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Transgenic patchouli plants produced by *Agrobacterium*-mediated transformation

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Abstract

A successful transformation procedure using *Agrobacterium* was established for the most important aromatic crop, patchouli (*Pogostemon cablin* Benth.). To avoid inhibition of *Agrobacterium* infection by patchouli oil accumulated in leaf tissues, complete plants regenerated *in vitro* which possessed no or trace amounts of patchouli oil in leaf tissues were used as an explant source. Conditions for transformation were examined using two *A. tumefaciens* strains containing a different chimeric plasmid. Leaf explants were infected with *A. tumefaciens* strain EHA101/pIG121-Hm carrying β-glucuronidase (GUS) and hygromycin phosphotransferase (HPT) genes. Following co-cultivation for 3 days and selection by 50 mg l^{-1} hygromycin B, greenish calli with adventitious shoots were selected, from which putative transformants with roots were regenerated. Histochemical assay showed that GUS expression is detected in every organs of transformants, which was confirmed by the detection of high activity of GUS. Using another strain LBA4404/pBI 121-PaCP1 encoding the coat protein precursor gene of patchouli mild mosaic virus (CP-P) and neomycin phosphotransferase (NPTII) gene, putative transformants were also obtained after co-cultivation for 7 days and selection by 100 mg l^{-1} kanamycin. Using total DNAs from the transformants, the full length of CP-P was detected by PCR reaction. Comparing between two strains examined, it was noted that prolonged co-cultivation period and higher dose of a selection drug were indispensable for successful infection with LBA4404/pBI 121–PaCP1.

Abbreviations: BA – benzylaminopurine; GUS – β-glucuronidase; 4-MUG – Methylumbelliferyl-β-D glucuronide; NAA – naphthaleneacetic acid; X-gluc – 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

Introduction

Patchouli (*Pogostemon cablin* Benth.) is an aromatic crop which yields an essential oil containing various sesquterpenes and hydrocarbons such as; patchouli alcohol (patchoulol), patchoulene, bulnesene, guaiene, caryophyllene, elemene and copaene (Lawrence, 1981; Sugimura et al., 1990; Hasegawa et al., 1992). The essential oil is one of the most important naturally occurring perfumery

raw materials because of its characteristic woody fragrance and fixative properties by which the scent is fixed and make it last longer on the skin. Areas of commercial cultivation are mainly located in Indonesia, which accounts for over 80% of world patchouli oil production (Nakajima et al., 1994).

Our field survey showed that patchouli plants grown in Indonesia and Taiwan are infected with patchouli mottle virus (PaMoV) and/or patchouli mild mosaic virus (PaMMV) at a high percentage (Natsuaki et al., 1994; Sugimura et al., 1995). Virus-free patchouli clones were produced by meristem-tip culture and cultivated in the fields, resulting in significant increases in leaf biomass and essential oil yields (Sugimura et al., 1995). However, the reinfection of virus-free patchouli is inevitable during successive cultivation. To eliminate virus disease persistently, it is essential to produce virus-resistant patchouli. The induction of the virus-resistant trait by sexual crossing is not possible, since the cultivar does not produce flowers even under the photoperiodical control (Hefendehl and Murray, 1979). A possible alternative is to produce transformants with the viral coat protein gene. The transgenic plants expressing viral coat protein gene were reported to display delayed or attenuated symptoms following infection (Hackland et al., 1994; Lomonossoff, 1995). In our previous reports, we determined the complete nucleotide sequence and the genome organization of both RNA-1 and RNA-2 of PaMMV (Ikegami et al., 1998, 2001). The RNA-2 species encodes coat protein precursor (CP-P) gene of 1.8 kbp. Actually, the CP-P is translated *in vivo* as a polyprotein and cleavaged into two protein species of 44K and 22K by protease encoded in the RNA-1.

In spite of its economic importance in the flavor and fragrance industry so far there are no reports available on genetically modified patchouli plants. In this paper, using two *Agrobacterium tumefaciens* strains containing a different binary plasmid we examined the effect of several factors such as explant source, co-cultivation period and selection dose of a drug. As a result, two kinds of transgenic patchouli plants with β-glucuronidase (GUS) gene and the polyprotein gene of PaMMV coat protein were produced in this study.

