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TISSUE CULTURE AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF HEMP (*CANNABIS SATIVA* L.)

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SUMMARY

Hemp (*Cannabis sativa* L.) is cultivated in many parts of the world for its fiber, oil, and seed. The development of new hemp cultivars with improved traits could be facilitated through the application of biotechnological strategies. The purpose of this study was to investigate the propagation of hemp in tissue culture and to establish a protocol for *Agrobacterium*-mediated transformation for foreign gene introduction. Stem and leaf segments from seedlings of four hemp varieties were placed on Murashige and Skoog medium with Gamborg B5 vitamins (MB) supplemented with 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 μM kinetin, 3% sucrose, and 8 $g l^{-1}$ agar. Large masses of callus were produced within 4 wk for all cultivars. Suspension cultures were established in MB medium containing 2.5 μM 2,4-D. To promote embryogenesis or organogenesis, explants, callus, and suspension cultures derived from a range of explant sources and seedling ages were exposed to variations in the culture medium and changes to the culture environment. None of the treatments tested were successful in promoting plantlet regeneration. Suspension cells were transformed with *Agrobacterium tumefaciens* strain EHA101 carrying the binary vector pNOV3635 with a gene encoding phosphomannose isomerase (PMI). Transformed callus was selected on medium containing 1–2% mannose. A chlorophenol red assay was used to confirm that the PMI gene was expressed. Polymerase chain reaction and Southern hybridization detected the presence of the PMI gene. Copy number in different lines ranged from one to four.

Key words: callus; suspension culture; *Agrobacterium tumefaciens*; mannose selection; phosphomannose isomerase; regeneration; transgenic hemp.

INTRODUCTION

Cannabis sativa L. is among the earliest cultivated plants and is thought to have originated in Central Asia (Clarke, 1999). It is valued as a food, oil, fiber, medicinal, and recreational drug source and, consequently, has been dispersed throughout the world. Hemp (*Cannabis sativa* L.) traditionally has been grown as a fiber crop and there is a renewed interest in expanding its cultivation as a fiber and seed crop in Canada. Hemp seeds possess high-quality oil and protein (Johnson, 1999). Hemp varieties are now developed and cultivated to produce high yields of fiber, seed, and oil, while possessing negligible amounts of Δ^9-tetrahydrocannabinol (THC), the psychoactive compound, within the resin. However, the confusion of hemp with marijuana varieties, which contain greater amounts of THC, continues to hinder the widespread cultivation of this crop (Forapani et al., 2001).

The development of new hemp cultivars with improved traits could be further facilitated using biotechnological strategies. The dioecious life cycle of many hemp varieties complicates breeding efforts towards improvement of specific traits, such as resistance to pests and diseases (Clarke, 1999). Development of a tissue culture system to regenerate hemp plantlets and an *Agrobacterium*-mediated transformation protocol would permit exploitation of a greater amount of genetic diversity for plant improvement and would facilitate clonal multiplication of plants with desirable traits.

There are only a small number of reports concerning tissue culture of hemp. Most of these studies were aimed at developing a cell culture system to obtain secondary metabolites, particularly the class of cannabinoids that are distinctive to the genus *Cannabis* (Turner et al., 1980). Callus cultures (Hemphill et al., 1978; Heitrich and Binder, 1982) and suspension cultures (Veliky and Genest, 1972; Itokawa et al., 1977; Hartsel et al., 1983; Loh et al., 1983; Braemer and Paris, 1987) have been established for extraction of secondary metabolites and biotransformation studies. Cryopreservation of hemp suspension cultures was developed as a means to preserve germplasm collections (Jekkel et al., 1989). A few reports have described tissue culture conditions intended for plantlet regeneration. Richez-Dumanois et al. (1986) propagated apical and axillary buds on stem explants in tissue culture and subsequently rooted the shoots. A report by Fisse et al. (1981) assessed organogenesis as a means of propagating hemp tissues. They did not observe any direct organ formation on explants and reported that *Cannabis* callus readily produced roots but was unreceptive to shoot formation. Mandolino and Ranalli (1999) have compiled an excellent review of the achievements with *in vitro*

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hemp cultures with the objective of regenerating hemp plantlets. There is only one account describing transformation studies with *C. sativa* using *Agrobacterium tumefaciens* to transform shoot-tips with a gene conferring resistance to infection by the fungal pathogen, *Botrytis cinerea* (MacKinnon et al., 2000). However, to our knowledge, there are no reports of somatic embryogenesis in hemp.

The objective of this study was to identify the conditions for callus and suspension culture growth of four fiber and seed hemp varieties, to promote regeneration of plantlets via somatic embryogenesis or organogenesis, and to develop an *Agrobacterium*-mediated transformation protocol to introduce the selectable phosphomannose isomerase (PMI) marker (Joersbo, 2001; Reed et al., 2001) into hemp cells.

