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

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Abstract

Visually, 'Black Mitcham' peppermint originating from meristem tip culture appeared identical to non-meristemmed 'Black Mitcham' in the field in 1995, the third full production year in the COARC field trial of meristemmed mint. Although extensive measurements of height, branching, leaf number, etc. were not made in 1995 compared to past years, a measure of height and branching in late June indicated no differences between meristemmed and non-meristemmed plants. Measurements of nitrogen and water usage also were comparable for meristemmed and non-meristemmed plants in 1995. Nevertheless, meristemmed 'Black Mitcham' continued to have lower yields at harvest compared to peppermint in plots planted with non-meristemmed 'Black Mitcham'. Although the dry weight of hay was comparable (only slightly higher in meristemmed plots), oil yield for meristemmed 'Black Mitcham' was only 78 percent of oil yield of non-meristemmed 'Black Mitcham', and the oil yield per unit of dry weight was substantially enhanced for non-meristemmed mint over that for meristemmed mint. The exceptional vigor observed in meristemmed 'Black Mitcham' in COARC field plots in 1993 lessened during 1994, and was not present at all in 1995. The only parameters that remained constant over these three years were oil yield (non-meristemmed peppermint continues to outperform meristemmed peppermint by 20 percent or more) and oil yield per unit of dry weight.

Plant growth differences in the greenhouse in North Carolina seem to mirror growth in the field in central Oregon, in that meristemmed and non-meristemmed plants are more similar in growth (e.g. stem length, leaf area) now than a year or more ago. Tests indicated a very low concentration of virus can now be detected in meristemmed material, both in material sent previously and material supplied in early spring 1995. Specifically, there are wide differences in the amounts of local lesions that appear on sap-inoculated indicator plants from these sources, indicating wide sap concentration differences. Although actual transmission of virus back into meristemmed peppermint cannot be totally excluded as a possibility, this is not likely from what is known of this class of virus (extremely slow growing, no known vectors, not spread by contact, etc.). These field and greenhouse observations are much more consistent with the probability that some virus was retained during the original meristem tip culture process, and that virus titer (concentration in plant) is only slowly re-establishing itself in the meristemmed plants. In other words, although the meristemmed plants initially appeared virus-free and behaved virus-free, they were not truly virus-free. Presumably, gradual increase of the virus in plants in both field and greenhouse explains the gradual loss of plant vigor.

This virus continues to seem similar to other Capilloviruses, which are relatively unfamiliar and unstudied plant viruses. These are known to exist in low concentration, are very slow growing and are difficult to handle. This one from peppermint is proving very difficult to purify in

quantity. This has slowed characterization and antibody development, although we are making progress.

Introduction

Meristem tip culture is a process in which a small part of differentiating plant tissues at the tip of the growing point of a bud is regenerated into a complete plant in a sterile environment and with the assistance of a supply of nutrients and plant hormones (Murashige and Skoog, 1962). Meristem tip culture generally preserves the genetic identity of the plant type and is not a cause of genetic variability, as may be seen for processes in which only undifferentiated embryonic cells or differentiated single cells (which revert to an undifferentiated state) are regenerated back into mature plants (Karp, 1991, Caligari and Shohet, 1992). Systemic plant pathogens move most quickly into growing point regions of plants via sugar-conducting tissues (phloem) or via water conducting tissues (xylem), and lag in their cell to cell movement into the rapidly growing tip because phloem and xylem are not yet differentiated. Thus, meristem tip culture commonly is used to regenerate propagation stock that is free of systemic pathogens. The meristem tip culture process requires some skill to avoid carrying along infected tissues, and pathogens vary in their ability to penetrate into the critical tissues to be taken during the process. Commonly, additional steps are included in the process that further limit systemic movement of pathogens, and/or which reduce the concentration or growth of pathogens in the plant. Such additional steps may include heat-treatment or treatment with chemicals that actively suppress pathogen growth. Thus, successful production of pathogen-free propagation stock requires experience with the plant materials and pathogens involved, and a means of verification that regenerated plants are indeed pathogen-free (Wright, 1988). Commonly, systemic plant pathogens cause at least a reduction in growth if not stronger, debilitating symptoms, although some viruses may only weakly affect plant growth. Meristem tip culture of peppermint has been reported on a number of times (Geslot, *et al.,* 1989, Holm, *et al.,* 1991, Mariska, 1987, Repcakova, *et al,* 1986, Rodov and Davidov, 1987), but in none of these reports were growth differences or pathogens discussed, which suggests that none were observed. Field observations from the early 1990s, noted that commercially-meristemmed 'Black Mitcham' peppermint in Montana grew more vigorously than rootstock propagated more traditionally In light of this, we acquired rooted cuttings of both meristemmed and non-meristemmed 'Black Mitcham', each from a different commercial greenhouse propagator, but for which each had a common original source or propagation stock. We began to compare these materials in 1992-93 in the field, and at the same time wished to pursue the hypothesis that some systemic pathogen may have been eliminated by the commercial meristem tip culture process. Our field trials from 1993 and 1994 confirmed Montana grower observations of strong growth enhancement and increased hay yield in meristemmed 'Black Mitcham', but also supported growers' experience that meristemmed 'Black Mitcham' under-performed with respect to oil yield (Crowe, *et al.,* 1995).
Systemic plant pathogens include many viruses, some bacteria (including highly fastidious types

