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Full Length Research Paper

Agrobacterium-mediated transfer of β-Glucoronidase gene (*gusA*) to water mint (*Mentha aquatica L.*)

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Water mint is a medicinal aromatic perennial herb belonging to the family Labiateae. It is distributed mostly in the temperate and sub-temperate regions of the world. Water mint is valued for its multipurpose uses in the field of pharmaceuticals, cosmetics and flavoring foods. *Agrobacterium*-mediated gene transformation has yielded a stunning array of transgenic plants with novel properties. Transgenic mint plants with reporter genes have been obtained only in peppermint. Therefore it would be interesting to extend transformation experiments to other mint species such as Water mint. Establishing a reliable direct regeneration system is necessary in plant transformation programs. *In vitro* direct regeneration of Water mint was obtained from leaf disks. Best results were obtained when explants were cultured onto MS medium supplemented with 4 mg/lit BAP. Transformed plants were obtained by co-cultivation of leaf disks with *Agrobacterium tumefaciens* strain GV3850 harbouring a binary vector pBI121 that carried *E. coli* β-glucuronidase as reporter gene and *nptII* as selective marker gene for kanamycin resistance. The presence and expression of transgenes in transgenics was evaluated by GUS histoenzymatic assay and PCR. An overall transformation frequency of 12% was achieved.

Key words: *Agrobacterium tumefaciens*, *Mentha aquatica*, transformation, β-glucuronidase gene.

INTRODUCTION

Medicinal plants have been the subject of man`s curiosity and purpose since time immemorial. The importance of medicinal plants in the treatment of chronic disease needs no elaboration. In fact, even with the tremendous advancement in the field of synthetic chemistry, almost 50% of the commercial drugs available in the market remain of plant origin. The herbal system was, however, pushed to the background with the advent of allopathic system. It is now back with a venegence and the age-old system of herbal medicine is being revived due to its long lasting curative effect, easy availability, natural way of healing and rare or no reported side effects. Due to growing world population, increased anthropogenic activities, rapidly eroding natural ecosystem etc., the natural habitat for a great number of plants are dwindling and many of them are facing extinction. New strategies are being therefore formulated for rapid multiplication and conversation of medicinal plants. Among these strategies, *Agrobacterium*-mediated transformation has emerged as an efficient method for genetic manipulation of plants (Srivastava et al., 2004). *Agrobacterium*-mediated plant transformation entails not only delivery and integration of engineered DNA into plant cells, but also the regeneration of transgenic plants from those genetically altered cells. Indeed, it has frequently been the plant tissue culture technology, rather than the transformation process itself, that has been the limiting step in achieving efficient genetic modification (Tzfira and Citovsky, 2008).

Mentha aquatica L. (English: water min, wild mint, marsh mint) is an aromatic perennial herb belonging to the family Labiateae, distributed mostly in temperate and sub-temperate regions of Eurasia. Water Mint grows in shallow margins of rivers and wet meadows, marshes and fens. Essential oil from *M. aquatic* contains mono

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and sesquiterpenes with a high degree of chemo-variation. A number of flavnoids have also been isolated from the plant. It is valued for its aromatic, stomachic, stimulant and anti spasmodic properties (Bhat, 2002).

GUS (β-glucuronidase), a kind of hydrolase that catalyzes many chemical reactions of GUS-like zymolytes, has been widely used for gene engineering and genetic transformation, especially in the experiments made use of its transient expression to determine and optimize various factors that influence transformation efficiency (Yongong, 2009).

MATERIALS AND METHODS

Plant material and explants source

Water mint plants collected from Avicenna medicinal plants garden of Bu Ali Sina university (Hamadan, Iran). Apical and axillary buds were used as source of plant micropropagation.

Tissue culture and plant propagation

For micropropagation of Water mint, buds were surface disinfected in a solution of 2% sodium hypochlorite with tween-20 (1ml/l of solution) for 20 min. and then washed three times with sterile deionized water. The buds were placed onto the surface of MS medium consisting of MS (Murashige and skoog 1962) salts, 1 mg l^{-1} pantothenic ac, 1 mg l^{-1} pyridoxin, 1 mg l^{-1} nicotinic acid, 0.01 mg l^{-1} biotin, 100 mg l^{-1} myo-inositol, 8g l^{-1} agar, 30 g l^{-1} sucrose supplemented with 8 mg l^{-1} BAP, 1 mg l^{-1} NAA and 1 mg l^{-1} TDZ. The pH was adjusted to 5.8 before autoclaving at 115°C for 15 min. Explants were maintained at 25±2°C under a photoperiod of 16 h of cool-white flourescenet light (16 $\mu Em^{-2}s^{-1}$) and subcultured every two weeks (Faure et al., 1998).

