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Agrobacterium tumefaciens-Mediated Genetic Transformation of *Eclipta alba*

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Abstract: *Eclipta alba* (Linn.) Hassk. (Asteraceae) is a high value medicinal plant which possesses diverse medicinal properties. It is an important herb for the treatment of various disorders, and is primarily used as a hepatoprotectant. Its major biochemical constituents include wedelolactone and dimethyl-wedelolactone (coumestans), which possess anti-hepatotoxic properties. Due to its numerous medicinal properties, it is in high demand by the pharmaceutical industry and therefore requires urgent biotechnological interventions for its improvement. Therefore, the present study was constructed with the aim of developing an efficient genetic transformation protocol for *E. alba*, which will help in the mass production of the active compounds found in *E. alba*. *Agrobacterium tumefaciens* strain LBA 4404, containing vector pBI121, was used to genetically transform the plant, and the effect of various factors such as infection type, light cycle effect, effect of pH, among others, on the genetic transformation efficiency was analyzed. Regenerated transformed shoots were confirmed using the standard Polymerase Chain Reaction PCR method. Kanamycin-resistant and beta- glucurosidaseGUS-positive shoots indicated the development of transgenic shoots in *E. alba*. Amplification of *nptII* and *uidA* genes confirmed the integration of t-DNA transgenic shoots. In conclusion, various factors affecting the transformation efficiency were analyzed, and a reliable *A. tumefaciens*-mediated genetic transformation protocol was developed.

Keywords: 16srRNA; EHA 105; GUS; kanamycin; LBA 4404

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1. Introduction

Plants have been used by humans for food, flavouring, healthcare and a variety of other purposes since ancient times. Many civilizations' ancient written records provide substantial proof of the use of medicinal plants; for example, ayurvedic writings detail the use of medicinal plants for treating a variety of diseases [1]. Medicinal plants contain phytoconstituents that provide a supply of bioactive molecules needed for significant pharmacological activity with no adverse effects. They can be used to develop new classes of possibly safer medications or treatments for a wide range of diseases [2]. The use of medicinal plants as herbal medicine is increasing in modernized countries; as per one study, 25% of the UK population uses herbal medication to meet their healthcare needs [3]. Approximately 40% of chemicals used in the pharmaceutical industry are sourced directly or indirectly from plants because chemical synthesis of such compounds is either impossible or financially unsustainable [4]. Consumers worldwide have a favourable mindset towards herbal goods, believing them to be of "natural" rather than "synthetic" origin, and believing that such products are more likely to be safe than synthetic pharmaceuticals. Herbal

medications and its contents have been shown to improve long-term fitness and can be utilized effectively to treat human diseases or disorders [5].

Eclipta alba (Linn.) Hassk. (Asteraceae) is one such medicinal plant which possesses diverse medicinal properties and is primarily used as hepatoprotectant. It is also recognized as a nerve tonic, in addition to having hair-strengthening, anti-aging, and immunomodulator qualities [6]. Its main contents include coumestans, eclalbatin, alpha-amyrin, urosolic acid, oleanolic acid, daucosterol and stigmasterol-3-O-glucoside, as well as other therapeutically significant biochemical components [7].

Due to their diverse medicinal properties, medicinal plants are under high demand by the pharmaceutical industry and thus require urgent biotechnological interventions for their conservation, propagation, and improvement. As such, there is a need to conserve and genetically transform this important plant. Now-a-days, substantial advancement has been made in the practical application of in vitro culture techniques to address the aforementioned challenges in the safeguarding of valuable medicinal plants [7]. Further in vitro propagation techniques have been proposed as a viable approach for generating sufficient material for the commercial cultivation of valuable medicinal plants.

Furthermore, genetic engineering of medicinal plants is one of the most encouraging strategies to enhance the productivity and quality of these plants [8]. It will help in decreasing the stress on natural resources, and furthermore will promote sustainable development within the medicinal plant industry. Despite significant improvements in plant genetic engineering worldwide, research in this area remains relatively scarce; moreover, efforts have largely focused on model plants. Thus, there is a need to develop genetic transformation protocols for other important medicinal plants. Genetic transformation can help with the mass production of active compounds found in medicinal plants, thereby enabling more comprehensive utilization of their benefits [9].