Materials and Methods

Plant material. Four hemp varieties representing different life cycles and bred for either fiber or seed were chosen for this study. Varieties Uniko-B and Kompolti are both dioecious and bred for fiber. Varieties Anka and Felina-34 are monoecious and bred for seed and both fiber and seed, respectively. Seeds were sown in 5 cm^2 plastic containers containing moistened potting mix soil (Sunshine Mix no. 1, Sun Gro Horticulture, Bellevue, WA) at ambient room temperatures (21–24°C). Seedlings were placed under cool-white fluorescent lights with an intensity of 18 $\mu mol\ m^{-2}\ s^{-1}$ and a photoperiod of 12 h. Shoots were excised at the base of the stem at 4 wk, at which time they had attained a height of about 20 cm and had two to four pairs of true leaves. The tissues were immersed in 70% ethanol for 20 s followed by 10% commercial bleach (Javex®, containing 4.5% NaOCl) containing two drops of 0.1% Tween-20 per 100 ml for 1 min while stirring. Tissues were then rinsed three times with sterile distilled water and transferred to sterile Petri dishes lined with moistened filter paper. Leaf (0.5 cm^2) and stem segments (0.5 cm long) were excised after edges were discarded and transferred to agar medium in 100 × 15 mm Petri dishes.

Callus induction. Leaf and stem explants of varieties Anka and Uniko were placed on MB medium containing Murashige and Skoog macro- and micro-nutrients (MS; Murashige and Skoog, 1962) with Gamborg B5 vitamins (Gamborg et al., 1968), 0.1 $g\ l^{-1}$ *myo*-inositol, 3% sucrose, 8 $g\ l^{-1}$ bacteriological agar (Anachemia Canada Inc., Montreal, PQ); the pH was adjusted to 5.8 before autoclaving. A series of plant growth regulator combinations were evaluated to induce callus development and somatic embryo formation (μM): 2,4-dichlorophenoxyacetic acid (2,4-D; 2.5, 5, 9) with either kinetin (0.5, 1, 5), α-naphthaleneacetic acid (NAA; 2.5, 5, 10), 6-benzylaminopurine (BA; 0.5, 1, 5), or indolebutyric acid (IBA; 2.5, 5, 10); or IBA (2.5, 5, 10) with either BA (0.5, 1, 5), NAA (2.5, 5), or kinetin (0.5, 1, 5); or NAA (2.5, 5, 10) with either BA (0.5, 1, 5) or kinetin (0.5, 1, 5). Each treatment consisted of 10 Petri dishes, containing 10 explants each. The dishes were wrapped with Parafilm® and placed inside a dark drawer. After 1 mo, length and width of each callus mass was measured with a ruler and averaged to obtain callus diameter.

Callusing responses of different explant sources were evaluated using tissues from seedlings of varieties Felina and Uniko. The aerial structures (leaves, petioles, stem, and cotyledons) from each seedling were sterilized and sequentially cut into small segments as described above. The segments were carefully arranged in sequential order in a Petri dish containing MB with 5 μM 2,4-D and 1 μM kinetin (MB5D1K). Callusing was recorded over a period of 2 wk.

The rate of callus development on stem and leaf explants of all four hemp varieties on MB5D1K medium was recorded at 3–4 d intervals over a 1 mo. period. Ten explants were placed in each Petri dish and there were 10 dishes for each variety. At 4 wk, length and width of each callus mass were measured with a ruler and averaged to obtain callus diameter. Callus was maintained by transferring to fresh medium every 4 wk. The experiment was repeated three times over a 2 yr period using the same batch of seeds.

Suspension cultures. Suspension cultures were initiated by cutting 4-wk-old callus masses into small pieces and transferring 0.5–1 mg of tissue to 150-ml Erlenmeyer flasks containing MB supplemented with 2.5 μM 2,4-D and 3% sucrose; the pH was adjusted to 5.8 (MB2.5D). Cultures were shaken at 115 rpm under ambient laboratory conditions and with 12 $h\ d^{-1}$ light at an intensity of 10 $\mu mol\ m^{-2}\ s^{-1}$. Every 2 wk, three-quarters of the spent medium was replaced with fresh medium. By 4 wk, suspensions were established and transferred to 250-ml Erlenmeyer flasks by suctioning about 1 ml cell volume through a 3 mm diameter pipette tip and transferring to fresh medium. Suspension culture growth was measured at 3–4 d intervals over a 1 mo. period and fresh weights of tissues were recorded after filtration. Dry weight was obtained by incubating tissues at 40°C, until constant mass was achieved. The experiment was repeated three times over a 1 yr period.

Regeneration of plantlets. A range of treatments was evaluated to determine whether hemp callus could be induced to regenerate plantlets either through embryogenesis or organogenesis (Table 1).

