World Academy of Science, Engineering and Technology
International Journal of Agricultural and Biosystems Engineering
Vol:18, No:12, 2024

# Plant Regeneration via Somatic Embryogenesis and Agrobacterium-Mediated Transformation in Alfalfa (Medicago sativa L.)

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**Abstract :** Alfalfa is renowned for its nutritional and biopharmaceutical value as a perennial forage legume. However, establishing a rapid plant regeneration protocol using somatic embryogenesis and efficient transformation frequency are the crucial prerequisites for gene editing in alfalfa. This study was undertaken to establish and improve the protocol for somatic embryogenesis and subsequent plant regeneration. The experiments were conducted in response to natural sensitivity using various antibiotics such as cefotaxime, carbenicillin, gentamycin, hygromycin, and kanamycin. Using 3-week-old leaf tissue, somatic embryogenesis was initiated on Gamborg's B5 basal (B5H) medium supplemented with 3% maltose, 0.9µM Kinetin, and 4.5µM 2,4-D. Embryogenic callus (EC) obtained from the B5H medium exhibited a high rate of somatic embryo formation (97.9%) after 3 weeks when the cultures were placed in the dark. Different developmental stages of somatic embryos and cotyledonary stages were then transferred to Murashige and Skoog's (MS) basal medium under light, resulting in a 94% regeneration rate of plantlets. Our results indicate that leaf segments can grow (tolerate) up to 450 mg/L of cefotaxime and 400 mg/L of carbenicillin in the culture medium. However, the survival threshold for hygromycin at 12.5 mg/L, kanamycin at 250 mg/L, gentamycin at 50 mg/L, and timentin (300 mg/L). The experiment to improve the protocol for achieving efficient transient gene expression in alfalfa through genetic transformation with the Agrobacterium tumefaciens pCAMBIA1304 vector was also conducted. The vector contains two reporter genes such as β-glucuronidase (GUS) and green fluorescent protein (GFP), along with a selectable hygromycin B phosphotransferase gene (HPT), all driven under the CaMV 35s promoter. Various transformation parameters were optimized using 3-week-old in vitro-grown plantlets. The different parameters such as types of explant, leaf ages, preculture days, segment sizes, wounding types, bacterial concentrations, infection periods, co-cultivation periods, different concentrations of acetosyringone, silver nitrate, and calcium chloride were optimized for transient gene expression. The transient gene expression was confirmed via histochemical GUS and GFP visualization under fluorescent microscopy. The data were analyzed based on the semi-quantitative observation of the percentage and number of blue GUS spots on different days of agro-infection. The highest percentage of GUS positivity (76.2%) was observed in 3-week-old leaf segments wounded using a scalpel blade of 11 size- after 3 days of post-incubation at a bacterial concentration of 0.6, with 2 days of preculture, 30 min of bacterial-leaf segment co-cultivation, with the addition of 150 µM acetosyringone, 4 mM calcium chloride, and 75 µM silver nitrate. Our results suggest that various factors influence T-DNA delivery in the Agrobacterium-mediated transformation of alfalfa. The stable gene expression in the putative transgenic tissue was confirmed using PCR amplification of both marker genes, indicating that gene expression in explants was not solely due to Agrobacterium, but also from transformed cells. The improved protocol could be used for generating transgenic alfalfa plants using genome editing techniques such as CRISPR/Cas9.

**Keywords :** Medicago sativa l. (Alfalfa), agrobacterium tumefaciens, β-glucuronidase, green fluorescent protein, transient gene
**Conference Title :** ICAB 2024 : International Conference on Agriculture and Biotechnology
**Conference Location :** Istanbul, Türkiye
**Conference Dates :** December 23-24, 2024