Genetic transformation facilitates means to validate gene function and trait specific improvement in highly valuable medicinal plants without affecting their desirable genetic makeup [8]. Increasing interest in *E. alba* as a medicinal and oil yielding plant has opened avenues to develop an efficient genetic transformation system in order to transfer novel traits into the plant. Remarkably, only one singular instance of successful regeneration and transformation of *E. alba* has been documented [10]. Despite numerous micropropagation endeavors undertaken for *E. alba* [11–13], the notable absence of dedicated efforts towards developing a reliable and efficient protocol for the genetic transformation of *E. alba* remains conspicuous. Therefore, the present investigation aims to assess various parameters influencing genetic transformation, and to develop a dependable and reproducible *A. tumefaciens*-based genetic transformation technique for *E. alba.*

2. Materials and Methods

2.1. Explant Source, Preparation and Culture Conditions

The *E. alba* plants (young, healthy, free from any symptoms of disease) used in this study were initially collected from Tau Devi Lal herbal garden, District Yamunanager, Haryana, India, and maintained at the nursery of Maharishi Markandeshwar (deemed to be) University, Mullana, Ambala, Haryana, India, under standard conditions. Nodal segments were used to establish aseptic cultures using standard tissue culture protocol as mentioned by Datta et al. [13]. HiMedia Laboratories (Mumbai, India) supplied all frequently used chemicals, while Sigma Chemical Co. (St. Louis, MO, USA) supplied growth regulators, antibiotics and speciality chemicals. Unless otherwise indicated, all experiments were performed in 300 mL glass culture bottles (Kasablanka, Mumbai, India) with 50 mL of Murashige and Skoog medium [14] containing 58 mM sucrose and 0.7% (*w*/*v*) agar (MS media). The pH of the medium (used for the establishment, regeneration and genetic transformation of *E. alba*) was adjusted to 5.8 prior to autoclaving, and cultures were incubated at 25 ± 1 °C with a light intensity of 42 mol m^{-2} s^{-1} in a 16-h light/8-h dark cycle. The explants for the investigations were expanded leaves from elongated

microshoots grown on MS media [14] supplemented with 2.5 µM benzyladenine (BA) and 0.1 µM α-naphthaleneacetic acid (NAA).

2.2. Agrobacterium Strain, Vector and Kanamycin Sensitivity Determination

Disarmed strains of *Agrobacterium tumefaciens* strains EHA105 and LBA4404 containing the binary vector pBI121 with the *uidA* gene (*β*-glucuronidase) (GUS) as a reporter marker gene and the selection marker gene *nptII* (neomycin phosphotransferase II) were employed for the genetic transformation investigations. Both genes were located on T-DNA and were triggered by the CaMV 35S and nos promoters, respectively [15]. The binary vector was delivered into *Agrobacterium tumefaciens* disarmed strains EHA105 and LBA4404 by the freeze–thaw method [16]. The presence of the pBI121 plasmid was verified in the antibiotic-resistant bacterial colonies by PCR, using *nptII* gene-specific primers [17]. The transformed *A. tumefaciens* strains were kept at 28 °C on yeast extract peptone (YEP) agar medium (10 g/L bacto peptone, 10 g/L yeast extract, 0.5 g/L NaCl and 1.5 g/L agar at pH 7) containing 15 lg/mL rifampicin and 50 lg/mL kanamycin, and were used for the genetic transformation experiments. The kanamycin resistance (tolerance limits) of explants were assessed by incubating leaves on shoot regeneration medium (MS + 15.0 µM of NAA and 1.0 µM of BA) containing variable quantities of kanamycin (0–100 µg/mL) as per the protocol mentioned by other authors [18]. Antibiotic was sterilized using a 0.22 µm filter and incorporated into the medium succeeding autoclaving.

