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# Plant Regeneration of Peppermint, *Mentha piperita*, from the Hairy Roots Generated from Microsegment Infected with *Agrobacterium rhizogenes*

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## Abstract

Microsegments from peppermint, *Mentha piperita*, were placed on supplemented Murashige & Skoog medium and infected with *Agrobacterium rhizogenes*. The hairy roots resulted from peppermint were cultured on various media in order to regenerate plants. The hairy roots formed a callus on Gamborg B5 medium containing 1 $\mu$M 1-naphthaleneacetic acid (NAA), 10 $\mu$M N-(2-chloro-4-pyridyl)-N'-phenylurea (4-CPPU), and 10% coconut powder in the dark. Shoots were regenerated from the calli cultured on 1/2 Murashige and Skoog medium containing 1 $\mu$M NAA and 10 $\mu$M 4-CPPU during a 16-h photoperiod. All plants were recovered following rooting of the shoots in B5 medium without hormones. The polymerase chain reaction (PCR) analysis of the genomic DNA showed that all regenerated plants had the region from the *rolA* to *rolB* gene.

**Key words:** *Agrobacterium rhizogenes*, hairy roots, *Mentha piperita*, regeneration.

Mint plants belong to the Labiatae family and account for more than 25 species, not including the numerous varieties obtained by spontaneous hybridization (Kokkini, 1991; Banthorpe, 1996). Mint plants are crops of considerable commercial value and widely cultivated for their essential oil. This valuable product is mainly composed of monoterpenes and is largely used for the production of food, cosmetics and pharmaceuticals. Because the quality of oil depends on the composition of the monoterpene, it is of great interest to obtain a strain producing a better quality oil.

Technological advances in genetic engineering have resulted in the potential for utilizing biotechnology to improve mint. The transformations of mint plants using the soil bacterium *Agrobacterium tumefaciens* have been reported (Spencer *et al.*, 1990; Spencer *et al.*, 1993; Diemer *et al.*, 1998; Niu *et al.*, 1998; Diemer *et al.*, 1999; Krasnyanski *et al.*, 1999; Niu *et al.*, 2000; Diemer *et al.*, 2001; Mahmoud and Croteau, 2001). However, there have not been any reports using *A. rhizogenes*.

Following inoculation of a susceptible host, *A. rhizogenes* induces a disease known as 'hairy root syndrome'. This neoplastic disease is due to T-DNA, a portion of the Ri (root inducing) plasmid (Chilton *et al.*, 1982; Zambryski *et al.*, 1989). This transformation process delivers a valuable by-product, 'hairy roots', which are tissues capable of unlimited propagation in culture media. Hairy root cultures have several advantages that have promoted their use in plant biotechnology; i.e., fast growth, genetic and biochemical stability, and growth in hormone-free media. Because of these advantages, the hairy root culture serves as a model system for studying plant metabolism and physiology, and also as a technical alternative to cell suspension cultures for producing therapeutics and specialty chemicals.

To apply these advantages of the hairy roots to mint plants, the optimum conditions for plant regeneration from the hairy root must be determined for mint, because essential oils are produced mostly in the leaves and stem of the plant, but not in the hairy root. In this paper, we report the regeneration of *M. piperita* through the calli from the hairy roots and shoots.

The *M. piperita* used in this experiment was

**Table 1** Effect of growth regulators on callus induction

| Auxin[1] | Cytokinin[2] | Other | Callus formation[3] |
|---|---|---|---|
| 2,4-D | Kinetin | | + |
| NAA | 4-CPPU | | - |
| NAA | 4-CPPU | 10% coconut powder | ++ |

Growth condition: B5 medium with 1.0% sucrose, 0.5% gellan gum and pH 5.8 in the dark
[1] auxin concentration was 1 $\mu$M
[2] cytokinin concentration was 10 $\mu$M
[3] -: not induced, +: induced, ++: vigorously induced

cultured at the Experimental Farm of Shinshu University. Shoots of the plant were surface sterilized in 70% ethanol for 1 min followed by treatment with 10% sodium hypochlorite containing 0.1% Tween 80 for 20 min. Microcuttings of *M. piperita* were placed on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 30 g $l^{-1}$ sucrose and 12 g $l^{-1}$ agar, pH 5.8. The shoots were repropagated by axillary at 26 ℃ during a 24-h photoperiod (2500 lx, white fluorescent tubes).