Materials and methods

Callus induction and plant regeneration

Patchouli plants (*Pogostemon cablin* Benth.) were classified into three cultivars on the basis of both agronomical traits and chemical composition of the essential oils (Sugimura et al., 1990). Cultivar I was selected for this study, since it yielded an essential oil of high quantity. Leaves of patchouli plants grown in a greenhouse were surface-sterilized in a solution of 1% sodium hypochloride with 0.05% Tween-20 for 20 min and then washed with sterile water well. The leaf explants were placed onto a basal medium supplemented with 10^{-6} M naphthaleneacetic acid (NAA) and 10^{-6} M 6-benzylaminopurine (BA). The basal medium was composed of the following constituents: LS mineral salts (Linsmaier and Skoog, 1965), 100 mg l^{-1} myo-inositol, 0.4 mg l^{-1} thiamine·HCl, 30 g l^{-1} sucrose, 3 g l^{-1} Gellan gun. During 3–4 weeks of culture at 28 °C, shoots appeared from calli in the cut-edges of explants. For rooting, shoots were recultured onto the basal medium at 3–4-week intervals. The *in vitro* plants (about 7–8 cm in height), at the end of a culture passage, were used as the source of leaf explants for this transformation experiments.

Bacterial strains, plasmid construction and inoculation

Agrobacterium tumefaciens strain EHA101 harboring the binary vector plasmid pIG121-Hm (Akama et al., 1992) which carries the chimeric neomycin phosphotransferase (NPT II), β-glucuronidase (GUS) and hygromycin phosphotransferase (HPT) genes was used for transformation. Another chimeric plasmid pBI121–PaCP1 was constructed using a binary vector plasmid pBI121 which contained NPT II gene as a selection marker: a full length CP-P cDNA having the *Bam*HI site in both terminals was amplified by PCR. The plasmid pBI121 was digested with *Bam*HI and ligated with CP-P gene to integrate into the *Bam*HI site between CaMV 35S promoter and GUS gene of plasmid vector pBI121. The resultant plasmid (pBI121–PaCP1) was then introduced to *A. tumefaciens* strain LBA 4404 by electroporation. Leaf explants were prepared by dissecting away the margins of the leaf piece, and dipped in a

suspension of *A. tumefaciens* strain EHA101/ pIG121-Hm and LBA 4404/ pBI121–PaCP1 for 10 min. The inoculum density was 0.5 at OD_{600}. The excess amounts of bacterial suspension was blotted on filter paper.

Selection and regeneration of transgenic plants

Inoculated tissues were transferred onto a co-cultivation medium containing 10^{-6} M NAA, 10^{-6} M BA and 10 mg l^{-1} acetosyringone. Following co-cultivation for 3–7 days in dark at 28 °C, they were placed on a basal medium containing 300 mg l^{-1} cefotaxime for 5–7 days, and then transferred to a selection medium. A selectable antibiotics, 50 mg l^{-1} hygromycin B, was used for tissues inoculated with the strain EHA 101/pGI121-Hm, while 100 mg l^{-1} kanamycin monosulfate (potency: >750 μg mg^{-1}) was applied to tissues inoculated with the strain LBA 4404/pBI121–PaCP1. The Greenish calli with adventitious shoot buds were picked up 4 weeks after antibiotics selection, and subcultured on the selection medium for shoot development. Regenerated shoots were rooted on the basal medium containing an antibiotics. Uninoculated control plants were regenerated similarly under nonselective conditions and maintained under same culture conditions as the putative transformants.

Histochemical GUS assay

Histochemical localization of GUS gene expression was assayed by 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) in 50 mM phosphate buffer (pH 7.0) at 37 °C for 5–6 h according to a previous report (Jefferson et al., 1987). To remove chlorophyll, they were washed with 70% ethanol after incubation.

GUS activity assay

Leaves from shoots were homogenized in 50 mM phosphate buffer (pH 7.0) containing 10 mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl and 10 mM 2-mercaptoethanol. After centrifugation, the supernatant was mixed with 1 mM 4-methyl-umbelliferyl-β-D-glucuronide (4-MUG), and incubated for 30–90 min. The reaction product, 4-methyl-umbelliferone (4-MU), was measured fluorometrically ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 455$ nm).