Callus induction

Leaves with petiole base from the propagated shoots were used for callus induction. Explants were cultured on Ms medium with different concentrations of NAA (1, 2 and 4 mg/l) and BA (0, 0.5, 1, 2 and 4 mg/l). Explants were maintained in darkness at 25±2°C. Callus induction rate was calculated after 4 weeks.

Direct regeneration

Leaves including portion of petioles from the propagated shoots were used for direct regeneration without callus formation. Explants were cultured on MS medium supplemented with the concentrations of 0 and 0.5 mg/l NAA and different concentrations of BA (0, 0.5, 1, 2 and 4 mg/l).

Explants were maintained at 25±2°C under a photoperiod of 16h of cool-white fluorescent light (16 $\mu Em^{-2}s^{-1}$). Regeneration rate was calculated after 4 weeks.

Statistical analysis

For all experiments there were three repeats and in each repeats six explants. Factorial experiment was used for all tests. Data were analyzed by using SAS 9.1 software. Duncan`s procedure was used for analyzing of averages comparison.

Agrobacterium tumefaciens transformation

E. coli strain DH5α containing plasmid pBI121 and *Agrobacterium tumefaciens* strain GV3850 were used for experiments. The binary vector pBI121 contain a *gusA* reporter gene and a neomycin phosphotransferase (*nptII*) marker gene for kanamycin selection. Plasmid isolation from *E. coli* was carried out by using mini-prep method. Transformed *A. tumefaciens* harboring pBI121 were constructed by using freeze-thaw method (Weigel and Glazebrook, 2006). For preparation of *A. tumefaciens* competent cells 200 ml of LB medium was inoculated with 2 ml of an overnight culture of *A. tumefaciens* and incubated at 28°C with vigorous agitation until the culture grew to an OD_{550} of 0.5 to 1.0. The chilled culture of bacteria was centrifuged at 3000 g for 5 min at 4°C and the pellet was resuspended in 20 mM $CaCl_2$. After adding about 1µg of plasmid, the cells were freezed in liquid nitrogen and then thawed by incubating in 37°C water bath for 5 min. Fresh LB medium was added to the culture and incubated at 28°C for 2-4 h with gentle shaking to allow the bacteria to express antiobiotic resistance gene. The culture was centrifuged and resuspended in fresh LB medium and spreaded on LB agar plate containing 30 mg/l kanamycin and incubated at 28°C.

Selection of effective concentration of kanamycin

For selection of effective concentration of kanamycin, leaf explants were cultured on direct regeneration solid MS medium supplemented with different concentrations of kanamycin (0, 25, 50 and 100 mg/l). The survival and regeneration rate of explants were calculated 4 weeks later.

Plant transformation

Agrobacterium tumefaciens containing pBI121 was grown overnight in Luria Bertani (LB) medium supplemented with 50 mg l^{-1} rifampicin and 25 mg l^{-1} kanamaycin at 28°C. The pellet resuspended in liquid MS medium to an OD_{600} at 0.6. This culture was used for infection of leaf explants and callus tissues.

Parameters such as incubation time (10 and 20 min) and type of explants (leaf and callus) were analyzed for Agribacterium-mediated T-DNA delivery. After immersion of wounded callus and leaf explants with petiol base in the bacterial culture for 10 and 20 minutes with occasional shaking, excess bacterial culture was removed by blotting the explants on sterile filter paper. Then they were placed onto MS regeneration medium without antibiotics for a 4 day cocultivation period in darkness at 28°C. After this period, explants were washed with sterile distilled water containing 100 mg l^{-1} cefotaxime and placed onto selection regeneration medium. Explants were cultured onto MS medium supplemented with 50 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin, for inhibition of bacteria and selection of transformed plant cells, respectively.

GUS histochemical assay

Expression of GUS activity was performed for distinguish transformed from untransformed tissue by the presence of GUS enzyme that converts the substrate 5-bromo-4-chloro-3-indolyl-beta-D-glucoronide (X_Gluc) into an insoluble precipitate (Jefferson et al., 1987), with some modifications. Leaves and callus from transgenic shoots were washed with sterile distilled water containing 100 mg l-1 cefotaxime and then explants were tested for histochemical GUS expression in X-gluc solution consisting of 2mM X-gluc, 500mM phosphate buffer, 10mM EDTA and Triton x-100. After incubation 24 h at 37°C explants were soaked in 70% ethanol for elimination of chlorophylls. The explants were observed under

microscope.

