of condition ratings (1 = poor; 5 = good) for in vitro-grown shoots of four mint genotypes (*Mentha arvensis*, *M. spicata*, *M. suaveolens* hybrid, and *M. suaveolens* cv. *Variegata*) stored at 25 °C with a 16-h photoperiod for 18 months on Murashige and Skoog medium with 25%, 50%, or 100% MS nitrogen concentrations (MS-N).

Source	df	Mean square
Genotype (G)	3	5.2**
MS-N concentration	2	19.5***
G × MS-N	6	1.7*
Error	48	0.7

***Significant at $P \leq 0.05$, or 0.001, respectively.

at low temperatures, with environment having the greatest influence (Table 2, Fig. 2). After 24 months of storage, the best ratings were obtained for shoots stored at 4 °C with a 12-h photoperiod; at -1 °C and 4 °C shoots stored in darkness were generally near or below ratings of 2, and in some treatments all shoots were dead.

Cultures of *M. arvensis*, *M. suaveolens* hybrid, and 'Variegata' grown at 4 °C in the light were rated significantly better than those in the other cold treatments at all MS-N concentrations (Fig. 2 a, c, and d). *M. spicata* ratings were best at 4 °C in light with 100% and 50% MS-N (Fig. 2b). Both *M. suaveolens* genotypes did poorly (mean rating <1) in the dark conditions (Fig. 2 c and d), while *M. arvensis* and *M. spicata* plants were rated 1 or greater in the dark (Fig. 2 a and b).

Cultures stored at 4 °C with a 12-h photoperiod continued to have viable ratings (>2) at 30 months (Fig. 1a). The three-way interaction of factors remained significant and the environmental effect remained strong (Table 2). *M. spicata* and *M. arvensis* cultures were viable at 30 months under the three cold-storage conditions, but ratings were highest at 4 °C with light; *M. suaveolens* hybrid and 'Variegata' shoots were rated <1 except when grown at 4 °C in light (data not shown). The optimum N concentration varied with genotype, but 50% MS-N was suitable for all four genotypes (Fig. 1b). After storage for 48 and 54 months, all genotypes rated <2, and therefore these conditions were not acceptable for continued storage and survival of the plants.

Plants of the four genotypes varied in their ratings under the environments tested. *M. arvensis* had the highest ratings in the cold-storage environments and *M. suaveolens* hybrid and 'Variegata' had the lowest (Fig. 1c). In warm (25 °C) storage conditions *M. spicata* and 'Variegata' had the highest ratings of the four mints tested. The highest overall ratings for up to 30 months were in the 4 °C light environment (Fig. 1a). The response of shoots grown at 25 °C to a lower N concentration was similar to that of raspberry shoots grown in vitro on 6%, 25%, and 100% MS-N, where reducing N concentrations improved ratings significantly (Reed, 1993). Reducing N in the medium is also beneficial for storage of cultures of chilling-sensitive grape (*Vitis* sp.) (Moriguchi and Yamaki, 1989).

Duplicate cultures of mint were stored at NCGR in the early 1980s in 13 × 100 mm tubes at 4 °C in darkness for 1 year (Gunning and Lagerstedt, 1985). Most genotypes remained alive, but 50% or more were contaminated by bacteria or fungi (Reed, unpublished). Genotypic variation was evident in mint cultures (116 genotypes) stored in plastic, five-chamber, tissue-culture bags at NCGR at 4 °C in darkness from 1989-95; these cultures could be stored from 0.7 to 5.7 years before requiring subculture (Reed and Chang, 1997). The results presented here indicate that mint cultures can be stored best on MS medium with 50% N concentration at 4 °C with a 12-h photoperiod. This regime should provide a minimum of 24 to 36 months of storage before subculture is

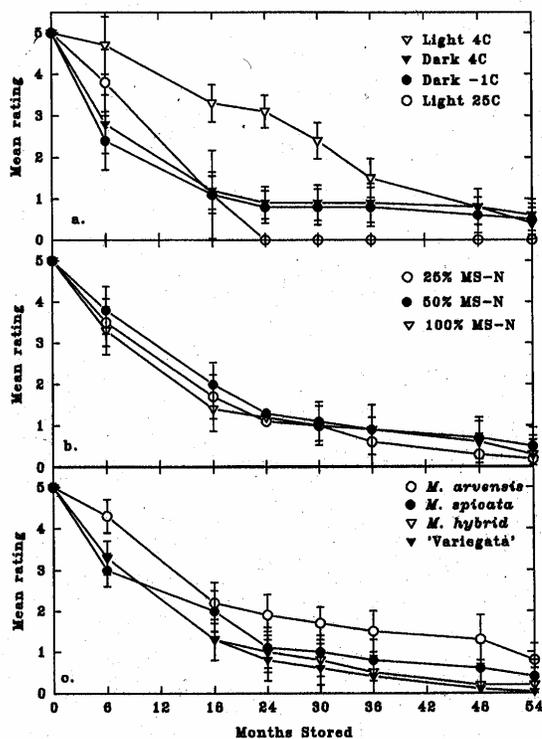


Fig. 1. Mean ratings (\pm sd) (0 = dead, 1 = poor; 5 = good) for in vitro-grown mint shoots stored in four environments for up to 54 months grouped by: a) storage environment (photoperiod, temperature), b) N concentration in the MS medium (25%, 50%, or 100%), and c) genotype (*Mentha arvensis*, *M. spicata*, *M. suaveolens* hybrid, and *M. suaveolens* cv. *Variegata*).

