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Changes in morphological phenotypes and essential oil components in lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.) transformed with wild-type strains of *Agrobacterium rhizogenes*

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ABSTRACT

Hairy roots were induced from leaf-derived calli of lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.) by infection with wild-type strains of *Agrobacterium rhizogenes*, A-5 (MAFF 02-10265) and A-13 (MAFF 02-10266). A-5-inoculated calli formed hairy roots more efficiently than A-13 ones. The transgenic shoots could be obtained from hairy root segments mediated by each *Agrobacterium* strain. However, different plant growth regulators were required for efficient adventitious shoot formation in each strain. In A-5, the most efficient adventitious shoot formation rate of 23.8% was observed in a medium with 4.4×10^{-6} M of 6-benzylaminopurine. On the other hand, a significantly higher rate of 13.2% was detected in a medium with 4.0×10^{-7} M of *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea in A-13. Most of the regenerated plants showed dwarfism with closed internodes and extensive lateral branching, which were typical characteristics of 'hairy root syndrome'. On the other hand, only nine of the 45 regenerated plants formed flower buds in early June, a delay of about one month compared with nontransgenic regenerated plants. The floral stalks and spikes of these plants were very short, resulting in a compacted form. Many regenerants showed a significantly lower productivity of essential oil than nontransgenic regenerants. Moreover, the relative percentage of the linalyl-cation-derived compounds, linalool and linalyl acetate, decreased in most of the regenerated plants. Compact plants with the ability of flower bud formation are assumed to be valuable not only for lavandin breeding, but also for clarifying the interaction between *rol* genes expression and essential oil production.

1. Introduction

Lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.) belongs to the Lamiaceae family and is a perennial species native to the Mediterranean region. Essential oil obtained by steam distillation of its floral spikes is widely used in pharmaceutical preparations, perfumery and cosmetics. It is a naturally occurring interspecific hybrid between true lavender (*L. angustifolia* Mill.) and spike lavender (*L. latifolia* Medik.), and shows increased vigor with respect to the essential oil content. Being of industrial interest for essential oil production, lavandin is extensively cultivated and is the most widely grown lavender, dominating the world's production of lavender oil (Upson and Andrews, 2004). The heterosis is also found in morphological traits, such as plant height, width and floral spike length, that are suitable for industrial cultivation, but not for potting (Upson and Andrews, 2004). Consequently, the introduction of a dwarf trait is desirable for gardening, although hybrid sterility prevents the use of conventional crossbreeding. In *Lavandula*, dwarf mutants were screened from somaclones regenerated from callus tissue (Tsuro et al., 2001). However, variants with desirable traits are obtained randomly, decreasing breeding efficiency.

Agrobacterium rhizogenes is a well known soil-borne bacterium that causes hairy root disease in dicotyledonous plants (Chilton et al., 1982). These bacterium-infected plant tissues form hairy roots by transferring T-DNA of the Ri plasmid into the plant genome. Regenerated plantlets that form hairy roots frequently show phenotypic and physiological alterations, e.g. dwarfness, leaf wrinkling, disrupted apical dominance and a confusing photoperiodic response (Gennarelli et al., 2009; Inoue et al., 2003; Kiyokawa et al., 1992; Koike et al., 2003), which comprise the 'hairy root syndrome'. These phenotypic changes have a negative effect in several crops; on the other hand, a new variety could be created in a selected genetic background for ornamentals. In particular, dwarfness may be of interest for genetic improvement of lavandin. In a previous experiment, by inoculating wild-type *A. rhizogenes* with an alien binary plasmid, we could obtain transgenic lavandin plants with *gus* gene expression from hairy root segments (Tsuro et al.,

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2009). However, any phenotypic changes and variation in essential oil components have not been described in mature plants.

Here, we describe the comparative effect of wild-type strains of *A. rhizogenes* on hairy root formation and plant regeneration in lavandin. We also characterize the morphological phenotypes of matured regenerated plantlets. In addition, content and composition of essential oils in those plants are discussed.

2. Materials and methods

2.1. Plant material and bacterial strains

Lavandin plants used in this experiment were cultivated in a greenhouse at Meijo University (Nagoya, Japan). Callus induction from leaf explants was carried out according to Tsuro et al. (2009).

For inoculation, two strains of mikimopine producing wild-type Japanese *A. rhizogenes*, A-5 (MAFF 02-10265) and A-13 (MAFF 02-10266) (Daimon et al., 1990; Horikawa and Iida, 1993), were used. Both bacteria were cultured in liquid YEB medium (Vervliet et al., 1974) on a rotary shaker (150 rpm) at 28 °C for 16 h before infection.

2.2. Hairy root induction and plant regeneration

Leaf-derived calli were immersed in an *Agrobacterium* suspension (OD_{600} = 0.1) supplemented with 100 mM acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) for 10 min, and then blotted onto sterilized filter papers. Co-cultivation was conducted on Murashige and Skoog (1962) (MS) solid medium supplemented with 5×10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D) at 25 °C under dark conditions for three days. Calli were then transferred to 1/2 MS solid medium (MS macronutrients at half strength) supplemented with 500 mg l^{-1} carbenicillin, and cultured at 25 °C in the dark, to induce hairy root and eliminate bacteria.