TABLE 1

SUMMARY OF TREATMENTS EVALUATED TO PROMOTE HEMP PLANTLET REGENERATION THROUGH EMBRYOGENESIS OR ORGANOGENESIS

Tissue source[a]	Treatment
Callus, rhizogenic callus	Subculture from MB plus combinations[b] of 2,4-D, NAA, IBA, kinetin, and BA to growth regulator-free MB each month.
Callus	Subculture from MB5D1K containing casein hydrolyzate (0, 100, 250, 500 $mg\ l^{-1}$), L-glutamine (3.4 mM), or L-proline (15 mM) to growth regulator-free MB each month.
Callus, rhizogenic callus	Subculture to half-strength MB with BA (0, 1, 5, 10 μM) and place in light (12 $h\ d^{-1}$) or total darkness for 2 mo.
Callus, rhizogenic callus	Expose to MB plus BA (5 μM) containing silver nitrate (0, 11.7, 47, 70.6 μM) for 1 mo. in total darkness then to light for 1 mo., then subculture to growth regulator-free MB.
Tissue segments	Expose to MB containing thidiazuron (0, 0.5, 2.5, 5, 1 μM) for 4 d and then subculture to growth regulator-free MB.
Suspension cells	Expose to half-strength MB liquid with thidiazuron (0.5 μM) for 1 mo. and then subculture to growth regulator-free half-strength MB liquid.
Suspension cells	Expose to MB liquid with 2.5 μM 2,4-D and 10^{-10} M salicylic acid for 1 mo. and then subculture to growth regulator-free MB liquid.
Callus, rhizogenic callus	Subculture to half-strength MS with 1% activated charcoal.
Callus	Initiate and maintain on MB5D1K medium in the light (14 $\mu mol\ m^{-2}\ s^{-1}$) or in total darkness for 1 yr. Subculture to growth regulator-free MB every 2 mo.
Callus	Expose to MB5D1K medium and incubate at 4°C for 2 mo. Subculture to growth regulator-free MB every 2 wk.
Tissue segments	Expose to 1 M sucrose for 1–3 d and then subculture to growth regulator-free MB.
Suspension cells	Expose to half-strength MB with 5% sucrose for 2 mo. and subculture to half-strength MB every 2 wk.

[a] Explants were taken from 7-d-old or 4-wk-old hemp seedlings from four hemp varieties. A range of explant sources (hypocotyl, epicotyl, cotyledons, petioles, leaves, and immature flower buds) were used to initiate callus.

[b] Refer to Materials and Methods, callus induction section, for plant growth regulator combinations.

Hemp Transformation

Bacterial strains and plasmids. *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986) harboring the binary vector pNOV3635 was used for transformation. The plasmid pNOV3635 contains a coding region for PMI under control of the ubiquitin promoter from *Arabidopsis thaliana* (*Ubq3*) and the nopaline synthase terminator (NOS). The PMI gene for selection of transgenic plants is between the left and right T-DNA borders, and a spectinomycin gene resides outside of the borders for maintenance in *Escherichia coli* and *Agrobacterium* vectors. *Agrobacteria* were inoculated into 25 ml Luria-Bertani (LB) medium (Sambrook et al., 1989) supplemented with 150 mg l^{-1} spectinomycin and 50 mg l^{-1} kanamycin. The suspension was shaken at 28°C for 2 d. Bacteria were harvested by centrifugation. The resulting pellet was washed with hemp cell suspension medium (MB2.5D). The pellet was then resuspended in 5 ml MB2.5D containing 100 μ*M* acetosyringone to a final $OD_{600\,nm}$ 1.6–1.8. *Agrobacterium* was incubated in the medium for 10 min prior to inoculating plant cells.

Transformation procedure. Anka suspension cells (1 ml packed cell volume) were suctioned with a 3 mm diameter pipette tip and transferred with 4 ml of MB2.5D into a sterile Petri dish. The hemp cell suspension was inoculated with 5 ml *Agrobacterium* suspension for 30 min. Hemp cells were then collected on a 70 mm diameter filter paper (No. 1; Whatman Int. Ltd., Cambridge, UK) by vacuum filtration. The filter was placed in a Petri dish containing MB2.5D with 8 g l^{-1} agar and incubated in the dark for 3 d at ambient room temperature. Hemp cells were also incubated with *Agrobacterium* lacking pNOV3635. Throughout the transformation experiment, dishes were wrapped with Parafilm®. The transformation experiment was repeated four times with one to six replicate dishes each.

Effect of mannose on callus growth. The concentration of mannose that inhibited callus growth was assessed for the variety Anka to determine the selection criteria for transformation experiments. A series of concentrations of D-mannose (0, 1, 2, 3%) in MB5D1K, with and without sucrose, was tested. Six callus pieces, each about 0.5 cm^2, were aseptically placed in each Petri dish, with three dishes per treatment. Dishes were wrapped with Parafilm® and placed in the dark for 4 wk. Petri dishes were weighed at the beginning and end of the 4-wk period to determine callus growth over a range of sugar concentrations. The experiment was repeated three times. Data from one representative experiment was subjected to a one-way ANOVA with means separated using the Tukey–Kramer HSD statistical test ($P = 0.05$).

Selection of transformants. After the 3 d cocultivation period, cells were transferred to fresh filter paper and rinsed three times with a total volume of 200 ml MB2.5D. The filter containing cells was then transferred to MB2.5D with 8 g l^{-1} agar and 300 mg l^{-1} Timentin (SmithKline Beecham, Oakville, ON) and placed in the dark for 7 d to inhibit bacterial growth. Cells were then selected by transferring small callus clumps (about 0.3 cm^3) to MB2.5D with 1% mannose, 300 mg l^{-1} Timentin, and 8 g l^{-1} agar. Dishes were placed in the dark for 4 wk. Cell masses that continued to grow were transferred to MB2.5D with 2% mannose and 150 mg l^{-1} Timentin for 4 wk. The transformation frequency (number of independent events obtained per number of targets for which transformation was attempted) (Reed et al., 2001) was determined for representative dishes of each experiment. Twenty callus clumps (representing callus lines) were selected for replicates of each transformation experiment and maintained on MB2.5D with 2% mannose with subcultures made every 4 wk.

