EVALUATION OF PEPPERMINT FIELD PERFORMANCE FROM PLANTS REGENERATED FROM MERISTEM TIP CULTURE, AND INVESTIGATIONS OF VIRUS INFECTION

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Abstract

In 1994, its second full production year, field-grown meristemmed 'Black Mitchum' peppermint performed differently than in 1993, when compared with a non-meristemmed treatment in a field trial at the OSU-COARC. The difference between years may be partly attributed to better meeting the fertility and soil moisture needs of meristemmed plants in 1994. Plants were not moisture-stressed near harvest, and plots were fertilized according to stem and soil nitrate levels. Both visual ratings and detailed measurement of plant growth indicated little difference in the growth and growth habit between meristemmed and non-meristemmed plants in 1994. Nevertheless, in 1994, plants in meristemmed plots required supplementary nitrogen, whereas in 1993 they became nitrogen deficient, which may have contributed to reduced oil production, enhanced stem mass over leaf mass, reduced branching, etc., in 1993. In 1994, when yield performance was compared without the influence of rolling, oil yield was 9 percent greater in the meristemmed treatment an early harvest in late July than in non-meristemmed treatment, but two weeks later in early August, oil yield was 20 percent greater in non-meristemmed mint than in meristemmed mint. Harvested biomass of meristemmed mint was 20 percent and 7 percent greater than the biomass of non-meristemmed mint at each harvest date. Nevertheless, due to variability in data, none of these difference were statistically significant (P<0.05). Based on 1993 observations, half of all plots were rolled in late June, theoretically to induce branching and enhance more foliage production on meristemmed plants. Whereas rolling resulted in many measurable and statistically significant growth changes in plants (P<0.05), these were not necessarily greater branching, increased leaf number or size, or enhanced oil yield. For the meristemmed treatment, rolling significantly reduced both oil yield and biomass (P<0.05) for both harvest dates compared to the non-rolled meristemmed treatment. For the non-meristemmed treatment, rolling significantly enhanced yield at the early harvest date (P<0.05), but significantly reduced oil yield at the later harvest date (P<0.05), but the effect on biomass was negligible for both dates. It is recommended that future management of meristemmed peppermint focus on the potential advantages of early harvest, rather than rolling.

Molecular biological analyses indicated that non-meristemmed 'Black Mitchum' peppermint taken from the COARC field trial contained a double-stranded RNA (dsRNA) entity which was not found in meristemmed 'Black Mitchum' peppermint taken from the same trial. Such dsRNA is

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typical of single-stranded RNA virus replication. When plant sap from either meristemmed or non-meristemmed plants was rubbed into a range of indicator plant species, sap from non-meristemmed plants produced local lesions on several species, whereas sap from meristemmed plants produced no such symptoms. Such results further suggest the presence of a virus in non-meristemmed plants. No virus has yet been characterized, and it has not yet been proved that the putative virus is responsible for differences in growth and field performance measured in 1993 and 1994.

Introduction

Meristem tip culture (also called shoot tip culture) of plant materials was formalized in the 1950's and 1960's. In this process, a few cells from a growing point (meristem) of a plant together with cells from the first one-or two-leaf primordia below it are excised from a "mother" plant prior to development of the conductive tissues in the leaf primordia. A new plant is then regenerated from these excised cells, usually with the assistance of added plant hormones (Murashige and Skoog 1962). Some initial differentiation of cells in the leaf primordia is required, but maturity of tissues is not desired. Plants regenerated in this manner retain the genetic identity of the mother plant, but all or most systemic pathogens, such as viruses and certain bacteria and fungi commonly associated with mature vascular tissues, can be excluded (Wright 1988). This process works because the mature vascular tissues allow long-distance transport of water and nutrients around the plant and also provide the avenue for rapid and long-distance movement of the systemic pathogens.