After six weeks of culture, hairy roots were excised from calli and cut into short segments (about 1 cm in length), and placed on MS medium containing 250 mg l^{-1} carbenicillin, supplemented with 4.4×10^{-6} M of 6-benzylaminopurine (BA) or 4.0×10^{-7} M of *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU), the best shoot and multiple shoot induction medium for *Lavandula* from Tsuro et al. (1999, 2000). For shoot induction, they were then cultured at 25 °C under continuous fluorescent light (3000 lux). Adventitious shoots that formed on the surface of hairy-root-derived calli were excised and transferred onto 1/2 MS medium without any antibiotics or plant growth regulators, and cultured in the same conditions as for shoot induction for rerooting. The subcultures for shoot and root induction were conducted every two weeks.

Regenerated plantlets were transplanted on vermiculite moistened with 1/2000 Hyponex® solution in top-sealed plastic boxes (5 cm × 5 cm × 10 cm), and cultured at 25 °C under continuous fluorescent light (3000 lux) with gradually opening cover seals for two weeks. After acclimatization, plants were transferred to soil in pots (⌀ 15 cm × 12.5 cm) and cultivated in an outside facility from March to April 2008.

2.3. Morphological observations

Comparative morphological observations were conducted for cuttings propagated from regenerated plants, three to four cuttings from each plant, and 20 nontransformed plants regenerated from leaf-derived calli. After one year of cultivation, on 1 May 2009, the plant height and the degree of internode thickening were evaluated in each plant. In addition, the first day of flower bud formation was recorded in the second year of cultivation in 2010.

2.4. PCR and Southern blot hybridization analysis

Total genomic DNA of regenerated plants was extracted using the procedure of Tsuro et al. (2009). To confirm the presence of Ri T-DNA in regenerated plants, *rol*C-gene-specific oligonucleotide primers (rolF, 5′-CACCAATCTTCCACCGTACC-3′; rolR, 5′-CAGCTACTGCCATCACTCCA-3′) were designed from the whole nucleotide sequence of the pRi1724 plasmid in the mikimopine producing Japanese *A. rhizogenes* (MAFF 03-01724) (Accession No. NC_002575 of Moriguchi et al., 2001). A DNA fragment of *rol*C gene (288 bp) was amplified by PCR analysis. The amplification was carried out under the following temperature program: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, and final extension of 72 °C for 7 min. Amplified DNA fragments were electrophoresed on agarose gel and visualized by EtBr dyeing.

For genomic Southern blot analysis, an *Nco*I restriction site was confirmed between the *rol*B and *rol*C genes in T-DNA from the above-mentioned sequence of Ri-plasmid (Accession No. NC_002575 of Moriguchi et al., 2001). Each 20 μg DNA sample was digested with *Nco*I and separated in 0.8% agarose gel. The separated DNA was transferred to a Hybond-N^+ nylon membrane (Amersham Bioscience, UK) and hybridized with the DIG-labeled *rol*C gene fragment amplified from the plasmid. Hybridization and detection of the chemiluminescent signal were performed with a DIG Luminescent Detection Kit (Roche Diagnostics, Germany) according to the manufacturer's protocol.

2.5. Essential oil analysis

Full-bloomed florets with caryx (2.5 g F.W.) were removed from spikes, and immediately soaked in 50 ml of *n*-hexane. After two days of extraction, the extract was dehydrized with anhydrous Na_2SO_4 and concentrated to 1 ml under gentle vacuum using a rotary evaporator at 30 °C. The individual essential oil components in the *n*-hexane extracts were separated using a Yanaco gas chromatograph, model GC 8000 Top, equipped with a flame ionization detector (FID). Helium gas was used as a carrier at an injection pressure of 100 kPa. The split mode was adopted at a flow rate of 20 ml min^{-1}. One microliter of sample with ethyl heptanoate as an internal standard was injected on a TC-WAX capillary column (GL-Science, Japan; 30 m × 0.25 mm i.d.), then analyzed with the following temperature program: the initial temperature was kept at 80 °C for 10 min and increased to 190 °C at 2 °C min^{-1} and then increased to 220 °C at 20 °C min^{-1}, and the final temperature was kept at 220 °C for 10 min. The injector temperature was 240 °C and the detector temperature was 280 °C. The identification of the most important components of lavandin oil, α-pinene, β-pinene, limonene, 1,8-cineole, camphor, linalool, linalyl acetate, lavandulyl acetate, lavandulol and borneol, was carried out with a Hewlett-Packard GC–MS (model 5890 series II equipped with a mass spectrometer selective detector 5971) by comparison with authentic samples and mass spectra collected by Tsuro et al. (2001, 2004). After identification of the essential oil components, the essential oil content in 1 g of floret (F.W.) and the relative ratio of essential oil components were calculated.

2.6. Statistical analysis

The differences in hairy root formation rates between the mediated *Agrobacterium* strains and the adventitious shoot formation rates between the combination of cultured media and mediated *Agrobacterium* strains were analyzed statistically by Fisher's exact probability test. In the comparative evaluation of plant height, the mean values of the *Agrobacterium* strain-mediated regenerants and nontransgenic regenerants were analyzed statistically by *t*-test. The