PMI assays. PMI assays were performed by placing 0.6 cm^2 callus masses into wells of a 24-well ELISA plate (Becton Dickinson and Co., Lincoln Park, NJ). Each well was filled with 600 μl of assay medium consisting of MB2.5D with either 1% mannose or 3% sucrose, 0.1 g l^{-1} of the pH indicator chlorophenol red (CPR, Sigma-Aldrich Chemical Co., Milwaukee, WI), and 8 g l^{-1} agar. The pH was adjusted to 6 prior to autoclaving, resulting in a red-orange colored medium once dispensed into wells. Plates were incubated in the dark at ambient room temperature for 3 d and color changes in the wells were recorded. Between five and 13 callus lines from each of the four transformation experiments were evaluated. Cells capable of metabolizing the sugar source release acidic by-products into the medium, reducing the pH and causing a visible color change from red to yellow (Kramer et al., 1993).

To determine if there was contamination of hemp callus with *Agrobacterium*, all callus lines were incubated on LB medium at 28°C for 1 wk and examined for bacterial growth.

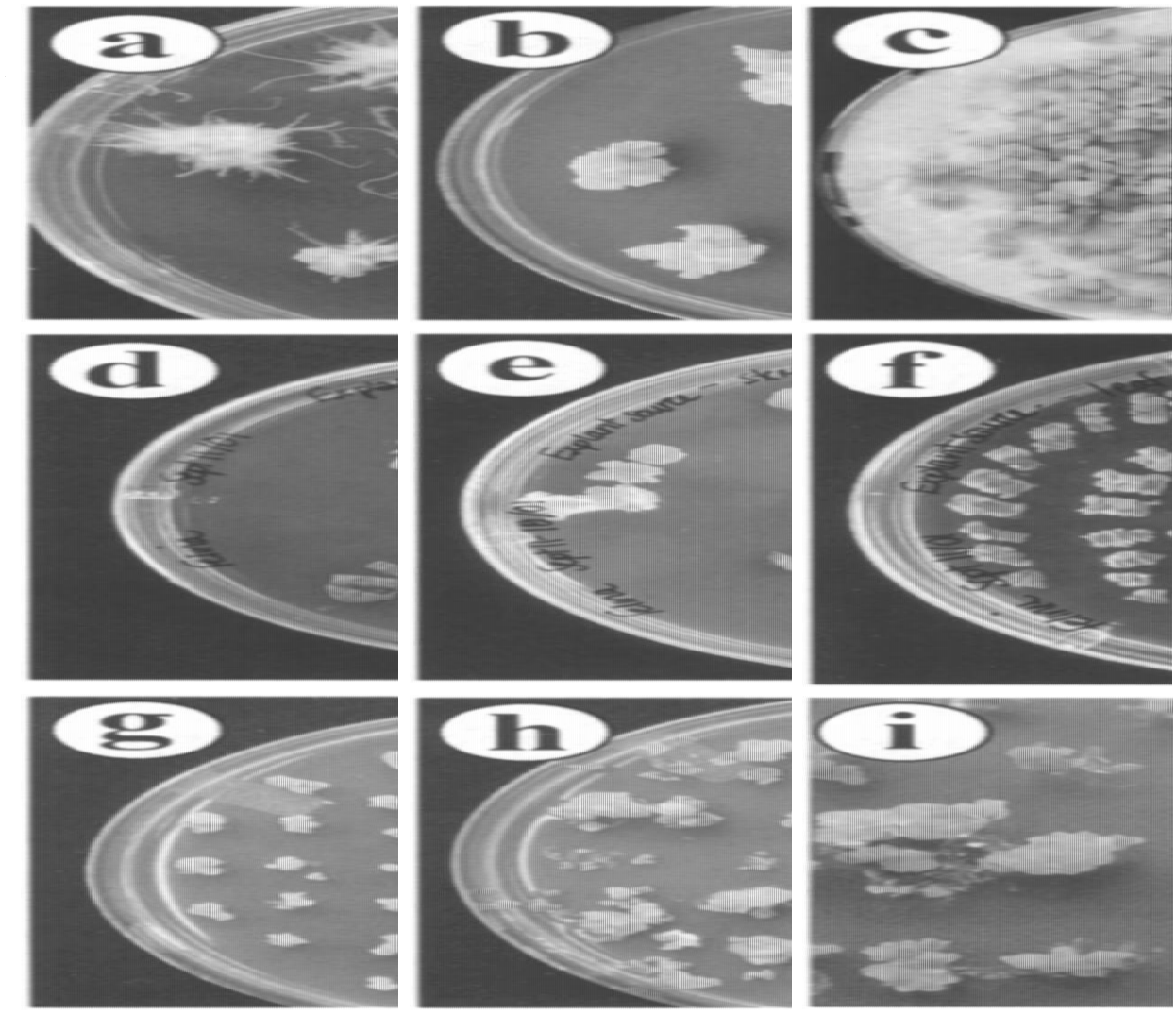
Molecular analyses. Genomic DNA was extracted using a modified protocol from Schluter and Punja (2002). Callus samples (100 mg) were ground with 25 mg polyvinylpolypyrrolidone (PVPP), approximately 100 mg sterile silica sand, 200 μl DNeasy AP1 buffer, and 4 μl RNase (Qiagen, Valencia, CA) in a 1.5 ml microfuge tube with a plastic pellet pestle (Kontes Glass Company, Vineland, NJ) attached to a hand-held drill, until a homogeneous mixture was obtained. Another 200 μl of buffer AP1 was added to the mixture, vortexed, and DNA was isolated following the Qiagen kit procedure. Primers used were described by Negrotto et al. (2000), amplifying a product of approximately 550 bp in size. Primers consisted of two 18-nucleotide sequences: PMI-1 5′-ACAGCCACTCTCCATTCA-3′ and PMI-2 5′-GTTTGCCATCACTTCCAG-3′, and were purchased from the Nucleic Acid-Protein Service Unit at the University of British Columbia (Vancouver, BC). Each 25 μl reaction for PCR contained 5 μl of template DNA, 50 m*M* $MgCl_2$, 20 m*M* Tris, 50 m*M* KCl, 200 μ*M* of each dNTP, 0.2 μ*M* of each primer, and two units of *Taq* polymerase (Invitrogen, Burlington, ON). Amplification was carried out in a DNA Thermal Cycler 9700 (PE Applied Biosystems, Mississauga, ON). PCR conditions were those chosen by Negrotto et al. (2000), with settings adjusted to 3 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 55°C and 45 s at 72°C, with a terminal elongation step of 5 min at 72°C.