Table 2. Analysis of variance of condition ratings (1 = poor; 5 = good) for in vitro-grown shoots of four mint genotypes (*Mentha arvensis*, *M. spicata*, *M. suaveolens* hybrid, and *M. suaveolens* cv. *Variegata*) stored either at 4 °C in darkness; 4 °C with a 12-h photoperiod; or at -1 °C in darkness for 24 and 30 months on Murashige and Skoog medium with 25%, 50%, or 100% MS nitrogen concentrations (MS-N).

Source	df	Mean square	
		24 months	30 months
Genotype (G)	3	20.1***	19.5***
Environment (E)	2	97.6***	47.5***
G × E	6	5.9***	6.1***
MS-N concentration	2	0.7	0.2***
G × MS-N	6	0.9	1.3
E × MS-N	4	1.4	0.6***
G × E × MS-N	12	1.6**	2.2***
Error	144	0.6	0.7

***Significant at $P \leq 0.01$, or 0.001, respectively.

required. Cold-sensitive genotypes could be stored for 18 months at 25 °C on 50% MS-N medium.

Literature Cited

- Ashmore, S.E. 1997. Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. Intl. Plant Genet. Resources Inst., Rome.
- Buckley, P.M., T.N. DeWilde, and B.M. Reed. 1995. Characterization and identification of bacteria isolated from micropropagated mint plants. *In Vitro Cell. Dev. Biol.* 31P:58-64.
- Chambers, H.L. and K.E. Hummer. 1992. Clonal repository houses valuable mint collection in Corvallis, Oregon. *Diversity* 8:31-32.
- Engelmann, F. 1991. *In vitro* conservation of horticultural species. *Acta Hort.* 298:327-332.
- Gunning, J. and H.B. Lagerstedt. 1985. Long-term storage techniques for *in vitro* plant germplasm. *Proc. Intl. Plant Prop. Soc.* 35:199-205.
- Moriguchi, T. and S. Yamaki. 1989. Prolonged storage of grape nodal culture using a low concentration of ammonium nitrate. *HortScience* 24:372-373.
- MSTATC. 1988. MSTATC: A software program for the design, management, and analysis of

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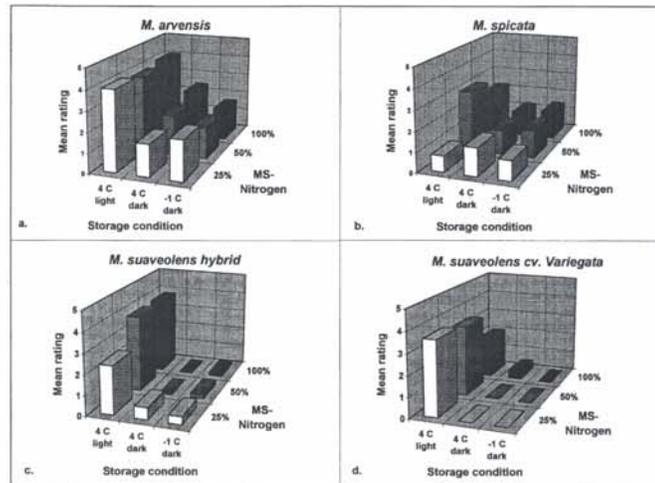


Fig. 2. Nitrogen concentration \times storage condition \times genotype interaction for mint plants stored in vitro for 24 months. (a) *M. arvensis*; (b) *M. spicata*; (c) *M. suaveolens* hybrid; (d) *M. suaveolens* cv. *Variegata*.

agronomic research experiments, Michigan State Univ., East Lansing.
 Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
 Reed, B.M. 1991. Application of gas-permeable bags for in vitro cold storage of strawberry germplasm. *Plant Cell Rpt.* 10:431-434.
 Reed, B.M. 1993. Improved survival of in vitro-

stored *Rubus* germplasm. *J. Amer. Soc. Hort. Sci.* 118:890-895.
 Reed, B.M., P.M. Buckley, and T.N. DeWilde. 1995. Detection and eradication of endophytic bacteria from micropropagated mint plants. *In Vitro Cell. Dev. Biol.* 31P:53-57.
 Reed, B.M. and Y. Chang. 1997. Medium- and long-term storage of in vitro cultures of temperate fruit and nut crops, p. 67-105. In: M.K. Razdan and E.C. Cocking (eds.), *Conservation of plant*

genetic resources in vitro, vol. 1. Science Publ., Enfield, N.H.
 Westwood, M. 1989. Maintenance and storage: Clonal germplasm. *Plant Breed. Rev.* 7:111-128.
 Withers, L.A. 1991. In-vitro conservation. *Bio. J. Linnean Soc.* 43:31-42.
 Withers, L.A., S.K. Wheelans, and J.T. Williams. 1990. In vitro conservation of crop germplasm and the IBPGR databases. *Euphytica* 45:9-22.