Meristem tip culture has proven most beneficial for development of pathogen-free planting materials for vegetatively propagated crops (bulbs, cloves, tubers, corms, crowns, rootstock, etc.) in which vascular pathogens commonly are retained in the planting material from generation to generation. In most cases, when vascular pathogens are so eliminated, plant materials respond by growing without disease symptoms and/or with greater vigor. It should be understood that many viruses manifest no outward symptoms other than a reduction of plant vigor. Not uncommonly, if increased vigor results following meristem tip culture, this is circumstantial evidence for presence of a vascular pathogen in the stock from which meristem tip cultured plants were derived, even when clear disease symptoms were not apparent.

There are potential complications with selection and handling of the excised cells which theoretically could upset the genetic stability normally desired with meristem or shoot tip culture. Normally, if care is taken to excise leaf primordia along with the meristem, then there is little worry about genetic changes in the regenerated plants compared to the mother plant, but such excision can be a delicate procedure. In the process of avoiding differentiated vascular tissues, there is some risk of excising only the meristem cells, or only leaf primordial cells, rather than the two together. Usually if this is done, plants fail to regenerate when handled in a manner which favors normal meristem tip culture. There is a small possibility that meristem cells or leaf primordial cells alone may form undifferentiated callus, survive the culture conditions, and then
proceed to form a somatic embryo. If plants eventually are regenerated from the undifferentiated callus and somatic embryo, these normally will also be genetically identical to the mother plant. Sometimes plants which pass through the somatic embryo stage may exhibit temporary growth differences from the mother plant type (based on hormonal imbalances), which may eventually disappear. However, somatic embryos may also sometimes exhibit permanent genetic changes due to chromosomal aberrations or changes in ploidy, such as increases in the number of full sets of chromosomes (Caligari and Shohet 1992). [Such genetic changes may be intentionally induced with other treatments of plant cells, such as somoclonal variation techniques, so that fresh genetic types can be selected (Karp 1991).]

In the experience of most meristem tip culture technicians, the likelihood of genetic shifts occurring with standard meristem tip culture and regeneration procedures is quite small. There is a much greater likelihood of failure to eradicate known or suspected viruses, as some viruses seem better able to penetrate beyond the mature vascular tissues, and there is a tendency for the technician to take too large a cut rather than too small a cut during the excision process. When viruses are well identified, every plant regenerated after meristem tip culture needs to be tested to verify virus eradication prior to use of the plants as propagative stock.

This research project was developed in response to observations that 'Black Mitchum' peppermint, which had been commercially meristem tip cultured in Montana, was growing more vigorously when field planted in Montana in comparison to plantings established from field-grown roots or non-meristemmed rooted cuttings. A review of the scientific literature indicated that peppermint and other mint species had been repeatedly meristem tip cultured and regenerated in various laboratories around the world, following standard practices for inducing roots and shoots in culture (Holm et al 1989, Geslot et al 1989, Mariska et al 1987, Repcekova et al 1986, Rodov and Davidova 1987). None of these reports indicated a growth enhancement following this procedure. None of these investigations used standard commercial U.S. varieties.

Results from the 1993 OSU-COARC field trial (Crowe 1994) verified that commercially developed 'Black Mitchum' peppermint grew and yielded in the field much differently after meristem tip culture and regeneration than did plants of the this same variety which were not manipulated in this manner. However, under normal management, the relative vigor resulting from meristem tip culture did not immediately result in improved oil yields. Instead, plants which were meristem tip cultured grew more vigorously and developed greater total dry weight by harvest, but most of this weight was in stem growth rather than in leaves which bear the great majority of the oil glands. Meristemmed plants may have been over-mature at harvest. It is logical to assume that with adjusted management, the increased vigor in meristemmed plants might be channeled differently, perhaps into greater leaf area, leaf gland number, and oil yields.
Materials and Methods