For Southern hybridization, hemp genomic DNA was digested with *Hin*dIII (Gibco BRL Life Technologies, Burlington, ON) and electrophoresed on a 0.8% agarose gel. DNA fragments were transferred to a nylon membrane (Hybond-XL, Amersham Biosciences, Piscataway, NJ) by capillary transfer with 0.4 *M* NaOH (Koetsier et al., 1993). Hybridization was performed according to the Amersham protocol for Hybond-XL membranes. The DNA was hybridized to a ^{32}P-labeled 550 bp PMI fragment obtained by PCR amplification of plasmid DNA. Blots were exposed to X-ray film (Kodak X-OMAT) at −80°C with an intensifying screen for 3–24 h.

FIG. 1. Hemp tissue culture and selection of transformed cells. *a*, Rhizogenic callus after 1 mo. on MB medium supplemented with NAA or IBA, instead of 2,4-D, as an auxin source. *b*, Callus growth on leaf explants on MB5D1K medium. *c*, An established suspension culture of the hemp variety Kompolti, showing cell aggregates. *d–f*, Callusing responses of different explant sources on MB5D1K medium. *d*, Arrangement of stem and cotyledon explants at day 0. *e*, Hypocotyl and epicotyl explants with comparable callus at 2 wk; cotyledons showed a poor ability to callus. *f*, Callus developing around petioles and leaf midveins, followed by cut edges. *g–i*, Selection of Anka cells transformed with pNOV3635 on MB2.5D with 300 mg l^{-1} Timentin and 1% mannose after 4 wk. *g*, Nontransformed cells are arrested in growth. *h*, Transformed cells distinguished by their increased size compared to untransformed cells. *Dishes* are 9 cm diameter (*a–h*). *i*, Transformed callus on mannose medium, forming large, pale yellow callus protruding from small, dark yellow parental callus (*bar* = 5 mm).

Results and Discussion

Callus induction. Based on previous work (Loh et al., 1983; Mandolino and Ranalli, 1999), a combination of MS salts with B5 vitamins (MB) was chosen in this study to promote callus and suspension culture growth. However, *Cannabis* explants have been found to respond favorably to both MS medium (Itokawa et al., 1977; Fisse et al., 1981) or B5 medium (Heitrich and Binder, 1982; Braemer and Paris, 1987). Callus developed on leaf and stem explants of hemp varieties Anka and Uniko for all treatments containing 2,4-D within 4 wk after plating. Overall, treatments containing 2,4-D supplemented with the cytokinins BA or kinetin promoted the greatest callus growth and best appearance (data not shown). Other treatments in which 2,4-D was replaced with IBA or NAA as auxin sources induced an initial callusing stage followed by development of a mass of rootlets covered in fine root hairs after 4 wk (Fig. 1*a*). The promotion of rhizogenesis by NAA was also noted by Fisse et al. (1981). The combination of 5 μ*M* 2,4-D and 1 μ*M* kinetin (MB5D1K) was chosen in this study to promote prolific growth of pale yellow, friable callus (Fig. 1*b*);