DNA isolation and PCR analysis

Total genomic DNAs were extracted from leaves of putative transgenic plants according to a previous report (Honda and Hirai, 1990). Specific oligonucleotide primers for detecting GUS and CP-P gene sequences (Hamill et al., 1991; Ikegami et al., 1998) were used to identify the presence of these genes in the genomic DNAs by PCR (for GUS gene detection, 5′-GGTGGGAAAGCG CGTTACAA G-3′ and 5′-GTTTACGCGTTGC TTCGCCA-3′; for CP-P gene detection, 5′-TAT GGCCAAGGCTTAATG-3′ and 5′-GCTAGT GACCTACGAA-3′). For PCR, samples were kept at 92 °C for 3 min, followed by 30 cycles at 92 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min. Amplified DNA was electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining.

Antimicrobial assay

Patchouli oil (a pure grade oil which was kindly provided by Dr N. Toi, Kao Corporation, Japan) was passed through a filter of 0.2 μm pore size, and added to LB medium containing 1% Bactotryptone, 0.5% yeast extract and 1.0% NaCl. *A. tumefaciens* EHA101 was inoculated in the medium (inoculum density: 0.1 at OD_{600}). After 9-h incubation with vigorous agitation at 37 °C, the microbial growth was assayed by assessment of medium turbidity at OD_{600}.

Results and discussion

Callus induction and plant regeneration

Calli with many adventitious buds were induced from the cut-edges of leaf tissues on the basal medium containing 10^{-6} M NAA and 10^{-6} M BA, and multiple shoots were formed from a single callus mass 3–4 weeks after the start of culture (Figure 1). The frequency of multiple shoot fomation was more than 90% of inoculated explants. In contrast, when 10^{-5} M NAA and 10^{-6} M were supplemented, the percentage of shoot initiation decreased up to 18% of total explants inoculated. In the combination of 10^{-6} M NAA and 10^{-5} M BA, a few number of shoots were formed from a callus mass. These proliferative responses to the

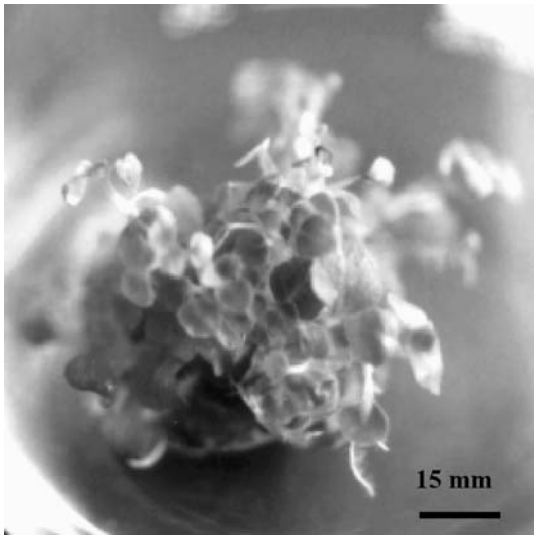

Figure 1. Multiple-shoot formation from a callus.

supplemented plant growth regulators were supported by a previous report showing plant regeneration from protoplast-derived calli of patchouli (Kageyama et al., 1995).

Transformation mediated with A. tumefaciens *EHA101/pIG121-Hm*

When leaf tissues from *in vitro* propagated plants were inoculated with *A. tumefaciens* EHA101/pIG121-Hm and co-cultivated for 3 days, it was noted that calli with green spots were initiated on a selection medium with 50 mg l^{-1} hygromycin. The green spots were stainable with X-gluc, indicating that meristematic zones are transformed (Figure 2a). Isolated whitish calli turned into brownish masses during culture, but still survived without shoot regeneration. Shoots were regenerated from greenish calli on a selection medium and grown on the basal medium. As shown in

