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The Optimized Method of *Agrobacterium* Mediated Transformation in *Nitraria schoberi* (Ghar-e-Dagh in Persian)

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ABSTRACT

Ghar-e-Dagh (*Nitraria schoberi*) is a native perennial plant profoundly resistant to salinity and drought. This plant, having a well-developed deep rooting system can act as sand dunes stabilization agent and also, due to its special alkaloids may perform a very significant role in pharmaceutical industries. Considering the importance of genetic engineering as a novel method to generate genetic variation in practicing precise and purposeful breeding of plants in terms of increasing their resistance against biotic and abiotic stresses and also, to increase the production of specific secondary metabolites, the present investigation was performed as a prerequisite of any transformation. In this research, the issues of pre-culture time, co-culture time, acetosyringone and kanamycin concentrations were investigated. The results obtained suggest that the interaction of pre-culture and co-culture times and also, acetosyringone concentration is significant at 1% probability level. The mean comparisons also showed that the maximum transformation rate was gained in 48 hours pre-culture time, at 100 µM acetosyringone density and 72 hours co-culture. The ultimate confirmation of β- glucuronidase (GUS) gene presence in transgenic plants was performed by performing polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) and histochemical assay. The method presented here can be used for further genetic engineering purposes of *N.* schoberi.

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Introduction

Ghare-e-Dagh with the scientific name of *Nitraria schoberi* belongs to *Nitraricaceae* family. It is an endemic plant of the Mighan desert region in the central province of Iran (Jafari *et al.,* 2018). *N. schoberi* is expanded in a vast area of saline and sandy lands in the central regions of Iran. It is a resistant plant to draught and can easily develop and survive in both sandy lands and dunes and properly thrives over arid regions. Having the privilege of high resistance to salinity and being properly tolerant to high temperatures, has gained especial gravity of importance to this plant species compared to the other halophytes (Ahkani, 2002; Parida and Das, 2005; Shakeri *et al*., 2011; Naseri, 2014). As a medicinal plant fully enriched with a considerable amount of fatty acids and phenolic components, *N. schoberi* with its great potentiality reserve of effective natural antioxidants. It can perform a very productive role in the protection of cells against damages generated by free radicals. It can also be very helpful in the production of certain chemical components like nitrarine and nitroxin in pharmaceutical industries (Shakeri *et al*., 2011). The fruit of this plant with its specific capability in medicinal productivity has shown a promising

application as a new medicine with anti-bacterial, anti-oxidant, anti-fungal and anti-inflammation properties. They can be used to cure different diseases or as a protecting agent against other diagnosed insufficiencies accompanied by inflammation (Sharifi-Rad *et al*., 2014). Genetic engineering or recombinant DNA technology is one of the most important applications of plant biotechnology which targets is the development of genetic diversity in plant communities and subsequently choosing superior plants in terms of their desirable qualities. Since reaching an effective regeneration method from transgenic tissue is considered a prerequisite to use genetic engineering technology. In this study, it has been endeavored to optimize the implementation process of this innovative and effective science by evaluating different treatments proved to be effective in *N. schoberi* transformation process. To our knowledge, this is the first report of gene insertion into *N. schoberi.*

Materials and Methods

Plant material, strains, and plasmid

Seeds of *N. schoberi* plants were collected from the Myghan desert (coordinates: 39_3401000N, 4900100E), Markazi Province, Iran, by Herbarium of International Desert Research Center, University of Tehran. The binary vector pBI121 having β- glucuronidase (GUS*)* as a reporter gene and *A. tumefaciens* strain LBA4404 used for transformation procedure.

General procedures

Agrobacterium tumefaciens strain was grown in YEP medium (LB supplemented with rifampicin (25.5 mg/L) at 28ºC with shaking (150 rpm). pBI121 plasmid was mobilized into *Agrobacterium* by freeze-thaw method (Sambrook and Russell, 2001). Six primers were used for PCR and RT-PCR analysis during the procedure (Table 1).