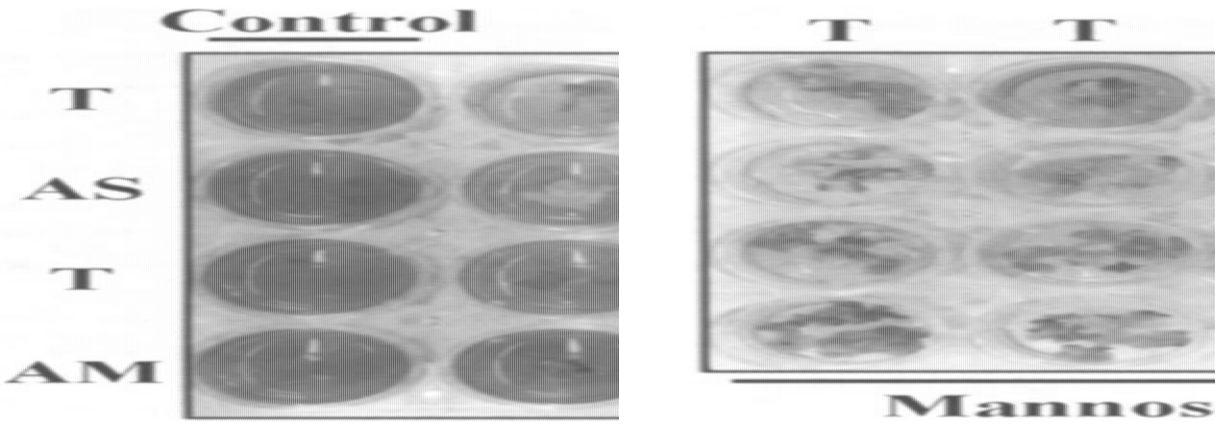


FIG. 4. Chlorophenol-red PMI assay after 3–4 d. Medium is composed of MB2.5D, 8 g l^{-1} agar, and 0.1 g l^{-1} CPR with either 3% sucrose or 2% mannose. Control wells do not contain callus. T, Transformed Anka callus harboring the PMI gene grew on both sugar sources, turning the medium pale yellow. AS, Anka callus metabolized sucrose and acidified the medium, turning it a pale yellow color. AM, Anka callus incubated with *Agrobacterium* lacking the PMI plasmid did not acidify the medium. Well diameter in each dish is 1.5 cm.

other studies have reported using a combination of 2,4-D and kinetin for callus initiation (Hartsel et al., 1983; Braemer and Paris, 1987) and establishment of suspension cultures (Braemer and Paris, 1987).

Callusing responses on MB5D1K medium of different explant sources from varieties Felina and Uniko were similar. However, callus proliferation was dependent on the type of explant chosen, and was greatest on stems, petioles, and leaves, and poor on cotyledon explants (Fig. 1*d–f*). Fisse et al. (1981) and Mandolino and Ranalli (1999) also noted that cotyledon and root explants did not produce callus well. There did not appear to be any differences in the extent of callus formation among leaves of different ages taken from the same seedling (Fig. 1*f*).

The rate of callus development on MB5D1K for stem and leaf explants of four hemp varieties was similar. By 2 wk, almost 100% of the explants had produced callus and at 4 wk, the average callus diameter was in the range of 6.8–7.8 mm, indicating that all four hemp varieties responded similarly to MB5D1K medium (data not shown). Callus from all varieties developed white roots covered with fine root hairs if left longer than 4 wk without being transferred to fresh medium.

Suspension cultures. Differences among the four hemp varieties were more pronounced when tissues were transferred to liquid MB2.5D medium. The extent of callus mass and root proliferation in suspension culture differed with variety. Subcultures made every 2–4 wk favored development of small cell masses without rootlets (Fig. 1*c*). Suspension cultures were easily established for the varieties Anka, Kompolti, and Felina, while Uniko did not respond well to suspension culture conditions. Suspension growth experiments indicated that both fresh and dry weights of the varieties Anka and Kompolti more than doubled within the first 7 d of growth; however, Felina suspensions grew more slowly, and dry and fresh weights doubled by 10–14 d, respectively (Fig. 2). Anka suspensions produced the greatest fresh (8.50 g) and dry (0.63 g) weights over 28 d.

Regeneration of plantlets. None of the attempts to regenerate hemp plantlets, either directly from explants or indirectly from callus or suspension cultures (Table 1), were successful. Neither somatic embryos nor shoot bud initiation was observed in any treatment tested. However, callus and suspension cultures had a tendency to form roots. Fisse et al. (1981) and Hemphill et al. (1978) described hemp callus which readily formed roots with no evidence of shoot formation in response to different plant growth regulator combinations. MacKinnon et al. (2000) also described root development but did not observe shoot formation from hemp callus. Alternatively, they developed a method to regenerate plantlets from shoot-tips. Richez-Dumanois et al. (1986) developed a protocol to micropropagate hemp using apical and axillary buds. With the exception of Mandolino and Ranalli (1999), who described occasional shoot regeneration from callus, *de novo* shoot formation has not been reported for hemp. Reports of shoot or root formation from callus as well as plantlet regeneration from shoot-tips or buds suggest that hemp is capable of differentiation; further research should be focused on this aspect to achieve an efficient and reliable hemp regeneration protocol.

Hemp transformation. For our genetic transformation studies, we used the PMI, *manA*, gene isolated from *E. coli* (Miles and Guest, 1984) as a selectable marker. The PMI selection strategy makes use of a sugar (mannose) as a selection agent (Joersbo, 2001; Wright et al., 2001) and is based on endowing transformed cells with a metabolic advantage (Joersbo, 2001). Most explants are incapable of growth on mannose as a carbon source (Joersbo, 2001) since this sugar is converted by an endogenous hexokinase to nonutilizable mannose-6-phosphate. In contrast, cells expressing the PMI gene are capable of converting mannose-6-phosphate to fructose-6-phosphate, which is readily metabolized (Negrotto et al., 2000; Joersbo, 2001; Reed et al., 2001). This selection strategy has been recently applied to several crops, including sugar beet (Joersbo et al., 1998), cassava (Zhang et al., 2000), maize (Negrotto et al., 2000), wheat (Wright et al., 2001), and rice (Lucca et al., 2001).