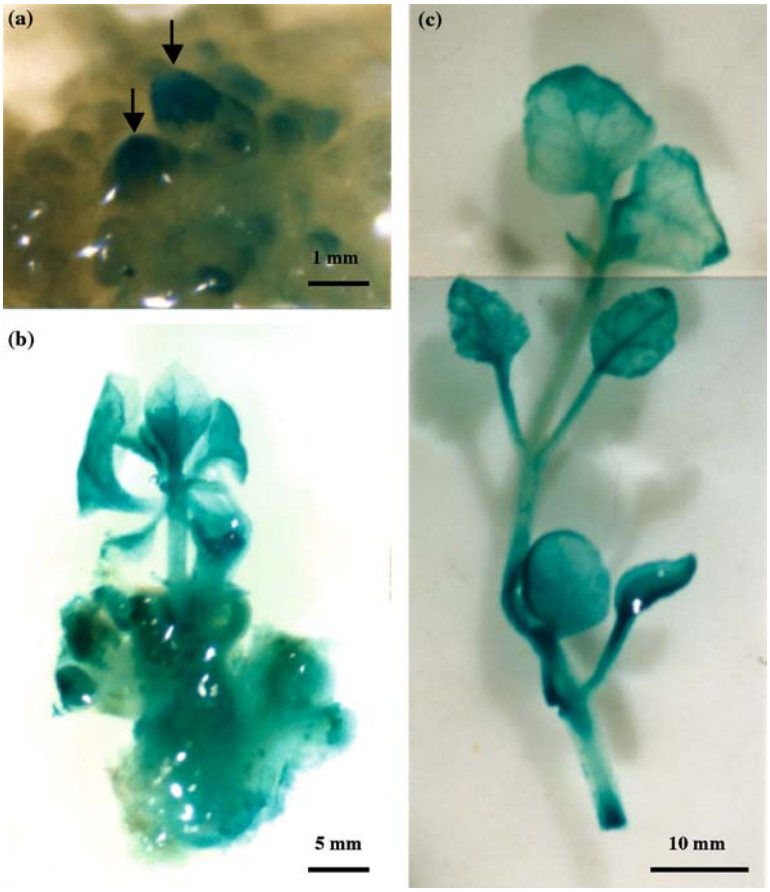

Figure 2. Regeneration of plant from the transformed callus. callus with adventitious buds (*a*), callus with a shoot (*b*) and a complete plant (*c*) were stained with X-gluc for histochemical detection of GUS gene expression. Arrows indicate transformed adventitious buds.

Figures 2b and c, a complete shoot regenerated from a callus mass was totally stained with X-gluc. It is evident that selected green calli were composed of transformed cells from which shoot proliferation took place under a selection pressure by hygromycin.

GUS activity assay of a putative transformant was carried out using a fluorescent substrate, 4-MUG. No GUS activity was detected in leaves of an uninoculated control plant, whereas a putative transformant showed a high level of activity (Figure 3). This result indicates that GUS was actively synthesized in shoot cells. In addition, the presence of GUS gene in putative transformants was analyzed using PCR with primers designed to amplify the 1.2 kb of GUS gene (from 400 to 1599). Electrophoretic pattern of the amplified DNA suggested that the 1.2 kb gene is possibly inserted in genomic DNAs of putative transformants (Figure 4), whereas no corresponding band appeared in control.

Since patchouli plants are not capable of flowering (Hefendehl and Murray, 1979), progeny analysis of transformants is not possible. As an alternative stability evaluation of transformants, obtained putative transformants were successively propagated by *in vitro* cutting and GUS activity was assayed using leaf tissues of the 4th generation propagated *in vitro*. No loss of GUS activity was observed by histochemical detection, suggesting the production of stable transformants.

Transformation mediated with A. tumefaciens *LBA4404/pBI121-PaCP1*

It is known that *A. tumefaciens* LBA4404 is less virulent than EHA 101. Preliminary experiments showed that prolonged co-cultivation period for

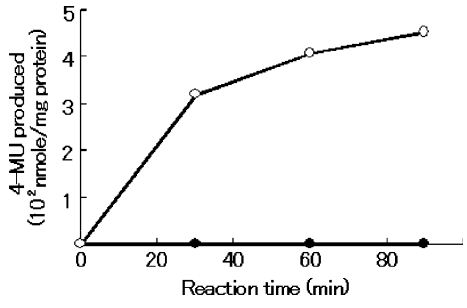

Figure 3. GUS activity of leaves from a transgenic patchouli. ○: transformant, ●: uninoculated control.