*A. rhizogenes* MAFF0301724 was used for infection of the mint. Internodes (2 cm parts) from the *in vitro* grown plants were pierced into Gamborg B5 (B5) medium (Gamborg *et al.*, 1968) with 10 g $l^{-1}$ sucrose and 5 g $l^{-1}$ gellan gum (Wako Pure Chemicals Industries, Ltd., Osaka, Japan), and inoculated with a needle soaked in the inoculum.

For the elimination of bacteria, the hairy roots were cultured in 1/2 B5 medium containing 500 mg $l^{-1}$ cefotaxime, 10 g $l^{-1}$ sucrose and 5 g $l^{-1}$ gellan gum at 26 ℃ in the dark for 1 week, then propagated in the 1/2 B5 medium without cefotaxime and gellan gum, at 80 rpm for 3 weeks (**Fig. 1A**). By cultivating the hairy roots at 26 ℃ on 802 medium containing 10 g $l^{-1}$ peptone(Becton Dickinson and Company, Sparks, MD), 2 g $l^{-1}$ Yeast extract(Becton Dickinson), 1 g $l^{-1}$ $MgSO_4 \cdot 7H_2O$, 15 g $l^{-1}$ agar (Nacalai tesque, Kyoto, Japan) and 2 g $l^{-1}$ glucose according to the procedure recommended by the Institute for Fermentation(Osaka, Japan), it was confirmed that the bacteria were eliminated from the hairy roots.

For inspection of the callus formation, the effect of the growth regulators on the callus induction was tested. After 4 weeks, the degree of callus formation was estimated. The callus formation was the most effective on B5 medium containing 1 $\mu$M 1-naphthaleneacetic acid (NAA, Nacalai tesque), 10 $\mu$M N-(2-chloro-4-pyridyl)-N'-phenylurea (4-CPPU, Sigma-Aldrich, St. Luis, MO), and 10% coconut powder (Research Institute of Industrial Bioscience, Iwata, Japan) in comparison with the other two (**Table 1** and **Fig. 1B**).

**Table 2** Effects of photoperiod on callus induction

| Photoperiod(h) | Weeks of culture | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| 0 | + | ++ | ++ | ++ |
| 16 | - | + | ++ | ++ |
| 24 | - | + | + | + |

Medium: B5 medium with 1 $\mu$M NAA, 10 $\mu$M 4-CPPU, 10% coconut powder, 3.0% sucrose, 0.5% gellan gum, pH 5.8
'-': not induced, '+':induced, '++': vigorously induced

Additionally, for testing the effect of the photoperiods, it was indicated that a longer photoperiod showed less induction of callus formation (**Table 2**).

Faure *et al.* (1998) reported *in vitro* shoot regeneration from peppermint leaf disks. They used MS medium as the basal medium. In our experiment, MS medium was also used for the examination of shoot formation. Shoot formation was tested for various concentrations of medium salt, NAA and 4-CPPU, and was also in the presence of coconut powder (10%). A portion of the formed calli was transplanted onto testing medium. After 6 weeks, the degree of shoot formation was estimated (**Table 3**). Shoots favorably generated on 1/2 MS medium with 1 $\mu$M NAA, 10 $\mu$M 4-CPPU, 1% sucrose, 0.5% gellan gum, and pH 5.8 at 26 ℃ during a 16-h photoperiod (2500 lx, white fluorescent tubes) (**Fig. 1C**). At the same proportion of NAA: 4-CPPU, shoots were generated in the section with 1 $\mu$M NAA and 10 $\mu$M 4-CPPU while shoot formation did not occur in the section with 0.1 $\mu$M NAA and 1 $\mu$M 4-CPPU. The same phenomenon was observed between the section with 0.1 $\mu$M NAA and 10 $\mu$M 4-CPPU and the section with 0.01 $\mu$M NAA and 1 $\mu$M 4-CPPU. These results suggested that the amount of 4-CPPU was important for shoot formation.