2.3. Genetic Transformation

Co-Cultivation and Infection

Fresh pure cultures of *A. tumefaciens* (both EHA 105 and LBA 4404) grown overnight on YEP medium containing 50 µg/mL kanamycin and 15 µg/mL rifampicin were used. The cells were spun (5000× *g*, 2 min) and then resuspended in YEP medium with 100 µM acetosyringone to reach the desired OD600. Leaf explants cultivated on shoot regeneration medium for 0–5 days were wounded using various methods, including piercing with a sterile injection needle, hammering with a surgical blade, and rubbing with abrasive paper or glass beads. Then, in petri plates, they were infected with the aforementioned *A. tumefaciens* suspension for various time intervals (0–30 min). After infection, the explants were dried out using sterile absorbent paper to eliminate leftover bacterial cells and media. They were then cultured in an antibiotic-free co-cultivation medium for a period ranging from 1 to 5 days. The vessels containing cultures were wrapped with parafilm and maintained in various light periods (24 h light, 16-h light/8-h dark and 24 h darkness).

2.4. Regeneration of Transformed Leaf Explants

Following co-cultivation, the leaf specimens were washed with autoclaved milli-Q water with 500 µg/mL of cefotaxime. After drying the explants on sterile absorbent paper, they were subsequently shifted to vessels containing MS medium, which had been supplemented with 15.0 µM of NAA, 1.0 µM of BA, 50 µg/mL of kanamycin and 500 µg/mL of cefotaxime. Throughout the experiment, the cultures underwent periodic sub-culturing on the same medium every 25–30 days. This process was continued until successful regeneration of transformed tissue was achieved.

2.5. Measurement of GUS Activity

Regenerated shoots exhibiting resistance to kanamycin were used to conduct a GUS assay. The purpose was to assess GUS expression, following the procedure outlined by Jefferson et al. [15]. For the measurement of GUS activity, 20 explants were evaluated for every treatment (treatments as mentioned in Table 1, including infection time, pre culture, OD value, etc.). Explants exhibiting a blue coloration were considered positive.

Table 1. Influence of various factors on genetic transformation efficiency of *Eclipta alba*.

Factor	Variable	% *GUS* Expression
A. tumefaciens strain	LBA 4404	52.3 ± 0.91 [a]
	EHA 105	48.5 ± 0.4 [b]
Pre-culture	0 d	36.8 ± 0.35 [f]
	1 d	45.7 ± 0.20 [d]
	2 d	56.8 ± 0.26 [a]
	3 d	51.2 ± 0.2 [b]
	4 d	48.3 ± 0.20 [c]
	5 d	43.7 ± 0.28 [e]
Method of injury	Intact	36.7 ± 0.15 [e]
	With hypodermic needle	55.6 ± 0.36 [a]
	With surgical blade	48.5 ± 0.1 [b]
	With carborundum	44.5 ± 0.35 [c]
	With glass beads	40.1 ± 0.35 [d]
Acetosyringone	0	43.5 ± 0.20 [d]
	100	58.6 ± 0.2 [b]
	150	63.4 ± 0.25 [a]
	200	57.4 ± 0.15 [c]
pH of co-cultivation medium	5.2	51.4 ± 0.25 [d]
	5.4	58.6 ± 0.15 [a]
	5.6	53.3 ± 0.3 [b]
	5.8	52.8 ± 0.2 [c]
Optical density O.D Value	0.2	34.6 ± 0.25 [e]
	0.4	42.6 ± 0.25 [d]
	0.6	56.3 ± 0.20 [a]
	0.8	53.8 ± 0.2 [b]
	1.0	49.8 ± 0.15 [c]
Co-cultivation period	1 d	43.5 ± 0.30 [d]
	2 d	54.6 ± 0.25 [a]
	3 d	49.3 ± 0.25 [b]
	4 d	46.2 ± 0.37 [c]
	5 d	43.1 ± 0.26 [d]
Infection Time	5 min	42.4 ± 0.3 [c]
	10 min	46.6 ± 0.25 [b]
	15 min	53.3 ± 0.26 [a]
	20 min	42.3 ± 0.15 [c]
	30 min	38.8 ± 0.20 [d]
Photoperiod	24 h light	46.3 ± 0.36 [c]
	24 h dark	51.7 ± 0.36 [b]
	16 h light/8 h dark	56.5 ± 0.35 [a]

Statistically validated with Duncan's multiple range test ($p < 0.05$). Values sharing a common letter within the column are not significant at $p < 0.05$ (each factor analyzed separately). Values are the means of three experiments, consisting of three replicates each (10 explants in each replicate).

