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Cannabis sativa L. genetically transformed root based culture *via Agrobacterium rhizogenes*

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Abstract

It is an increased interest in the therapeutic potential of *Cannabis sativa* L. (marijuana) for treatment of multiple sclerosis and HIV neuropathy. Because of limitation in cultivation of this plant, an efficient hairy root induction system for *Cannabis sativa* L. was developed in the present study. *Agrobacterium rhizogenes* mediated transformation performed by two different co-cultivation mediums and four different bacterial strains including A4, ATCC15834, MSU440, and A13 (MAFF-02-10266). Genomic DNA from putative transgenic hairy root lines and the control root was extracted using a modified CTAB protocol. Molecular analysis of transformed root lines was confirmed by polymerase chain reaction using specific primers of the *rolB* gene. The transformation frequency by Murashige and Skoog co-cultivation medium resulting in hairy root induction frequencies of 42.3%, 46.3%, 68.6% and 39.3% by A4, ATCC15834, MSU440, and A13 strains, respectively. There was no significant difference between MS or ½ MS co-cultivation mediums. This study established a reliable protocol for induction of hairy roots of *C. sativa*. The best *A. rhisogenes* strain was MSU440. It was observed no significant difference between MS and ½ MS co-cultivation mediums on transformation frequency.

Keywords: *Agrobacterium rhizogenes, Cannabis sativa* L., genetically transformed roots

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Introduction

Hemp (*Cannabis sativa* L.) (marijuana) produces about 80 cannabinoids, including Δ^9 -tetrahydrocannabinol (THC), which is the main psychotropic component and specifically binds to G-protein-coupled receptors called cannabinoid (type-1 (CB1) and type-2 (CB2)) receptors (1). Cannabinoids, the active components of *C. sativa* L., have received renewed attention in recent years because of their various pharmacologic activities such as cell growth and proliferation inhibition, anti-inflammatory effects, anti-emetic, appetite stimulant and tumor regression. It uses gradually in the treatment of multiple sclerosis, spinal cord injury, anti-convulsing and HIV neuropathy and chronic pain situations (2, 3, 4). It revealed that cannabinoid receptors have an important role in regulating ectoderm-derived neural progenitor/stem cell and mesoderm-derived hematopoietic progenitor/stem cell fate decisions. Schmuhl *et al.* reported that plant-derived cannabinoids CBD to promote mesenchymal stem cell migration *via* CB2 receptor activation and G protein-coupled receptor 55 inhibition to prompt osteoblastic differentiation. They also investigated the antitumor and anti-inflammatory activities of CBD in human prostate cancer cell lines (5). *Sativex*® is a *Cannabis*-based pharmaceutical product containing THC and CBD, delivered in an oral mucosal spray. It has been approved in patients with multiple sclerosis (MS) as adjunctive treatment for neuropathic pain as a cost-effective treatment (6,7).

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Genetic transformation by Ri Plasmid of *Agrobacterium rhizagenes* has been found to be an effective indirect approach for accumulation of high secondary metabolites in plant cell. The *A. rhizogenes* transfers a DNA segment from its Ri plasmid into the plant genome (8). Hairy roots have been obtained from many medicinal plants which exhibit susceptibility to *A. rhizogenes* infection (9-14). Hairy root-based cultures have several advantages such as genotype and phenotype stability, fast *in vitro* growth in absence of phytohormones under sterile conditions. These cultures have been used to study root physiology along with bio-synthetic pathway elucidation for secondary metabolites production. Due to hairy roots biosynthetic capacity to produce secondary metabolites similar to the native plant roots or even higher they have been called as 'phytochemical factories' (15). Farag and Kayser reported that *C. sativa* callus cultures when transferred to B5 medium supplemented with various concentrations of auxins (NAA, IBA and IAA) resulted to initiation of adventitious roots (so called hairy roots). They reported that emerging hairy root cultures were isolated from callus cultures and subsequently transferred to solid B5 medium with 4 mg/l NAA for further proliferation (16).