Shoots generated root on hormone free B5 medium within 1-2 weeks (**Fig. 1D**). Plantlets with well developed shoots and roots were transferred to

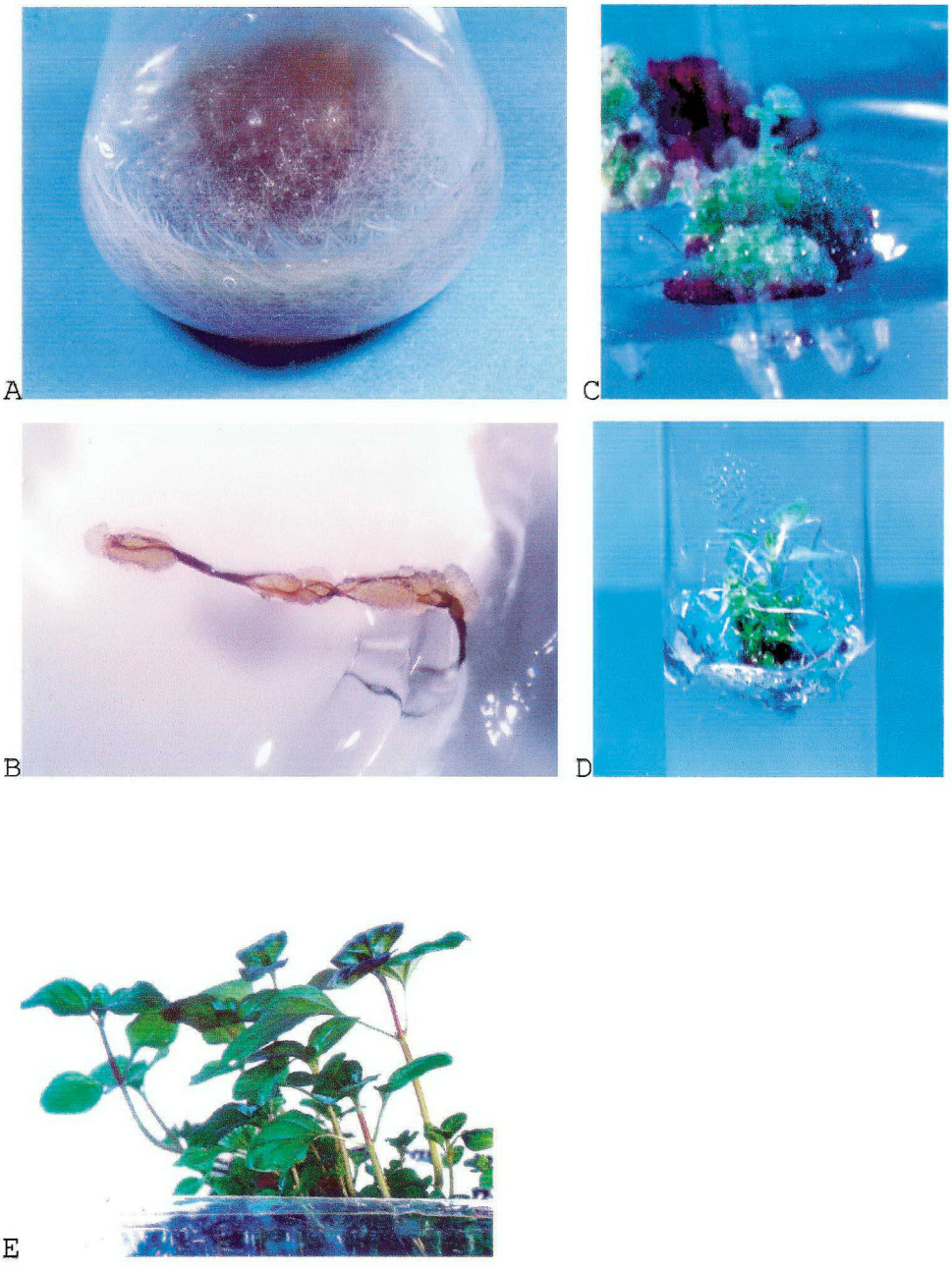


**Fig. 1** Plant regeneration of *M. piperita*. (A) Hairy roots cultured in 1/2 B5 medium for 3 weeks. (B) Callus from hairy root on B5 medium containig 1 $\mu$M NAA, 10 $\mu$M 4-CPPU, and 10% coconut powder after 4 weeks. (C) Shoot from calli on 1/2 MS medium containing 1 $\mu$M NAA, 10$\mu$M 4-CPPU after 6 weeks. (D) Plantlets with well-developed roots and shoots grown on hormone-free B5 medium after a 4 week cultivation. (E) Plantlets on vermiculite immersed in water after 2 weeks in a capped containner for acclimatization.