Field Performance Trial

The field trial established in 1992 and reported on for 1993 (Crowe 1994) was continued with some modifications. Rooted cuttings of 'Black Mitchum' peppermint were derived from plants which had been commercially meristem tip cultured (M plants, specific procedures undefined). Plants were received from StarIde Farms, Inc., Ronan, Montana. Similarly, rooted cuttings from non-meristem tip cultured plants (Non-M plants) were obtained from Plant Technology, Inc., Albany, Oregon, which was the source of plants from which Starkle Farms developed meristem tip cultured plants. Planting was on July 1, 1992. In each of 25-foot x 25-foot square plots, 250 rooted cuttings were planted 1 per foot into 10 25-foot opened furrows spaced 2 feet apart. Four plots of each treatment (M and Non-M) were arranged in a randomized, complete block experimental design. Plots were separated by 6-foot unplanted alleys. Plots were immediately irrigated with solid set irrigation, and regularly irrigated and fertilized equally as per commercial practices through the course of the study. [Growth in 1992 was discounted due to different handling procedures used by each supplier.] Plots were hand-weeded through the early, fall of 1992, at which time a half-rate of Sinbar herbicide was applied. Beginning in 1993, all weed control, fertility, and irrigation was as per standard commercial practice for the peppermint in central Oregon. No insect or mite control was required in either 1992 or 1993. Because of the presence of a trace of verticillium wilt, plots were not fall or spring tilled, but were fall propane flamed in 1993 and 1994.

In 1994, plots required minimal weed control beyond application of Sinbar herbicide and occasional hand weeding. Spider mites were treated twice with Comite. Based on soil nitrate and stem nitrate analyses, meristemmed plots were fertilized more than non-meristemmed plots: All plots received 200 lb N/a on April 15 and 100 lb N/a on July 7, and meristemmed plots received an additional 50 lb N/a on May 31 and 100 lb N/a on July 13. Total N was 300 lb N/a for nonmeristemmed and 450 lb N/a for meristemmed plots. Water usage was monitored with soil gypsum blocks, and water management was determined to be the same for both meristem and non-meristem treatments. Plots were split into rolled and non-rolled sub-plots on June 29. The roller consisted of a set of closely spaced tires mounted on a bar pulled by a tractor. Plots were split again and harvested on July 25 and August 4, which was 3.5 and 5 weeks after rolling, respectively. In 1994 (in contrast to 1993), plots were irrigated between harvest dates. Main plots were meristem vs non-meristem treatments, and split plots were rolling vs non-rolling and the two harvest dates.

At several dates in June, July, and August, 20 plants per plot were sampled randomly and partitioned so that various data on lengths, nodes, branch and leaf number and distribution, and weights of various parts could be determined. Plant growth was monitored by partitioning of plant samples collected in June, July, and August. Data collected included the following:
1. Length of the main stem.
2. Length of the main stem below the first leaves.
3. Length of the main stem above the first leaves.
4. The number of main stem nodes.
5. The number of main stem nodes below the first leaves.
6. The number of main stem nodes above the first leaves.
7. The number of leaves on the main stem.
8. The node position where the stem bends (following rolling or lodging).
9. Bud development on main stem.
10. The number of branches.
11. The number of branch nodes with leaves.
12. Then number of leaves on all branches.
13. The number of buds on all branches.
14. The number of leaves on the main stem and on all branches.
15. Weight of all branches, stems and foliage.
16. Weight of main and branch stems.
17. Weight of all leaves.

Plots were harvested with a 40-inch-wide plot forage harvester, and sub-samples were collected for oil distillation in research stills located at the Central Oregon Agricultural Research Center. Oil was distilled within 10 days of harvest from hay which had been air-dried from about 10 pounds of fresh hay. Distillation was in small research stills, with exit temperature of condensate held between 110-120°F and as close to commercial procedures as could be followed (Hughes 1952). Oven dry weight of harvested hay was calculated based on the total fresh harvested weight. Oil samples were collected in small glass bottles, topped off with nitrogen and stored in a refrigerator until shipped to an oil company for oil compositional analysis. Data were analyzed by analysis of variance.