At present, a range of commercially important high-value bioactive molecules such as therapeutic and cosmetic products are being produced by hairy roots from various plant species at industrial level. It can produce high-value plant-derived compounds with safe and rapid process compared to field cultivation and chemical synthesis, as well as competitive cost compared with conventional production (8, 15, 17).

In the present study, we report the hairy root production of *C. sativa* from the leaf and stem explants with four different strains of *A. rhizogenesis*. We evaluate the strains of *A. rhizogenes* A4, ATCC15834, MSU440, and A13 (MAFF-02-10266) for the first time on this plant. Also the age and type of the explants in the present study have not investigated before. Based on high demand and introducing new application of *Cannabis* secondary metabolites in recent years (5), this *in vitro* transgenic organ culture can use as a future source of THC and CBD production.

Materials and methods

Seed sterilization, germination and plant material and bacterial strains

Seeds of *Cannabis Sativa* were surface-sterilized with 70% (v/v) ethanol for 1 min and 2% (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. The seeds were then placed on Murashige and Skoog (MS) (18) medium solidified with 0.7 g/l agar and germinated in growth chamber at 24 ºC under a 16 h photoperiod regime with fluorescent light.

Preparation of A. rhizogenes strains

Five strains of *A. rhizogenes* (ATCC 15834, ATCC 31798, MAFF-02-10266, and MSU440) (All of them were provided by the microbial bank at NIGEB, Tehran, Iran) were used in this study. From each strain, a single bacterial colony was inoculated in 15 mL of liquid LB medium containing 50 mg rifampicin L^{-1}, 5 g tryptone L^{-1}, 5 mg yeast extract L^{-1} and 10 mg NaCl L^{-1}, pH 7.2 to an optical density of 0.8, at 28 °C, 120 rpm on a rotary shaker incubator for 24 h. The bacterial suspensions were centrifuged at 3500 rpm for 12 min. The pellets were re-suspended in 25 mL MS liquid medium supplemented with 100 µM acetocyringone after sterilization using 0.22 µm syringe filters at 28 ºC in the dark.

Co-cultivation and establishment of hairy root cultures

Leaf and stem explants from 3-week-old *in vitro* seedlings were used for transformation. The explants were immersed in bacterial inoculation medium for 5 min and then blotted on sterile filter paper and incubated in a co-cultivation medium consisted of MS or ½ MS salts and vitamins along with 50 mg sucrose L^{-1} and 100 µM acetocyringone. After two days of co-cultivation, the explants transferred to hormone-free MS media supplemented with 300 mg cefotaxime L^{-1} to eliminate the *A. rhizogenes*. Control explants were treated without bacterial inoculation. After 3-4 weeks, hairy roots appeared from the explants. The induced hairy roots were separated from the explants and cultured on selection medium at 25 ºC in dark. Rapidly growing hairy root cultures were obtained after repeated transfer to fresh selection medium.

DNA extraction and PCR amplification

Genomic DNA from putative transgenic hairy root lines and control root line was extracted using a modified CTAB protocol for DNA extraction from medicinal plant species (19). Polymerase chain reaction was performed using approximately 50 ng genomic DNA as a template and *rolB* gene-specific primer to determine the integration of T-DNA from Ri plasmid. The *rolB* primers were: 50 -GCTCTTGCAGTGCTAGATTT-30 (forward primer) and 50 -GAAGGTGCAAGCTACCTCTC-30 (reverse primer).

The PCR reaction mixtures contained, in a final volume of 25 µL of 1 × PCR buffer, 3 mM $MgCl_2$, 1 mM of each dNTP, 0.4 µM of each specific primer, 1 U of *Taq* DNA polymerase (NGene Biotech. Co.), and 20 ng genomic DNA or 10 ng pRi plasmid DNA used as positive control. The PCR was carried out under the following conditions: 94 ℃ for 5 min, 30 cycles of three steps (94 ℃ for 1 min (denaturation), 58 ℃ for 1 min (annealing) and 72 ℃ for 1 min (amplification)), and 72 ℃ for 10 min for final extension. PCR products were separated by electrophoresis on 0.8% agarose gel in 0.5 × TBE buffer, stained by ethidium bromide and imagined by UV document scanner.