**Table 3** Effect of the concentrations of growth regulators on shoot formation in Murashige & Skoog basal medium

| Medium salt[1] concentration | NAA ( $\mu$M) | 4-CPPU ( $\mu$M) | Coconut powder | Shoot Formation[2] |
|---|---|---|---|---|
| 1 | 1 | 10 | | + |
| 1 | 0.1 | 10 | | + |
| 1 | 0.01 | 10 | | - |
| 1 | 0.1 | 1 | | - |
| 1 | 0.01 | 1 | | - |
| 1 | 0 | 1 | | - |
| 1/2 | 1 | 10 | | ++ |
| 1/2 | 1 | 1 | 10% | - |

Growth condition: medium with 1.0% sucrose, 0.5% gellan gum and pH 5.8 at 26 ℃ during a 16-h photoperiod

[1] MS medium salts

[2] -: not induced, +: induced, ++: vigorously induced

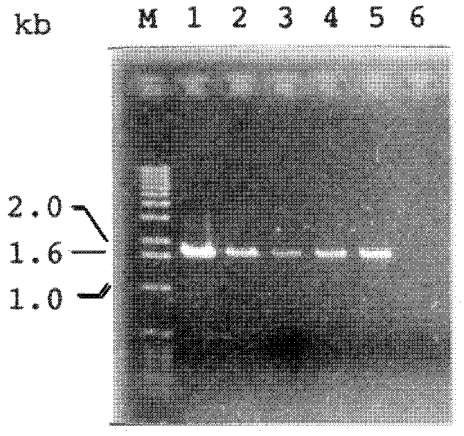


**Fig. 2** PCR analysis of regenerated plants from hairy roots. Genomic DNA was prepared from regenerant leaves according to the method of Doyle and Doyle (1987). The PCR mixture contained 1.25 $\mu$g DNA, 0.1 $\mu$M of each primer, 0.2 mM dNTPs, 1 x PCR buffer, and 0.625 units of Taq DNA polymerase (Ampli Taq DNA polymerase, TaKaRa Bio inc., Kyoto, Japan) in a final volume of 25 $\mu$l. The primers used to amplify the region between *rolA* and *rolB* were 5'-GTGCTTT-CGCATCTTGACAG-3' and 5'-TCTCGCGA-GAAGATGCAGAA-3'. The amplification reaction was performed as follows: preheat at 94 ℃ for 5 min; 30 cycles at 94 ℃ for 1 min, at 58 ℃ for 30 s, and at 72 ℃ for 1.5 min; and a final extension at 72 ℃ for 7 min. The PCR products were separated on 1.0% agarose gel by electrophoresis. M, size marker (1kb DNA Ladder, Invitrogen Corp., Carlsbad, CA); 1, Ri plasmid; 2-5, regenerated plants; 6, uninfected peppermint.

sterile vermiculite immersed in water. After 2 weeks in a capped containner for acclimatization, they were directly exposed to green house conditions (**Fig. 1E**). The plantlets grew well to produce flowers.

The regenerated plants showed the hairy root syndrome; short internodes, wrinkled leaves, and increasing rooting ability. To detect the integrated DNA fragments, the polymerase chain reaction (PCR) was performed with the genomic DNA of the regenerated plants. The amplified fragments were fractionated on 1.0% agarose gel (**Fig. 2**). An about 1.6 kb fragment, which included the region from the *rolA* to *rolB* gene, was detected in all the regenerated plants (**Fig. 2**, lane 2-5). No DNA fragement was amplified in the uninfected peppermint (**Fig. 2**, lane 6).

In this paper, we established the method of regeneration from the hairy roots of peppermint. The appearance of hairy root syndrome does not seem to be disadvantage for obtaining the essential oils from the regenerants of Mentha. The regenerants were dwarfing and allowed to facilitate treatment of plants *in vitro*. Further experiments are now in progress to apply this method to other *Mentha* species, and to introduce a specific gene into the peppermint with a binary vector.

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