Table 1. Oligonucleotides (primers) used in PCR and RT-PCR. The name and the sequences of each primer were mentioned.

Name	Oligonucleotides sequence	Accession number
35S	5'-GCGAACAGTTCATACAGAGTCT-3'	AF485783
NOSR	5'-GTGAAGCTTCCCGATCTAGTAACAT-3'	AF485783
virGf	5'-ATGATTGTACATCCTTCACG-3'	X91231
virGr	5'-TGCTGTTTTTATCAGTTGAG-3'	X91231
GUSF	5'- ATGTTACGTCCTGTAGAA ACC-3'	AF485783
GUSR	5'- CTAAAGAGAGGTTA AAGCCGA-3'	AF485783
Tub1	5'- GCTTTCAACAACTTCTTCAG-3'	M20405
Tub2	5'- GGGGCGTAGGAGGAAAGC-3'	M20405

Preparation of the explants and bacteria

The seeds were washed thoroughly with tap water and surface-sterilized with 70% (v/v) ethanol for 2min and Mercury (II) chloride1% for 7 min and then rinsed 3 times (5 min each) with sterile distilled water to remove any traces of the surfactants. The seeds were then germinated on MS medium (Murashige and Skoog, 1962) solidified with 6/5 g/L plant agar, and maintained for 30 days in a growth chamber at 25± 2°C with a 16/8 h (light/dark) photoperiod under a photon flux of 6000 Lux, provided by fluorescent lamps. Single colonies of the *A. tumefaciens* harboring pBI121 were grown in the LB medium supplemented with 20 mg/L kanamycin and allowed to grow overnight at 28°C with constant shaking (180 rpm) to mid-log phase. The bacterial culture was then transferred to a fresh medium cultivated till OD600= 0.4 with a liquid medium. The bacterial cells were collected by centrifugation (6000 rpm for 5 min) and re-suspended in ½MS medium (half-strength MS salts) for the subsequent inoculation step.

Transformation and selection

After germination, the cotyledones were cut and placed on the pre-culture medium including MS solid medium with 2 mg/L 6-Benzylaminopurine (BAP) and 1mg/L 1-Naphtaleneacetic acid (NAA) for 24 or 48 h. After that, the explants

were immersed in the bacterial suspension including ½MS medium with 60 g/L sucrose, 10 g/L glucose, 100 or 200 µM acetosyringone (pH 5.2) for 7 min with constant shaking. Then the explants were placed on the sterile filter paper to remove the excess moisture and subsequently were placed on the MS solid medium with 2 mg/L PAP and 1 mg/L NAA in the Petri dishes for co-cultivation at 25ºC for 48 or 72 h in the dark. After co-cultivation, the explants were washed with the sterile water containing 200 mg/L cephatoxime to inhibit the growth of *A. tumefaciens* attached to the explants and then transferred to the MS solid medium with 2 mg/L BAP, 1 mg/L NAA,15 or 30 mg/L kanamycin and 200 mg/L cephatoxime.

After shoot initiation, the explants were transferred to MS solid medium with 15 or 30 mg/L of kanamycin and 200 mg/L of cephatoxime. The regenerated shoots (about 3 cm length) were excised from the explants and transferred to MS solid medium with 1 mg/L of 3-Indolebutyic acid (IBA), 1 mg/L of Indole-3-Butyric acid and 200 mg/L of cephatoxime for rooting. All the above media contained 3% (w/v) sucrose and 8 g/L agar. The pH of the media was adjusted to 5.6 - 5.8 prior to the addition of agar and autoclaving at 121°C for 20 min. All the explants were cultured at 25 ± 2°C and 16 h of day time with the light intensity of 2000 Lux.