that do not grow free of plant cells), and a few fungi. As a virus or viruses seemed most likely to be involved, this group was first considered. Last year we reported: finding double-stranded virus RNA (proof of presence of most plant viruses) from non-meristemmed peppermint, but which could not be detected from meristemmed peppermint, electron microscope pictures of a virus from meristemmed peppermint that could not be seen in non-meristemmed peppermint, and positive responses (local lesions) from sap-inoculated indicator plants using nonmeristemmed 'Black Mitcham' but not meristemmed peppermint, when sap from these two sources were rubbed into the indicator plants. Only one virus seemed to be present (Crowe, *et al.,* 1995). We considered these steps proof that a virus was present in non-meristemmed 'Black Mitcham' in our test plots, that absent from meristemmed 'Black Mitcham' in adjacent plots. These steps do not constitute proof that the virus detected was responsible for the growth differences, as (at least theoretically) other undetected agents could be involved. In 1995, it was proposed to purify the virus, develop antiserum to it for diagnostic use, and confirm Koch's postulates by re-inoculating virus-free peppermint to assess the response of reinfection, i.e., would such reintroduction cause the mint to lose vigor and enhance oil yield?

Methods and Materials

Plots established in 1992 from rooted cuttings were continued through 1995. Sources of 'Black Mitcham' are described in previous reports (Crowe, *et al.,* 1995). In 1993 and 1994, nitrogen usage was greater for meristemmed than non-meristemmed 'Black Mitcham', as determined by petiole and soil nitrogen analyses. In 1995, nitrogen use again was monitored. All other field management was similar for both meristemmed and non-meristemmed 'Black Mitcham'. Plots were harvested and mint was distilled as per earlier years (Crowe, *et al.,* 1995). Plots were not further divided into additional treatments as in 1994, and there was only one time of harvest.

Oil was distilled in mini-stills from dry, long-stemmed hay (Hughes, 1952), oil was collected into vials, and topped off with nitrogen then refrigerated prior to shipment to Wm. Leman, Inc, Bremerton, IN, for compositional analysis.

In the laboratory, various methods were used in an attempt to increase the concentration of the virus in the sap of either peppermint or non-peppermint species. High plant concentration facilitates purification of virus and production of animal antibodies toward development of a reliable antiserum for virus detection.

In mid-1995, additional field-grown material from Oregon field plots was supplied to the lab in North Carolina for inspection and determination of virus infection status as per techniques described earlier (Crowe, *et al.,* 1995). It was thought that field-grown meristemmed material might be re-infected based on reduction in vigor in 1994 and early 1995.

Results

Nitrogen usage on meristemmed and non-meristemmed 'Black Mitcham' was comparable in 1995. On this basis, fertility was kept identical for both treatments in 1995. Stem height and branching number (determined as per Crowe, *et al.,* 1995) were evaluated in the third week of June 1995, and no differences were observed. In general, all casual observation suggested growth was identical for both treatments.

Harvest was August 8, 1995. Harvest data is shown in Table 1. As in past years, meristemmed peppermint had less total oil and less oil per unit of dry harvested hay, although the trend toward more dry matter for meristemmed peppermint was reduced over previous years. Oil characters evaluated are shown in Table 2, along with percentage composition. No statistically significant differences ($p<0.05$) were found between meristem and non-meristemmed 'Black Mitcham' for any component.