PCR analysis

Total DNA was extracted from leaves using the cetyl trimethylammonium bromide (CTAB) procedure (Doyle and Doyle, 1990). About 200 mg of fresh leaves were crushed in liquid nitrogen and incubated at 65°C under shaking condition for 1 h with 1 ml of pre-warmed (65°C) CTAB extraction buffer (1 M Tris-HCl, 5 M NaCl, 0.5 M EDTA, 2% CTAB, 2% PVP and 1% β-mercaptoethanol). DNA was purified with chloroform: isoamyl alcohol (24:1) and precipitated with pre-chilled isopropanol and washed with 70% ethanol, dried and dissolved in 50 µl sterile distilled water. The presence of *gus* gene in the putative transgenic plants was analyzed through PCR with gus gene-specific primer. The gus specific primer sequences F: 5´-GGTGGGAAAGCGCGTTACAAG- 3´ and R: 5´-TGGATTCCGGCATAGTTAAA- 3´ were used. This primer amplifies a 320 bp fragment containing a portion of the GUS coding region. Each PCR reaction was performed in 25 µl reaction volumes consisting of 10X buffer reaction, 1 µl sample DNA, 2.5 mM $MgCl_2$, 15 pmol of each primer, 200 µM dNTPs and 1 U of Taq DNA polymerase (Cinnagen , Iran). PCR for amplification of GUS was carried out under the following condition: 94°C for 5 min (preheating), then 35 cycles of 94°C for 1 min (denaturing), 61°C for 1 min (annealing), 72°C for 100 s (synthesis) and 5 min at 72°C as final extension. The PCR products were checked on 1% agarose gel and detected by ethidium bromide staining. The PCR analysis was also carried out for transformed *A. tumefacien* to verify transformation.

RESULTS AND DISCUSSION

Plant propagation through apical and axillary buds

The cultured buds were evaluated after 4 weeks. Explants grew easily and rapidly as they were uncountable after two months. Plantlet development and rooting occurred without any problem (Figure 1A-C). Thus the protocol proposed by Faure et al. (1998) for peppermint and spearmint is also suitable for propagation of Water mint. In some cases, callus and adventitious roots were observed (Figure 1D). With consideration of easily rooting and propagation, this medium was used as main medium for micropropagation of Water mint.

Micropropagated shoots are suitable sources for preparing explants during transformation experiments. So using an appropriate medium for this purpose is important.

Callus induction

All hormonal treatments (except BA 0 mg/l and NAA 1, 4 mg/l) caused callus induction (Figure 2). However there is no significant difference between hormonal levels, qualitative observation showed that in equal concentrations of auxin and cytokinen, especially 2 and 4 mg/l, induced larger and more fragile callus (Figure 3A). This callus had better potential for regeneration. High proportion of auxin (2 mg/l NAA and 0 BAP) caused large white and crispy callus with adventitious roots (Figure 3B).

Among callus-derived tissues somaclonal variation may be resulted, further it is favorable to use leaves for transformation however inoculation of callus with *Agrobacterium*, increase transformation efficiency and regeneration than leaves (Niu et al., 1998).

Direct regeneration

Maximum rate of direct regeneration without callus formation was obtained with MS medium supplemented with 0 mg/l NAA and 4 mg/l BAP (Figure 4). This treatment was used during next experiments. When the concentrations of NAA and BAP were 0.5 mg/l and 2 mg/l respectively, callus-mediated regeneration was observed. Regeneration occurred mostly near petioles and wounded regions (Figure 5A-B).

Niu et al. (1998) also reported shoot or primary callus formation in peppermint was occurred rather uniformly from regions of the leaf that had been injured as a consequence of dissection during explants preparation.

Selection of effective concentration of kanamycin

Kanamycin inhibits protein synthesis by binding to the 30 s ribosomal subunit and preventing translation. Increasing concentration of kanamycin generally resulted in full or partial inhibition of regeneration. Successful plant transformation requires effective regeneration and selection systems (Katzung, 2004). By increasing concentration of kanamycin, number of regenerated shoots was reduced and in concentrations above 50 mg/l all the explants died out (Figure 6). Thus, the concentration of 50 mg/l kanamycin was chosen as the optimum. This effective concentration of kanamycin has also been reported for peppermint and cornmint (Niu et al., 1998; Kumar et al., 2009).