PCR analysis of the transformation

The leaf material from the transgenic and non-transgenic *N. schoberi* was harvested, lyophilized and grinded into a fine powder for extraction of genomic DNA. For the extraction of genomic DNA, the modified protocol of Dellaporta *et al., (*1983) was used. PCR amplification was used for initial evidence of the transgene (GUS) presence in the putative transgenic plants by using the genomic DNA and 35S/NOSR primers (Table 1). PCR was carried out as follows: An initial denaturation at 94ºC for 15 min followed by 40 cycles of denaturation at 94ºC for 45s, annealing at 54°C for 60 seconds, extension at 72ºC for 90 s and a final extension at 72ºC for 7 min. The resulting PCR products were separated by electrophoresis on 1% (w/v) agarose gel.

Semi-quantitative RT-PCR analysis

The isolation of *N. schoberi* total RNA was carried out using the Qiagen RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. DNase (Fermentas) treatment was given to the isolated RNA samples for the removal of any possible DNA contamination. The concentration of the RNA preparations was determined using NanoVue spectrophotometer (GE). The quality of the isolated RNA preparation was assessed by electrophoresis on a 1.3% agarose formaldehyde gel. The RNA bands after electrophoresis were visualized under UV light in a gel documentation unit. Semi-quantitative duplex reverse transcriptase PCR was performed by primers for amplification of GUS transcript (GUSF/GUSR) and primers for the Tubulin housekeeping transcript (Table 1).

Histochemical GUS assay

GUS expression in transformed and non-transformed mature leaves was determined histochemical according to Jefferson (1987). All tissues were rinsed three times in double-distilled water and stained in a 2-mM X-Gluc (5-bromo-4- chloro-3-indolyl-D-glucuronide, cyclohexylammonium salt) solution overnight at 37°C. After staining, the tissues were washed in 70 % ethanol until no visible chlorophyll remained.

Statistical analysis

The experiment was performed in triplicate with100 explants for each replication. Data were expressed as the mean of samples with standard deviation. Statistical differences were assessed based on the analysis of variance (ANOVA) using SPSS (version 18, USA). Differences were considered significant at a probability level of $p<0.05$.

Results and Discussion

One month after *in vitro* culturing of *N. schoberi* seeds and keeping them in the growth chamber, the cotyldonary leaves were inoculated with *A. tumefaciens* harboring pBI121 plasmid. The

inoculated plantlets were kept for 30 days in selective medium. Various new shoots appeared on some of the explants immediately 2 weeks after being planted. Some of these new shoots were green and the rest became white and eventually disappeared. It seems that the green shoots have received the T-DNA region of pBI121 containing the kanamycin-resistant gene and became transgenic. Given that, they were evaluated using molecular analysis. In order to perform a molecular evaluation of transgenic plants, genomic DNA was extracted from all green and young leaves of probably transformed plantlets (all those plants which remained green in selective medium) and also non-transformed plants as control. All stages from seed germination up to regeneration on the selective medium are shown (Fig.1)

Fig.1. Different stages from Germination of *N. schoberi* to regeneration in selective medium: A) in *vitro* culture of seed germination; B) Inoculation of cotyledonary leaves with *Agrobacterium*; C) Plantlet regeneration in selective medium supplemented with the antibiotic.

Then, PCR was conducted with NOSR/35S primers with the aim of proving the presence of T-DNA of the GUS gene. A set of virG primers (Table 1) was used for the detection of *Agrobacterium* contamination if any of that might have escaped the selection. The PCR of transgenic plant DNA with virG primers showed no band (data not shown). In the next step, as approval of GUS gene expression, RT-PCR was performed on the base of specific primers of the *GUS* gene (GUSR/GUSF). According to variance analysis, it was observed that the interaction effect of pre-culture time, acetosyringone concentration and co-culture time, were significant at a 5% probability level. The concentration of kanamycin had no significant effect. By reviewing mean comparison, it was determined that the treatment with 48 hours of pre-culture time, 100 µM acetosyringone and 72 hours for co-culturing, has shown the most transformation frequency up to 58% (Fig. 2). It is worth noting that the transformation frequency was calculated based on the number of plants whose transformation was approved by PCR and RT-PCR analysis in ratio to the number of inoculated explants with *A. tumefaciens*. PCR and RT-PCR analysis were done by 35S/NOSR and GUSF/GUSR primers (Table.1), respectively. The expected fragments which were amplified by PCR and RT-PCR were ~2000 bp and ~1700bp, respectively.