This virus continues to seem similar to other Capilloviruses, which are a group of relatively unfamiliar viruses. These are known to exist in low concentration and are difficult to work with. Dr. Lommel's lab is finding it a much more tedious and complicated procedure than normal to obtain sufficient virus to generate an antibody. In the event that we cannot obtain enough pure virus, we can cDNA clone the viral nucleic acid from a minimal amount of genomic RNA or from dsDNA (Jelkmann, *et al.,* 1989), which we have already established that we can obtain. The viral capsid protein gene will be identified in the clone and then overexpressed in a bacterial expression system (Giesman-Cookmeyer and Lommel, 1993). We can then obtain unlimited amounts of viral protein to immunize rabbits and create a virus-specific antibody.

Plant growth differences in the greenhouse seem to mirror growth in the field in central Oregon in that, compared to a year or more ago, meristemmed peppermint is becoming less vigorous and smaller-leafed (more like non-meristemmed peppermint held in the same greenhouse). It appears as if a very low concentration of this virus is showing up in meristemmed material, both the material sent previous to 1995 and in material supplied in early spring 1995. Specifically, there still are distinct differences in the amounts of local lesions that appear on sap-inoculated plants from meristemmed vs. non-meristem plants. This indicates that a wide sap concentration difference persists between meristemmed and non-meristemmed 'Black Mitcham' (even though the most concentrated is still quite low for purposes of purification). Capilloviruses are not known to be transmitted by arthropod vectors nor by mechanical means. As stated earlier, they seem to grow very slowly in all known hosts. Most likely, the finding that a trace of virus is appearing in meristemmed plants suggests that the original meristem tip culture process failed to eliminate all of the virus, and that it is slowly increasing again in these plants.

The finding that our meristemmed plants are not likely truly virus-free complicates eventual confirmation of the hypothesis that the detected Capillovirus in fact causes the observed reduction in vigor and increased oil yields for non-meristemmed plants in contrast to the distinctive growth of the meristemmed plants. This is because we have no verified virus-free

material to infect with the virus, nor is there additional material to leave virus-free for comparison purposes. Discussion of this situation, and potential resolution, will be addressed in the 1996 grant proposals.

Discussion

We remain confident that a virus has been detected in non-meristemmed 'Black Mitcham' peppermint, and that it may exist in our meristemmed material at a much reduced concentration. It seems likely that a small amount of virus remained in the original meristem tip cultured 'Black Mitcham', and this trace was propagated through to the rooted cuttings used in the field plantings, which were themselves the source of lab material used for virus detection. We argue that the original trace was enough less than the amount "normally" occurring in nonmeristemmed 'Black Mitcham' to account for the strong growth and performance differences seen during 1993, but that this trace has been increasing during 1994 and 1995. With this increase, the growth differences seen in 1993 have subsided substantially, although oil yield remains quite different. Nevertheless, this argument needs to be confirmed by completion of Koch's postulates, where we re-insert the virus into truly virus-free mint to re-establish symptomology.

Associating the presence of this difficult-to-handle virus with enhanced field performance of 'Black Mitcham' peppermint implies that its presence is an advantage to the peppermint industry. Our own tests to try to enhance performance of meristemmed 'Black Mitcham' suggests it is better harvested earlier in the season than non-meristemmed peppermint (Crowe, *et al.,* 1995). This was demonstrated even better by Leon Welty in Montana in 1994 and 1995 (Leon Welty, Montana State Univ., personal communication). It may prove important to ascertain the virus infection status and virus-free performance of other peppermint varieties and spearmint. May be especially important to ascertain the virus status of any mint regenerated from meristem tip culture (perhaps there are other pathogens one might wish to eliminate by this process!), or from single cell culture in various biotechnology applications. For these reasons, we still believe it important to develop an antiserum tool for rapid, easy, and positive detection and to characterize this virus.

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TABLE 1: Harvest data for meristem and non-meristem, COARC 1995

Treatment	Dry Weight lb/a	Oil Yield lb/a	Oil/Dry Wt. Ratio x 100
Non-meristem	5,688	72.9	1.36
Meristem	5,430	56.9	0.99
Stat. Sig.[1]	NS	p<0.06	p<0.04

1. Statistical significance level: NS = not significant at 10% level or lower; other p levels listed.

TABLE 2: Oil composition from meristem and non-meristem peppermint, COARC 1995

Treatment	Total Heads	Total Ketones	Total Menthol	Mentho furan	Menthone	Menthol	Esters	Pulegone
Non-meristem	8.8	30.2	39.6	22.0	25.6	32.3	4.5	1.2
Meristem	8.9	28.6	41.4	21.0	24.1	33.8	3.9	1.3
Stat. Sig)-	NS	NS	NS	NS	NS	NS	NS	NS

1. Statistical significance level: NS = not significant at 10% level or lower.