Statistical *analysis*

The experiments were laid on a completely randomized design (CRD) with three replications and 9 explants cultured in each Petri dish. The data collected were subjected to analysis of variance test. The means were compared using Duncan's multiple range tests. The data expressed as percentage were subjected to *arcSin* transformation before the statistical analysis.

Results

Effect of A. rhizogenes strains and co-cultivation medium

Seeds were germinated in one week, and leaf and stem explants from four weeks old *C. sativa* seedlings were used for co-cultivation (Fig.1a). Four different *A. rhizogenes* strains were examined for their transformation efficiency. In all experiments we used a

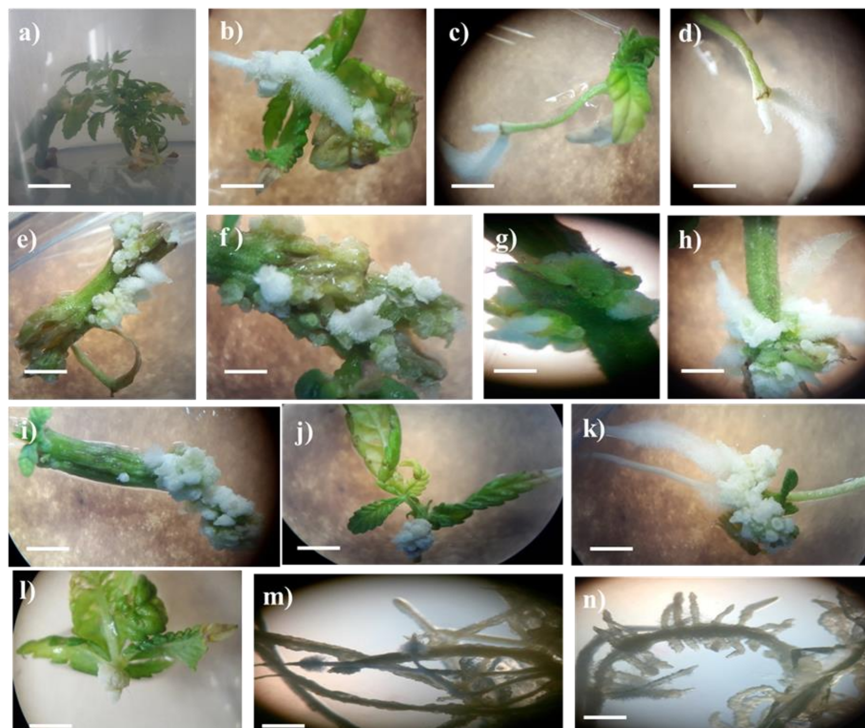

Figure 1 *Agrobacterium rhizogenes* mediated transformation in *Cannabis sativa* L. (a) One month old plants in MS medium (*bar* 2 cm) (b, c, d) Hairy root induction on explants after 4 weeks using strain MSU440 (*bar* 5 mm); (e, g, h) Hairy root induction on explants after 4 weeks using strain ATCC15834 (*bar* 5 mm) (j, k) Hairy root induction on explants after 4 weeks after 5 weeks of inoculation using strain A4 (*bar* 5 mm); (l) Hairy root induction on explants after 4 weeks using strain A13 (MAFF-02-10266) (*bar* 5 mm); (m,n) Growth of hairy root in MS medium (*bar* 5 mm).

phenolic compound acetosyringone to stimulation of *A. rhizogenes* T-DNA transduction. Some studies reported that an appropriate concentration of acetosyringone in the culture medium during hairy root induction can promote transformation (11). Hairy roots of *C. sativa* were initiated from stem and leaf explants after three to five weeks by four different *A. rhizogenes* strains (Fig. 1. b-l). Hairy roots were excised from explant tissues and cultured on agar solidified MS medium for more growth (Fig. 1. n,m).