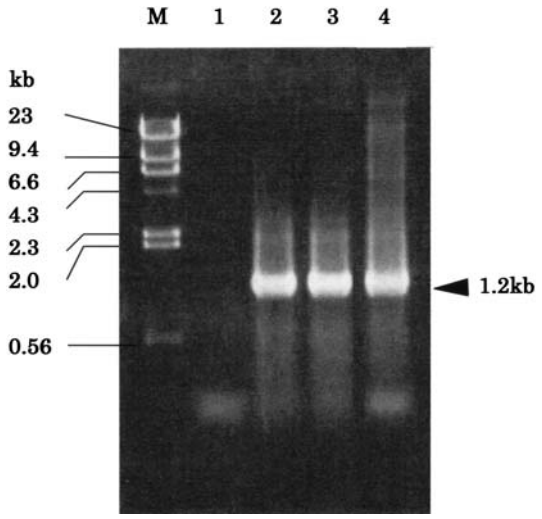

Figure 4. PCR analysis of genomic DNAs from transformants to detect GUS gene with 1.2 kbp. Lane 1: non-transformed control, Lanes 2–4: transformants, M: size marker.

7 days was required for successful infection. The callus formation and shoot regeneration were tolerant of kanamycin at low concentrations. Therefore, the addition of 100 mg l^{-1} kanamycin to a selection medium was indispensable for effective selection of transformants. Calli with adventitious shoot buds were selected in the presence of high dose of kanamycin and grown as mentioned above. Total DNAs from two putative transformants were used to allow amplification of the full length of CP-P gene. A 1.8 kbp band corresponding to CP-P gene was clearly detected in each transformant (Figure 5), showing possible insertion of CP-P gene into genomic DNAs of patchouli plants.

Agrobacterium-mediated transformation has often proved unsuccessful due to the accumulation of secondary metabolies in explants used, particularly complex mixtures such as the essential oils. Essential oils from various aroma crops were reported to have an antimicrobial activity (Morris et al., 1979). Therefore, it is likely that the essential oil accumulated in patchouli leaves shows inhibitory activity against *A. tumefaciens* infection. In fact, the growth inhibition of 0.1 and 1.0% patchouli oil was 60 and 86%, respectively, which is a negative factor for the establishment of *Agrobacterium* infection. For successful *A. tumefaciens*-mediated transformation, it is

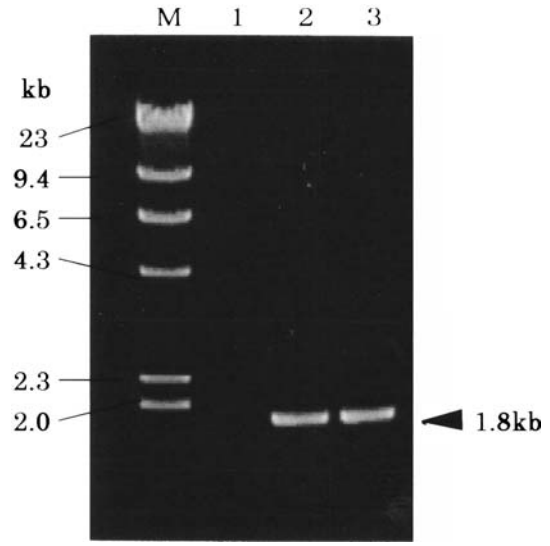

Figure 5. PCR analysis of genomic DNAs from transformants to detect PaMMV CP-P gene with 1.8 kbp. Lane 1: non transformed control, lanes 2 and 3: transformants, M: size marker.

important to select the leaves possessing low amounts of patchouli oil. The oil droplets in leaf tissues were observed by histochemical staining, resulting in no or trace amounts of the essential oil present in young leaves of the *in vitro* plants (data not shown). Such young leaves were used as explants and cultured on the medium supplemented with 10^{-6} M NAA and 10^{-6} M BA for shoot formation and *A. tumefaciens* infection. Two strains of *A. tumefaciens* with a different chimeric plasmid were used for the development of transformation system of patchouli plant. Successful introduction of GUS and CP-P genes was demonstrated using both strains, although conditions for transformation were significantly different. Pathological analyses of transformants with CP-P gene are under investigation in order to identify resistance to PaMMV infection.

The constituents of essential oils are of prime importance for flavor and fragrance uses. Compositional improvement of essential oils was demonstrated by transgenic manipulation of monoterpene biosynthesis in peppermint (Mahmoud and Croteau, 2001) and tomato plants (Lewinsohn et al., 2001). Although biosynthetic pathways of sesquterpenes in patchouli plants are not fully understood yet, metabolic engineering approaches may make it possible to increase the content of patchouli alcohol (a key component of patchouli oil) and to alter the constituents of patchouli oil, thereby providing the new essential oils with unique odor. The genetic transformation system presented here would be valuable tool for generating transformants with pest resistance and/or compositional improvement of the patchouli oil.