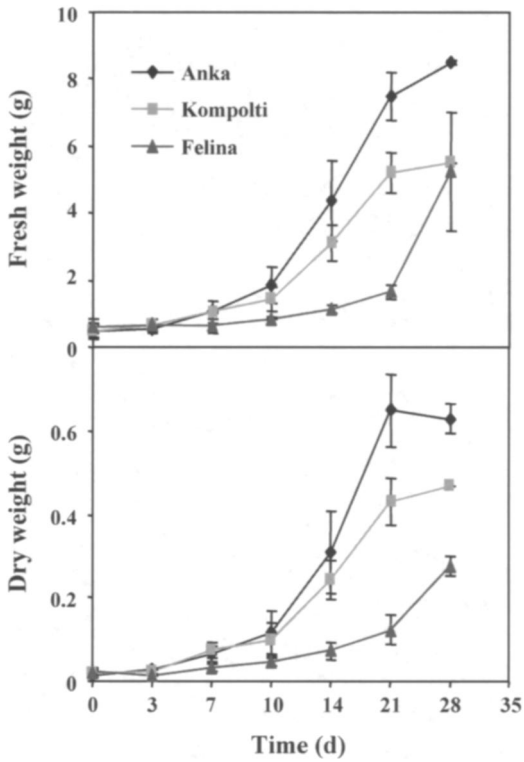


FIG. 2. Average growth rates of three hemp varieties in suspension culture (MB2.5D). Fresh and dry weights were measured at 3–4 d intervals over 28 d.

Medium supplemented with 1–3% mannose as a carbon source, with or without sucrose, significantly arrested hemp callus growth compared with medium containing 3% sucrose (Fig. 3*a*). Callus placed on mannose had a similar appearance to callus growing on sucrose, except that callus clumps were arrested in growth on the former carbon source and were easily distinguished at 4 wk by appearing much smaller and deeper yellow in color compared to callus growing on sucrose (Fig. 1*g–i*). Medium containing 1% mannose therefore was chosen for selection of transformed hemp cells.

After *Agrobacterium* infection and cocultivation, callus developing on MB2.5D with 1% mannose and 300 mg l^{-1} Timentin turned from pale yellow to a darker yellow color within 1 wk. By 4 wk, cells capable of metabolizing mannose were easily distinguishable by their color and larger size. Pale yellow callus emerged from darker yellow cell clumps, growing larger than other callus masses (Fig. 1*h*, *i*). These were transferred to fresh selection medium with 2% mannose and 150 mg l^{-1} Timentin for proliferation. An average

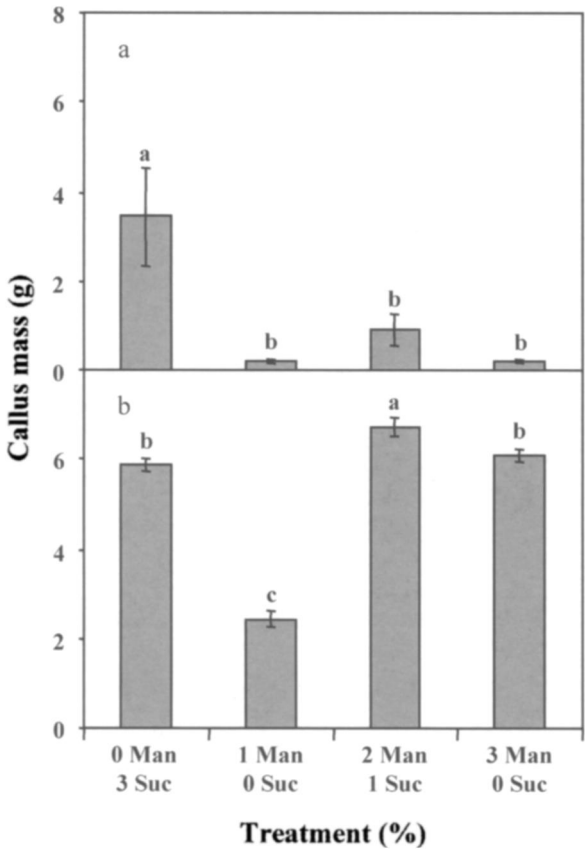


FIG. 3. Callus growth over a range of mannose (Man) and sucrose (Suc) concentrations (%). *a*, Anka callus. *b*, Transgenic Anka callus. Significance was tested with ANOVA. Means showing the same *letter* are not significantly different ($P = 0.05$).

transformation frequency of 31.23% (±0.14) was obtained for all transformation experiments, with a range of 15.1–55.3%. This is within the range (2–45%) of other reports using mannose selection (Reed et al., 2001).

Growth of Anka callus transformed with the PMI gene was evaluated on mannose-containing medium and compared with that of untransformed Anka callus. Transformed callus was capable of growing over a range of mannose concentrations that would otherwise have inhibited untransformed callus growth. Comparisons among transgenic callus cultures revealed that callus growth was not as good on 1% mannose medium and was greatest on 2% mannose with 1% sucrose (Fig. 3*b*). This suggests that 1% mannose is insufficient to promote a high rate of callus growth and that cells are capable of utilizing sucrose in the presence of mannose.