All of the *A. rhizogenes* strains led to hairy root induction on explants and in some case caused the formation of tumorigenic calli. Tumor induction occurred in all strains with differing frequencies (data not shown). The results revealed that the stem explants is the best explant for *A. rhizogenes* mediated transformation in *C. sativa* (Fig. 2). The stem explants were significantly susceptible to infection by each strain of *A. rhizogenes*. The highest rate of infection occurred in MSU440 in the stem explants with 63.6%; whereas, MAFF-02-10266 strain infected only 39.3% of the stem explants (Fig. 2). Leaf explants showed lower rate of hairy root induction in compare to stem explants. To determine the optimal co-cultivation medium for *A. rhizogenes* mediated transformation of *C. sativa*, full MS and ½ MS co-cultivation media were evaluated using stem explants and *A. rhizogenes* MSU440 which revealed in the previous experiment as the best choices. In the full strength MS co-cultivation medium, the rate of transformation was 63.6% and in the ½ MS co-cultivation medium was 68.3% (Fig. 3).

PCR analysis

The PCR analysis of hairy roots and control resulted in the extension of expected fragment in hairy root lines similar to that obtained in the positive control, whereas there was no extension in the DNA isolated from normal roots (Fig. 4). 10 transgenic hairy root lines among the obtained hairy root lines presented in Fig. 4. To confirm hairy roots were not contaminated by *A. rhizogenes*, PCR analysis using specific primers to *vir*G was done and amplification of *vir*G was not detected (data not shown). This result indicates transformation of T-DNA from Ri plasmids occurred in the putative hairy roots lines.

Discussion

All of the *A. rhizogenes* strains led to hairy root induction on explants and in some case caused the formation of tumorigenic calli. Previous reports indicated that *A. rhizogenes* strain and type of explants significantly affected on hairy root induction (11, 12). In some explants tumorigenic calli (galls) were induced. Galls formed at the infection site of explants may be due to T_R-DNA which is responsible for the auxins biosynthesis (8). As tumorigenic calli is not proper for large scale productions, our results indicated that strain MSU440 inducing high rate of hairy roots and low rate tumorigenic calli is more capable than the other bacterial strains in this study. The difference in virulence, morphology and growth rate can be partially described by the plasmid diversity harbored by the *A. rhizogenesis* strains.

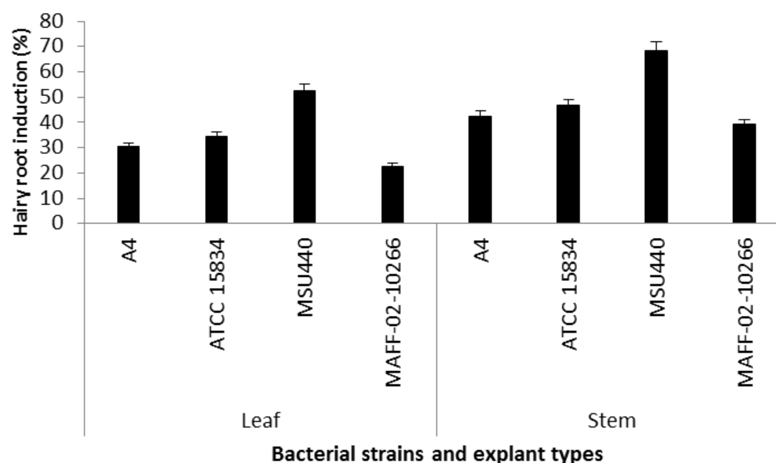

Figure 2 Effect of different *Agrobacterium rhizogenes* strains on hairy root induction percentage in *Cannabis sativa L.;* the data were obtained as a mean of three replications. The different letters denote a statistically significant difference at P ≤ 0.05, determined by Duncan test. Vertical lines represent standard errors.

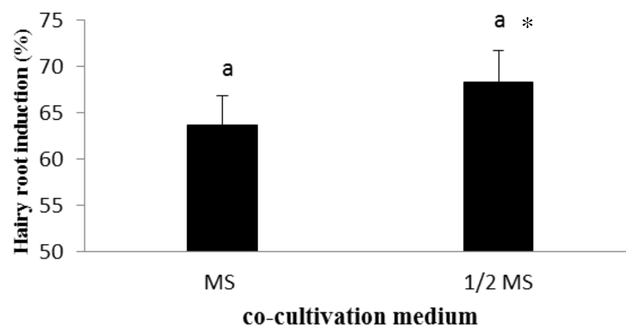

Figure 3 Effect of co-cultivation media on frequency of hairy root induction in *Cannabis sativa L.;* the data were obtained as a mean of three replications. The different letters denote a statistically significant difference at $p \leq 0.05$, determined by Duncan test. Vertical lines represent standard errors.
* There is no significant difference between two treatments statistically