With regard to the fact that we cannot show PCR and RT-PCR tests of all green plants present in selective medium, only 10 samples of the best treatment have shown (Fig. 3).

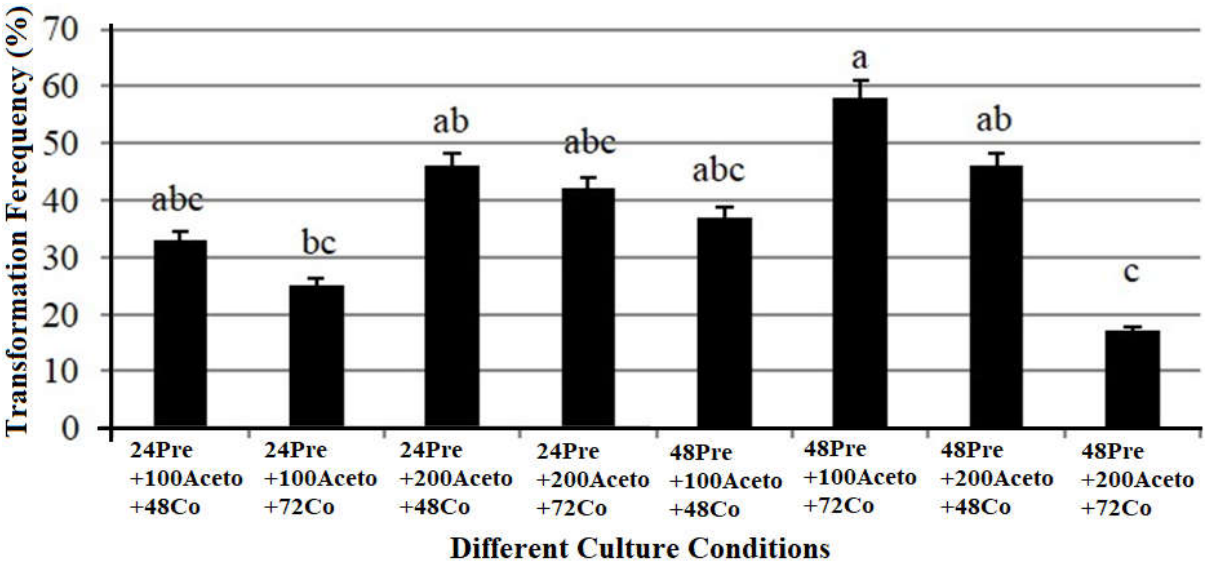

Fig. 2. Mean comparison of transformation frequency: Interaction within pre-culture time, acetosyringone concentration and co-culture time were compared.