GUS histochemical assay

Gus histochemical assay confirms transgenesis after preliminary selection of the transgenic plants by kanamycin (kumar, 2009). After the incubation of transgenic mentha leaves in X-Gluc solution for 24 h, blue color formation was occurred in the transgenic leaves beacuseas x-Gluc catabolized by the product of *gusA* gene. Color intensity varied in different transgenic explants (Figure 7A-D).

Intensity of blue color increased in wounded regions and near petioles. Gus assay was also performed for confirmation of *Agrobacterium tumefaciens* transformation (Figure 7E).

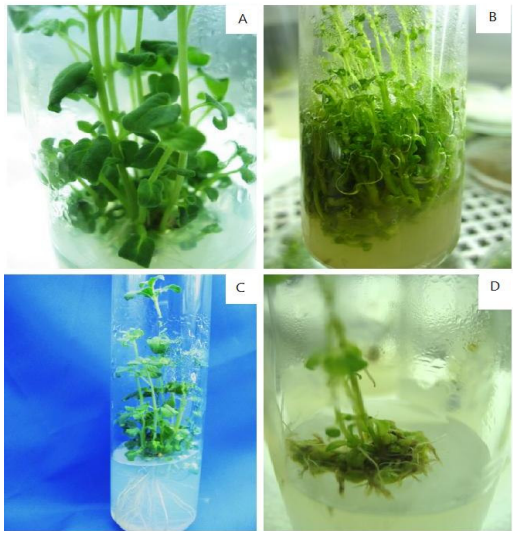

Figure 1A-B. Buds Explants propagation and plantlets development of Water mint. (C) Plantlets rooting of Water mint. (D) Adventitious roots of Water mint.

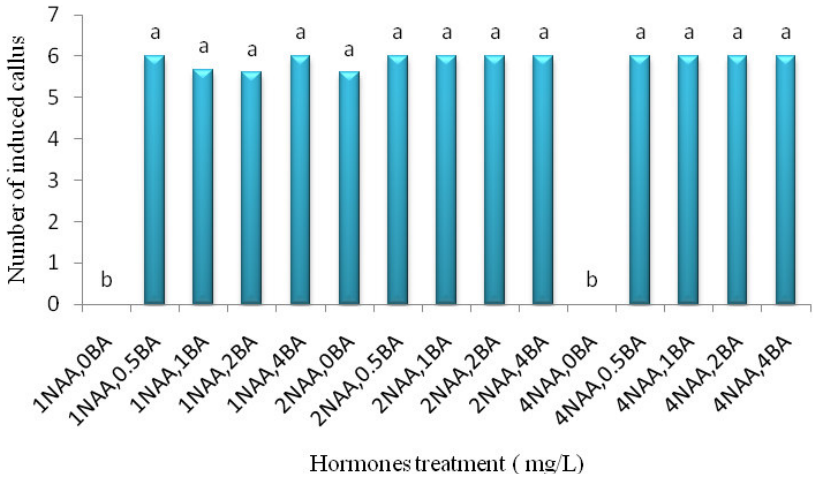

Figure 2. Effect of BA and NAA (mg/L) on callus induction in Water mint.

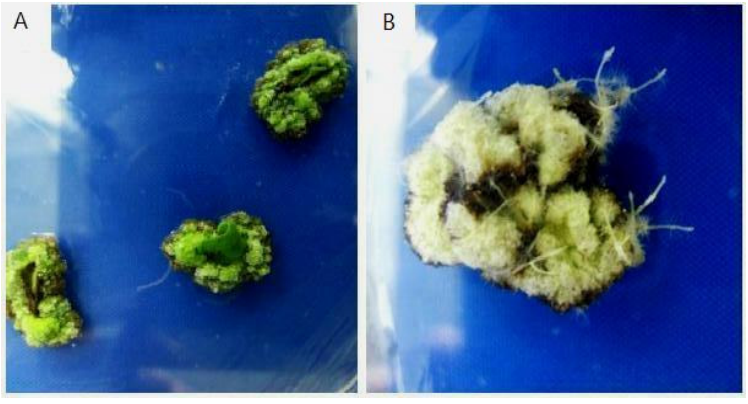

Figure 3A-B. Large and fragile callus of Water mint. B White and crispy callus with adventitious roots of Water mint.