**Determination of Viral Infection**

Established meristem tip cultured plants and non-meristem tip cultured plants from the COARC field trial were handled as follows to determine whether virus(es) may be present in non-meristemmed tissue: The primary test was isolation of double stranded RNA (Morris and Morris 1987). Only virus-infected plants contain dsRNA. Also, the distinct dsRNA profile is often diagnostic for the taxonomic assignment of the virus. Consequently, the simple detection of dsRNA is proof of a virus infection. This test may be repeated in the fall of 1994 with freshly meristemmed material from the few successful attempts to regenerate plants at the COARC, and such data may be available by the time oral reports are presented at the winter meetings of 1994/95. Direct electron microscope examination of the mint tissue (as proposed in 1993) for the presence of virus-like particles was precluded due to loss of plant stock from powdery mildew in the humid climate of North Carolina during the summer of 1994. Fresh material was shipped in the fall of 1994, and additional information may be available by the time of winter oral reports.
[Electron microscopy is generally quite definitive and the shape of the virus-like particle provides additional insight as to the type of virus.]

Traditionally, viruses may be determined to be present, and sometimes their identity can be deduced, by the reaction of a set of other plant species (called an indicator series) when sap of a plant suspected to be infected is mechanically rubbed into the leaves of the indicator plants (Hollings 1959). In this case, sap from both meristemmed and non-meristemmed 'Black Mitchum' from the OSU-COARC field trial was rubbed into such an indicator series.

A general isolation procedure was attempted which partially purifies the virus from the tissues. This purification is very gentle and works for almost any virus (Lane 1986). Once purified, the nucleic acid and protein components of the virion (virus particle) can be studied using standard techniques. Two such attempts at purifying the virus were not successful. Further attempts to purify the virus and to pass it from symptomatic (non-meristemmed 'Black Mitchum' with reduced vigor) to asymptomatic mint plants (meristemmed 'Black Mitchum' with increased vigor) were aborted as a result of the loss of stock peppermint materials in the greenhouse. As indicated above, new material is being shipped to continue this and other efforts.

Results

[Data on oil composition from field plots was not available at the time this report was prepared, but will be available at a later date for future reports.]

*Field Performance*

In 1993 plants in the meristem treatment appeared taller beginning early in the season and stems remained upright later in the season compared to the non-meristem treatment. In contrast, in 1994 plants in all plots looked similar throughout the season. Nevertheless, stem and soil nitrites dropped more rapidly in plots with meristemmed plants than in the non-meristemmed treatment. Because of this, in 1994 the meristem treatment ultimately was fertilized with 150 lb/a more nitrogen than the non-meristemmed treatment (450 vs 300 lb N/a), although water usage was similar (data not shown). As a result, meristemmed plants did not appear nitrogen-deficient at harvest as they had in 1993. In 1993, water deficiency in the larger, more vigorous meristem plants was partly associated with higher water demand of these plants together with an extended drying period during a three-harvest sequence. In 1994 plots were irrigated between the two harvests, soil did not dry excessively, and the comparable growth of meristem plants suggested little differential transpiration losses between treatments. Over all treatments, 1994 plot yields averaged 59.5 lb oil/a. Treatment averages for meristem vs non-meristem treatments, rolling vs non-rolling treatments, and harvest July 25 vs harvest August 4 treatments are not shown here. This is due to either non-statistical significance of treatment differences, or because extensive interactions among treatments require that all treatment
combinations be considered at the same time. Accordingly, mean oil yields are shown in Figure 1, and mean dry hay weight yields are shown in Figure 2 for all meristem X rolling X harvest date combinations.

Ignoring the effects of rolling, the early harvest date favored meristemmed mint over non-meristemmed mint by about 9 percent for oil (Figure 1: 64.3 vs 59.5 lb oil/a, respectively), with about 21 percent more dry hay was produced in the process in meristemmed vs non-meristemmed plots (Figure 2: 7,679 vs 6,314 lb dry hay/a, respectively). Further ignoring the effects of rolling, the later harvest date favored non-meristemmed over meristemmed mint by about 20 percent (Figure 1: 71.6 vs 59.8 lb oil/a, respectively), whereas the meristem plants produced 7 percent more hay than non-meristemmed plants (Figure 2. 6,353 vs 5,933 lb dry hay/a, respectively). Although this trend suggests that early harvest favored meristemmed 'Black Mitchum' peppermint and that later harvest disfavored meristemmed 'Black Mitchum' peppermint in comparison to nonmeristemmed 'Black Mitchum' peppermint, none of the above differences were found to be statistically significant for P<0.05.