References

Akama K, Shitaishi H, Ohta S, Nakamura K, Okada K & Shimura Y (1992) Efficient transformation of *Arabidopsis thaliana*: comparison of the efficiencies with various organs, plant ecotypes and *Agrobacterium* strains. Plant Cell Rep. 12: 7–11

Hackland AF, Rybicki EP & Thomson JA (1994) Coat protein-mediated resistance in transgenic plants. Arch. Virol. 139: 1–22

Hamill JD, Rounsley S, Spencer A, Todd G & Rhodes MJC (1991) The use of the polymerase chain reaction in plant transformation studies. Plant Cell Rep. 10: 221–224

Hasegawa Y, Tajima K, Toi N & Sugimura Y (1992) An additional constituent occurring in the oil from a patchouli cultivar. Flavour Frag. J. 7: 333–335

Hefendehl FW & Murray ML (1979) Genetic aspects of the biosynthesis of natural odors. Lloydia 39: 39–52

Honda H & Hirai A (1990) A simple and efficient method for identification of hybrids using nonradioactive rDNA as probe. Jpn. J. Breed. 40: 339–348

Ikegami M, Kawashima H, Natsuaki T & Sugimura Y (1998) Complete nucleotide sequence of the genome organization of RNA2 of patchouli mild mosaic virus, a new fabavirus. Arch. Virol. 143: 2431–2434

Ikegami M, Onobori Y, Sugimura Y & Natsuaki T (2001) Complete nucleotide sequence and the genome organization of patchouli mild mosaic virus RNA 1. Intervirology 44: 355–358

Jefferson RA, Kavanagh TH & Bevan MW (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901–3907

Kageyama Y, Honda Y & Sugimura Y (1995) Plant regeneration from patchouli protoplasts encapsulated in alginate beads. Plant Cell Tiss. Organ Cult. 41: 65–70

Lawrence BM (1981) Progress in essential oils. Perfum. Flav. 6: 73–76

Lewinsohn E, Schalechet F, Wilkinson J, Matsui K, Tadmor Y, Nam K-H, Amar O, Lastochkin E, Larkov O, Ravid U, Hiatt W, Gepstein S & Pichersky E (2001) Enhanced levels of the aroma and flavor compound S-linalool by metabolic engineering of the terpenoid pathway in tomato fruits. Plant Physiol. 127: 1256–1265

Linsmaier EM & Skoog F (1965) Organic growth factor requirements of tobacco tissue culture. Physiol. Plant. 18: 100–127

Lomonossoff GP (1995) Pathogen-derived resistance to plant viruses. Ann. Rev. Phytopathol. 33: 323–343

Mahmoud SS & Croteau R (2001) Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase

and menthofuran synthase. Proc. Natl. Acad. Sci. USA 98: 8915–8920

Morris JA, Khettry A & Seitz EW (1979) Antimicrobial activity of aroma chemicals and essential oils. J. Am. Oil Chem. Soc. 56: 595–605

Nakajima M, Toi N & Sugimura Y (1994) The Japanese market for perfumery products. Proceedings of 4emes Rencontres Techniques et Economiques Plantes Aromatiques et Medicinales, Nyons, France (pp. 71–75)

Natsuaki KT, Tomaru K, Ushiku S, Ichikawa Y, Sugimura Y, Natsuaki T, Okuda S & Teranaka M (1994) Characterization of two viruses isolated from patchouli in Japan. Plant Dis. 78: 1094–1097

Sugimura Y, Ichikawa Y, Otsuji K, Fujita M, Toi N, Kamata N, Rosario RMR, Luingus GR & Taga-an GL (1990) Cultivarietal comparison of patchouli plants in relation to essential oil production and quality. Flavour Frag. J. 5: 109–114

Sugimura Y, Padayhag BF, Ceniza MS, Kamata N, Eguchi S, Natsuaki T & Okuda S (1995) Essential oil production increased by using virus-free patchouli plants derived from meristem-tip culture. Plant Pathol. 44: 510–515