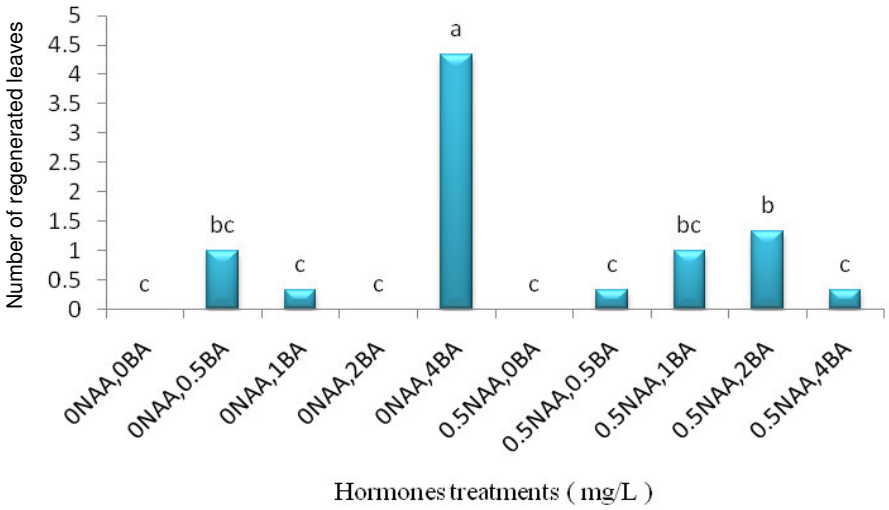

Figure 4. Effect of BA and NAA on direct regeneration from Water mint leaf.

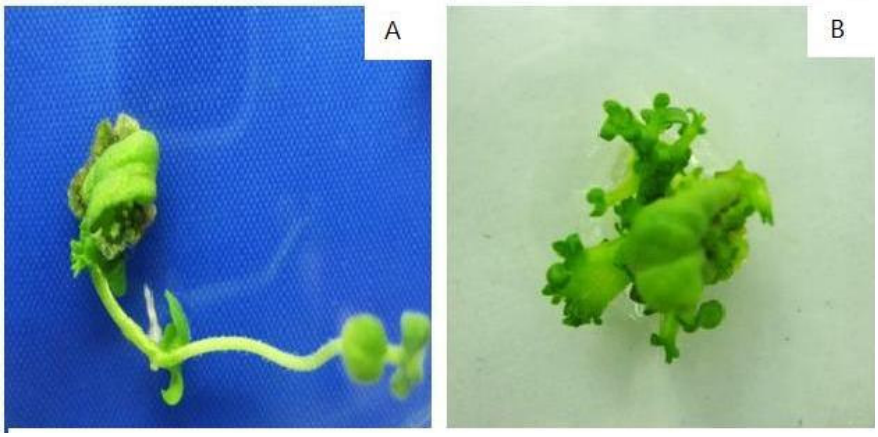

Figure 5A-B. Direct regeneration without callus formation from water mint leaves.

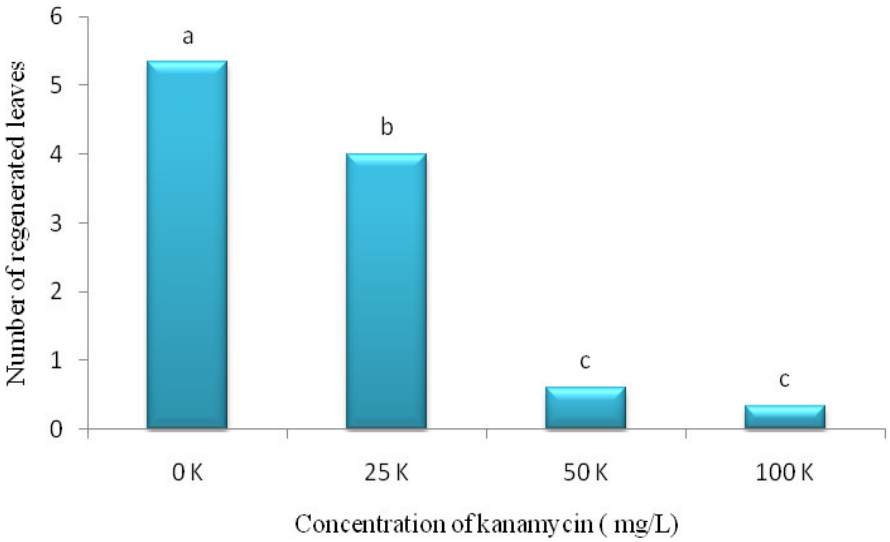

Figure 6. Effect of kanamycin on regeneration of Water mint leaves.