The effect of rolling cannot be separated easily from the effect harvest date and from meristem and non-meristem treatments, because the results are complicated by significant interactions among all treatments (P<0.05): The effect of rolling for the first harvest date was to eliminate any advantage of meristemming on oil yield for this date. On the other hand, non-meristemmed mint was favored by rolling on the first harvest date. Specifically, for the first harvest date, oil yield of rolled non-meristemmed mint exceeded all other treatment combinations by 14-22 percent (Figure 1. 73.0 lb oil/a for rolled non-meristem vs 57.5-64.3 for other treatment combinations, respectively). For the second harvest date, rolling greatly reduced oil yield compared to non-rolling for both meristem and non-meristemmed treatments (Figure 1.). For the second harvest, the superior treatment combination for oil yield was the non-rolled non-meristem treatment which was 38 percent better than the rolled non-meristem treatment, 20 percent better than the non-rolled meristem treatment, and 75 percent better than the rolled meristem treatment, respectively (Figure 1.). With respect to dry hay weight, differences were less pronounced (Figure 2.). Dry weight did not vary much for rolled or non-rolled treatment of non-meristemmed mint on either date (Figure 2.). Dry weight was enhanced somewhat in non-rolled meristemmed mint, but was reduced by rolling of meristemmed mint (Figure 2.). The two exceptional oil yields in the trial (Figure 1: 73.0 lb/a for the rolled non-meristem treatment on July 25, and 71.6 lb/a for the non-rolled non-meristem treatment on August 4) were not the result of greater biomass yields for those treatment combinations (Figure 2.)

Plant partition data are only briefly summarized here. In general, direct statistically significant differences (P<0.05) between meristem and non-meristemmed plants were few and were limited to mid-July or earlier. There were no stem length differences during 1994 in contrast to 1993, but meristem plants had more total leaves than non-meristem plants on June 20; and near mid-July, meristemmed plants had fewer branches than non-meristemmed plants. In general, the general
lack of statistically significant plant partition data support the relative lack of yield differences between meristem and non-meristem treatments in the absence of rolling.

There were general effects of rolling on plant growth irrespective of meristem and non-meristem treatments. Few of these effects were seen prior to the first harvest on July 25. Specifically, the position at which the stem was bent due to rolling on June 29 clearly was measured on July 18, but the only other non-interactive significant effect on that date was that total stem length was greater in rolled vs non-rolled treatments. On the other hand, many effects of rolling were observed at just prior to second harvest on August 4. Of the 17 parameters measured, 9 were statistically significant differences (P<0.05) on August 1 with respect to rolling vs non-rolling. Several of these plant partition parameters may relate to the general under-performance of plants in both rolled meristem and rolled non-meristem treatments compared to non-rolled treatments on August 4, but they are difficult to relate to yield in a straightforward manner. For example, on August 1 plants sampled from rolled plots had more leaves than plants from non-rolled plots, even though yields were higher for non-rolled plots.

There were several statistically significant interactions (P<0.05) among rolling X meristem treatments on plant partition parameters for both July 18 (one parameter) and August 1 (three parameters). Such differences again support the fact that statistically significant yield difference interactions were measured on the two harvest dates, but the plant partition parameter data offer no ready explanation of these yield differences. For example, the length of the stem above the first leaf was greater for the rolled non-meristem treatment compared to other treatment combinations on July 18, and this treatment had the superior oil yield for July 25. Similarly, the length of the stem above the first leaves was greatest for the non-rolled non-meristem treatment on August 1, and this treatment had the superior oil yield for August 4. Whereas length of the stem above the first leaf may prove to be an indicator of yield differences, it is not clear why this should be the case. Clearly, more work on plant partitioning as a tool for explaining yield differences is required. Increased sample numbers may be required.