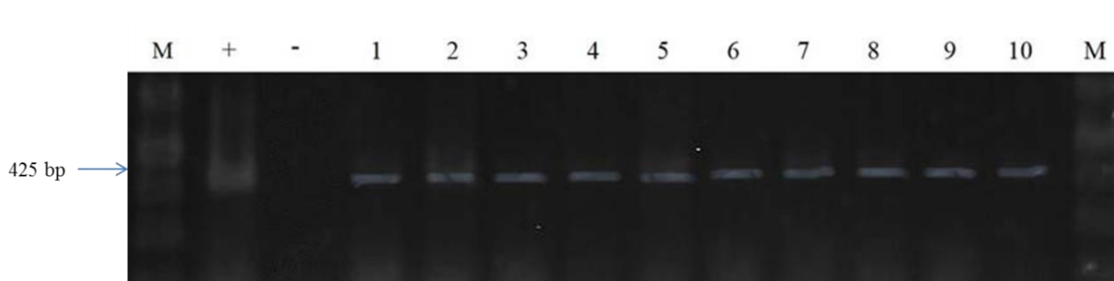

Figure 4 Molecular analysis of hairy roots; **a)** PCR analysis for detection of the *rolB* gene in hairy root lines of *Cannabis sativa L.;* M: Molecular size marker (1 kb ladder); 1–10: hairy root lines, (-): negative control (non-transformed root); (+): positive control (Ri plasmid).

It revealed that a medium with half concentration of mineral compounds co-cultivation medium is not significantly better than MS medium to achieve the high transformation frequency. In contrast, previous studies showed that the expression of *vir* G can be activated by low levels of PO_4 and suggested that a shortage of PO_4 could be a positive signal to transformation frequency. Similar results were reported in *Lilium formolongi* transformation by *A. tumefaciens* (20) and hairy root induction using *A. rhizogenes* (11-13). As the transformation efficiency was not significantly increased by decreasing MS salts, our results suggest that macro-elements in co-cultivation media have not inhibitory effects on the *A. rhizogenes* mediated transformation of *C. sativa.* The reduction of MS salts slightly increased the transformation rate but it was not a significant. The present study suggested that the effect of macro-elements in co-cultivation media may be act in a species dependent manner. In a different procedure Farag and Kayser reported 2.0 µg/g dry weight cannabinoid in root cultures which emerged from callus cultures induced by auxins (16). Also Wahby *et al.* used five-day-old plantlets for transformation of *C. sativa*. They used hypocotyls, cotyledonary node, cotyledons and primary leaves as explant for inoculation. They reported hypocotyl was the most responsive material for transformation (21).

Conclusion

This study, established a reliable protocol for induction of hairy roots of the important medicinal plant *C. sativa*. The hairy root production of *C. sativa* from the leaf and stem explants by 4 different strains of *A. rhizogenesis* was reported. It revealed that the best bacterial strain and explant were MSU440 and stem segment respectively. The use of hairy roots could have good potential in investigating the molecular regulation of genes encoding THC and CBD biosynthetic enzymes. The hairy root culture system is a potential approach for

the production of THC and CBD, because it has many good qualities, such as fast growth rate, easy culture and genetic manipulation, and an increased capability to synthesize by elicitation. Advances in plant transcriptomics and metabolomics joined with modeling of metabolic fluxes through *in silico* approaches and genetic engineering will allow hairy roots to become a powerful and sustainable phytochemical production system. Different strains of *A. rhizogenes,* cocultivation media and types of *C. sativa* explants were evaluated in this study. This study opens the way for biotechnological production of pharmacologically active THC using wild-type transformed or *rolB* transgenic root cultures of *C. sativa*. Further studies are in progress to evaluation of THC and CBD content in hairy root culture and commercialize this method by designing a cost effective bioreactor.

Conflict of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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