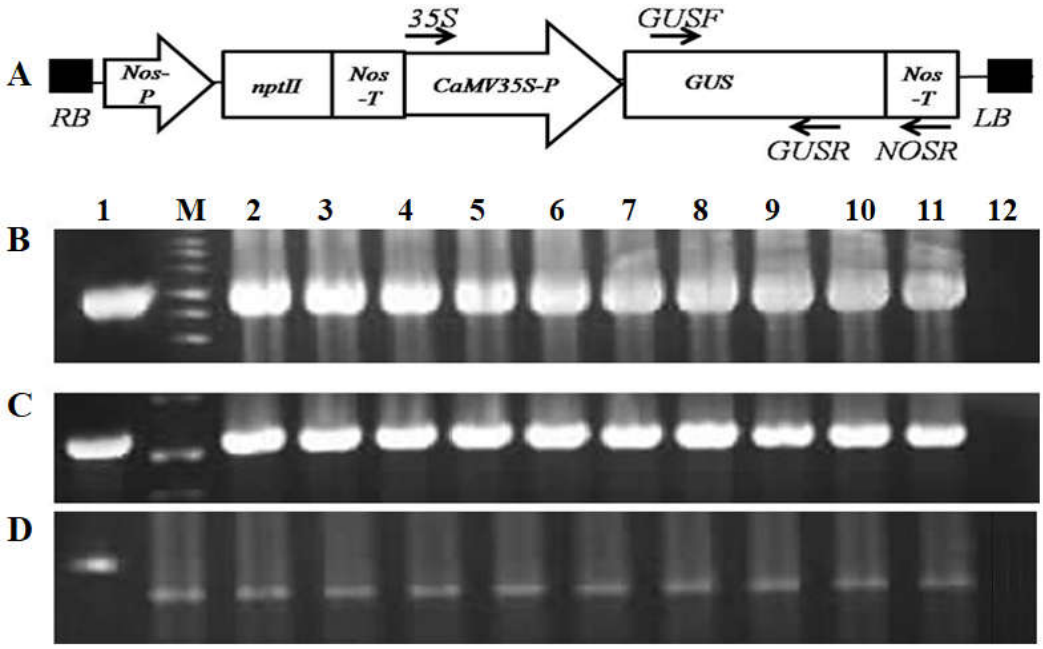

Fig. 3. Molecular analysis of extracted DNA from 10 green plants in the best treatment: A) Schematic figure of primer positions using in PCR and RT-PCR; B) PCR of transformed plants; C) RT-PCR of transformed plants; D) RT-PCR Tubulin housekeeping gene as control; line1= positive control (pBI121 plasmid); line 2-11= PCR products of 35S/NOSR primers and RT-PCR products of GUSF/GUSR primers; line12= PCR and RT-PCR of wild type plant; line M= Molecular Marker 1kb.

Three RT-PCR positive plants from each treatment were randomly selected and used for GUS activity analysis. In mature leaves from the phenotypically normal transgenic plants, *GUS* expression was observed blue areas, as expected, were not observed in the non-transformed tissues (Fig. 4).

Fig. 4 Histochemical GUS staining: A) Leaves from a non-transgenic plant; B) Leaves from transgenic plants.

The method of *agrobacterium*-mediated transformation, especially for plants which their tissue culturing is easy, is one of the common practices being implemented in gene transformation into the plants. One of the benefits of this method is its high efficiency in gene transferring without any remarkable reduction in the regeneration rate of transgenic plants and its less copy number of transgenes compared to direct gene transformation methods. The latter case led to the reduction of gene silencing probability after gene transcription (Gelvin, 2003). Regeneration conditions and transformation systems are two important factors to facilitate the transformation process. Different factors like genotype of plant, type of explants, cell density of the *Agrobacterium* culture, vacuum infiltration assisted inoculation, medium components (sucrose amount, mineral elements and concentration of plant growth regulators), environment conditions (light, temperature, relative humidity *etc*.), duration of co-cultivation and concentration of antibiotics used for selection and gene construct, concentration of acetosyringone and so on are considered to be effective to achieve this success (Cogbill *et al*., 2010; Babu *et al.* 2012; Bayer *et al*. 2014; Satish *et al.* 2017).

In the current study, the transformation frequency gained in different treatments ranges from 17 to 58 percent of which the highest degree belongs to the pre culture time of 48 hours, 100 µM acetosyringone and 72 hours for co-culturing.

Reviews carried out in scientific reports confirm the efficiency of the method presented in this investigation (He *et al*., 2004; Duan *et al*., 2012; Cho *et al*., 2014). The presented protocol is the first reported method to optimize the *Agrobacterium*-mediated transformation of *N. schoberi*. Up to our knowledge, there is no report about the *Agrobacterium*-mediated transformation of this species and even other species of *Nitraricaceae* family. So, this investigation can be the prerequisite to generate genetic variation which is highly based on DNA polymorphism and is independent of environmental factors (Shirmohammadli *et al.,* 2018).

Conflicts of interest

The authors have declared that no competing interests exist.

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