PMI assays. The chlorophenol red (CPR) assay was a quick, sensitive, and indirect method to confirm the expression of the PMI gene in hemp callus. The assay is based on the appearance of a color change by the pH indicator, CPR. Tissues capable of active growth in the presence of mannose will acidify the medium and cause a visible color change from red to yellow (Kramer et al., 1993). We used 0.1 g l^{-1} CPR dye, within the concentration range described by other researchers (Kramer et al., 1993; Lucca et al., 2001), which gave a red-orange color once dispensed into wells, instead of deep red. At higher concentrations, CPR dye had an inhibitory effect on hemp callus growth and no color change was detected (data not shown). Two representative assays are shown in Fig. 4. To evaluate control callus in the CPR assay, a 1 wk pre-incubation period on mannose-containing medium instead of sucrose was required. If control callus was grown on sucrose-containing medium, a change from red to yellow was observed (data not shown). This suggests that growth on sucrose allows hemp cells to store energy reserves that could be used for metabolic functions that would acidify the assay medium, giving a false positive reaction. Therefore, untransformed callus placed on MB2.5D with sucrose as an energy source acidified the medium, turning CPR a pale yellow color (*lane AS*), whereas untransformed callus maintained on mannose-containing medium and transferred to the PMI assay could not metabolize mannose and the assay medium remained red (*lane AM*). Transgenic callus that grew on medium containing sucrose or mannose (*lanes T*) changed the color to pale yellow. Control wells without any callus remained red-orange throughout the experiment. All callus masses absorbed a small quantity of CPR dye from the assay medium, but this did not affect subsequent callus growth.

Between five and 13 callus lines from each of four transformation experiments were evaluated for PMI activity. All but two callus lines turned the CPR medium yellow within 3 d. Presence of the PMI gene was later confirmed by PCR analysis (see below). In contrast, callus that had not been transformed did not induce a color change over 3 d. Presence of the PMI gene within the two lines not expressing PMI suggest that they may be low expressers and that enzyme activity may not be detected. Wright et al. (2001) also reported low-expressing transgenic tissue with mannose selection and suggested that the PMI selection system can encourage growth of both low- and high-expressing transgenic tissue.

All callus lines incubated on LB medium for 1 wk at 28°C showed no signs of bacterial contamination. Plates were kept for an additional 1 mo. at room temperature without the appearance of *Agrobacterium*.

Molecular analyses. PCR analysis was performed on 28 callus lines representative of all transformation experiments, including all callus lines from the CPR assay. All transformed lines tested by PCR were shown to contain an amplified sequence of about 550 bp, corresponding to the region between the PMI primers (Fig. 5). Untransformed callus did not produce any bands.

Eight callus lines that had tested positive for the presence of the PMI gene by PCR were analyzed by Southern hybridization. The PMI gene was detected in transgenic callus and not in untransformed control callus. A single *Hin*dIII restriction site is present inside of the right T-DNA border in the PMI-containing

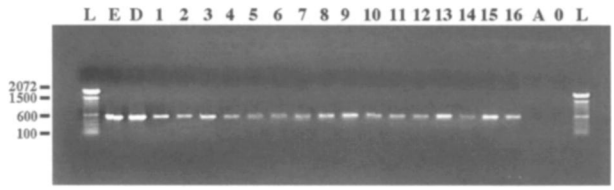


FIG. 5. Analysis of transformed hemp callus by PCR: L, 100 bp DNA ladder; *lane E*, DNA extracted from *A. tumefaciens* vector harboring pNOV3635; *lane D*, DNA extracted from *E. coli* vector harboring pNOV3635; *lanes 1–16*, DNA extracted from transgenic Anka callus lines showing an amplified sequence corresponding to about 550 bp; *lane A*, DNA from Anka callus incubated with *Agrobacterium* lacking pNOV3635; *lane 0*, negative control (no DNA).

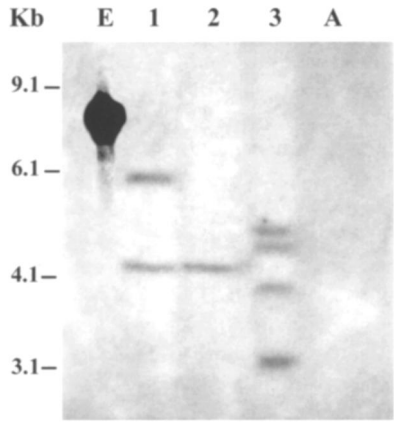


FIG. 6. Southern hybridization of three transformed hemp callus lines. Plasmid and plant DNA samples were digested with *Hin*dIII and the probe was made by PCR-amplification of the PMI gene (550 bp) and labeled with ^{32}P. *Lane E*, *Agrobacterium* plasmid DNA carrying pNOV3635; *lanes 1–3*, transformed hemp callus DNA; *lane A*, DNA from untransformed hemp callus. Molecular weight markers (kilobases) are indicated on the left.

plasmid. Genomic DNA samples showed one to four hybridizing bands, indicating that more than one T-DNA copy was integrated into the hemp genome in some lines (Fig. 6).

This study has described suitable tissue culture conditions for growth of callus and suspension cultures of four hemp varieties. Molecular and biochemical analyses showed that hemp callus was successfully transformed by *A. tumefaciens* harboring the pNOV3635 plasmid conferring resistance to mannose. This work will be useful for further studies towards the development of transgenic *Cannabis* varieties expressing horticulturally desired traits, such as pest and disease resistance, pending development of a suitable direct or indirect system to regenerate plantlets *in vitro*.

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