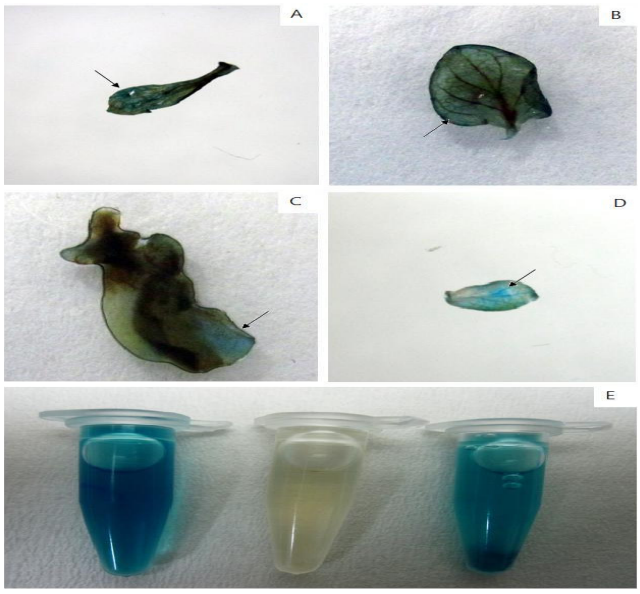

Figure 7A-E. Histochemical GUS assay for transgenic plants and formation of blue color. D Gus assay for transformed *Agrobacterium tumefaciens* (left and right) and non-transformed *A. tumefaciens* (middle).

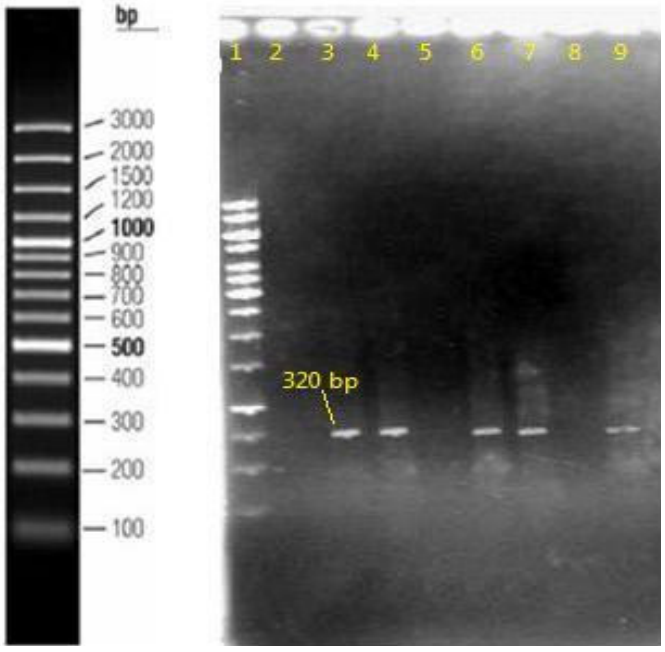

Figure 8. PCR analysis for transformed plants (3, 4, 6, 7), non-transformed plants as negative control (2, 5, 8), plasmid pBI121 as positive control (9) and 1kb ladder (1).

PCR analysis

Integration of the foreign gene into transgenic plants was confirmed through PCR analysis with gene-specific primers of *gusA*. Genomic DNA of transformed and untransformed (negative control) plants used as template for PCR amplification. Plasmid pBI121 harboring *gusA* gene was used as positive control. Bands of expected sizes (320 bp) were obtained for transformed plants. Control untransformed plants did not show any bond (Figure 8).

Transformation efficiency was 12 and 83% of transformed plantlets were obtained when incubation time was considered 10 minutes. There was no transformed plant among inoculated callus.

In conclusion, results demonstrate that concentration of 4 mg/l BAP without auxin or very low levels of auxin is suitable for direct regeneration of Water mint leaves. Leaves of Water mint are more suitable than callus for transformation and 10 min. incubation time is more efficient than 20 min. The efficiency of transformation was 12% and it is strongly suggested optimize other effecting factors such as during of co-cultivation time and another *Agrobacterium* strains to improvement this efficiency. The successful transformation of Water mint could lead to the genetic engineering of this medicinal crop to produce disease or herbicide resistance and improvement of oil and secondary metabolites quality and quantity.

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