**Virus Detection**

Molecular biological analyses indicated that non-meristemmed 'Black Mitchum' peppermint taken from the COARC field trial contained a double-stranded RNA (dsRNA) entity which was not found in meristemmed 'Black Mitchum' peppermint taken from the same trial. Such dsRNA is typical of single-stranded RNA virus replication. When plant sap from either meristemmed or non-meristemmed plants was rubbed into a range of indicator plant species, sap from non-meristemmed plants produced local lesions on several species, whereas sap from meristemmed plants produced no such symptoms. Species displaying local lesions included *Gomphrena globosa* and *Chenopodium amaranticolor*, two common indicator species. Such results further suggest the presence of a virus in non-meristemmed plants. No virus has yet been characterized, and it has not yet been proved that the putative virus is responsible for differences in growth and field performance measured in 1993 and 1994.
Discussion

Plant material in field plots described above originally was meristemed in 1991, propagated in greenhouses 1991 and 1992, planted in the field in 1992, and observed in the field from 1992 through 1994. Laboratory and greenhouse viral analyses of field material collected at random in the spring of 1994 strongly suggest the presence of a virus in non-meristemmed mint that seems absent from meristemmed mint. Although peppermint field performance differences measured in 1993 and 1994 are consistent with the presence or absence of a virus, it has not been proved that the virus is the causal agent, nor has the virus been characterized. The absence of a virus from meristemmed plants held in the field for two to three seasons suggests that plants have not become quickly or widely re-infected either in the propagation greenhouse or in the field in central Oregon. [In fact, if re-infection had been very rapid, or if other viruses were present in spite of meristem tip culture, both sources of plant materials would have appeared virus-infected and interpretation would have been less straightforward.]

With respect to yield in 1993, meristem plants distinctly under-performed compared to non-meristemmed plants grown in the COARC field trials. There was less oil and more biomass to harvest in meristemmed vs non-meristemmed that year. It further was observed in 1993 that meristemmed plants were nitrogen and moisture deficient when handled identically to non-meristemmed mint, which could have accounted for some of the under-performance. Effort was expended in 1994 to fertilize according to nitrogen usage, and not to place plants under unnecessary moisture stress near harvest. Whether due to better fertility and soil moisture or to other effects in 1994, yields were comparable for meristemmed and non-meristemmed mint which was not otherwise treated differently (i.e. rolled). This data still does not support the commercialization of meristemmed mint, because there was no yield advantage, plus meristemmed mint required more nitrogen than non-meristemmed mint.

The impact of rolling on meristemmed mint generally was negative, even though rolling in 1994 was included in an attempt to stimulate branching of meristemmed peppermint as a result of observations of poor branching in 1993. All positive yield effects of rolling were seen on non-meristemmed peppermint, and this only when combined with early harvest. When harvest was delayed, the yield of non-meristemmed mint was reduced with rolling.

On the other hand, meristemmed mint did out-yield non-meristemmed mint on the early harvest date. Even though this difference was not statistically significant (P<0.05), it may point to a need for even earlier harvest of meristemmed plants vs later harvest for non-meristemmed plants. In the future, additional testing of very early harvest or even double cutting may prove advantageous for meristem vs non-meristemmed mint. This concept fits with experience with double cutting of meristemmed mint in Montana in 1994, where early harvest and double-cut meristemmed mint performed substantially better than later-cut meristemmed mint (Leon Welty, Montana State University, personal communication). Further, anecdotal accounts suggest that non-meristemmed
mint often cannot sustain such double cutting and sufficiently develop in the fall to have the energy reserves necessary to maintain vigor over the winter and perform well in future years. If this is the case, being more vigorous, meristemmed mint may better tolerate double cutting.

References


Fig. 1 1994 Oil Yields for Meristem X
Rolling X Harvest Date Treatments

lbs oil/ac

Fig. 2 1994 Dry Hay Yields for Meristem X
Rolling X Harvest Date Treatments

lbs dry hay/ac (x1000)