2.6. Molecular Analysis

We isolated DNA from the leaves of both GUS-suspected transgenic shoots and non-transformed plants using the CTAB method described by [19] for PCR amplification of specific DNA fragments related to the *nptII* and *uidA* genes. The PCR mix included 20 ng of DNA, 1.0 U of *Taq* DNA polymerase, a 100 µM concentration of each dNTP, 2.0 µL of a 10X reaction buffer, 10 nmol of each primer and sterile Milli Q water to make a volume of 20 µL. The amplification procedure began with a denaturation at 94 °C for 5 min, followed by 30 cycles involving denaturation at 94 °C for one minute, annealing at 58 °C for 45 s, and extension at 72 °C for one and a half minutes, with a final extension at 72 °C for 5 min. A DNA segment 1500 bp long specific to the gene *uidA* underwent amplification using designated primer pairs (FP: 5′ GGTGGGAAAGCGCGTTACAAG 3′, RP: 5′ GTTTACGCGTTGCTTCCGCCA 3′). Similarly, a DNA fragment around 760 bp in length targeting *nptII* was amplified with the primer pair (FP: 5′GAGGCTATTCGGCTATGACTC 3′, RP 5′ATCGGGAGAGGCGATA CCGTA 3′). Plasmid DNA from pBI121 served as a positive control, while DNA from non-transformed *E. alba* shoots leaves acted as the negative control. To amplify a 16S rRNA segment of about 1500 base pairs of DNA [20], the procedure as mentioned by Aggarwal et al. [21] was followed, using bacterial genomic DNA as the positive control. The PCR results were analyzed on 1.0% (*w*/*v*) agarose gel and visualized through UV illumination (Quantum ST4, France) after staining with ethidium bromide.

2.7. Statistical Analysis

The trials were performed three times, each repeated three times. The results were statistically verified using analysis of variance (ANOVA) on the data, followed by mean comparisons through the DMRT test (where significance was set at $p < 0.05$). All these computations were carried out using GraphPad Prism 4 software.

3. Results and Discussion

Increasing interest in medicinal plants has opened up avenues to develop shoot regeneration and a genetic transformation system in order to transfer novel traits into the plants. However, the lack of an efficient genetic transformation protocol has been widely acknowledged as a hindrance to performing genetic engineering in many plants, including in *E. alba* [11]. *Eclipta alba*, a plant renowned for its distinct medicinal properties and utilization in various medical formulations, serves as a prominent example. Hence, the current research concentrates on the establishment of an efficient genetic transformation protocol for *E. alba*.

The fundamental approach to genetically modifying plants involves incorporating transgenes into the genomic DNA of the plant. In order to aid the transfer of DNA into host plant cells, several transformation methods have been developed [22]. The genetic transformation method involving *A. tumefaciens* is preferred over direct transformation methods due to its simplicity, reliability and ease of analysis for generating transgenic plants [23]. The present study explored the variables that influence the efficiency of delivering T-DNA into the *E. alba* plants through the utilization of *A. tumefaciens*. This research represents one of the limited instances where factors affecting the genetic transformation of *E. alba* have been investigated.

Selection of transformed tissue using a suitable antibiotic is one of the most critical steps in the development of any genetic transformation protocol. Kanamycin, an antibiotic derived from aminoglycosides, is frequently employed to identify cells possessing the *nptII* gene. Susceptibility to kanamycin has been found to differ among different plant species, and numerous plant species have displayed hindered regeneration across a range of concentrations [24,25]. Therefore, the kanamycin sensitivity of *E. alba* leaves was assessed through the cultivation of leaf explants on shoot regeneration medium (MS + 15.0 µM NAA + 1.0 µM BA) enriched with kanamycin (0–100 mg/L). At concentrations exceeding 50 mg/L, kanamycin significantly inhibited growth and completely arrested the regener-

ation of *E. alba* (Figure 1). This assessment of kanamycin sensitivity is expected to prove valuable in subsequent evaluations of transformants during genetic transformation investigations. These findings align with earlier research on various plants, including *E. alba* [10,26,27]. The cefotaxim concentration was kept at 500 µg/mL for the elimination of residual bacteria from the cultures. This concentration of cefotaxim was effective for the complete elimination of *A. tumefaciens*.

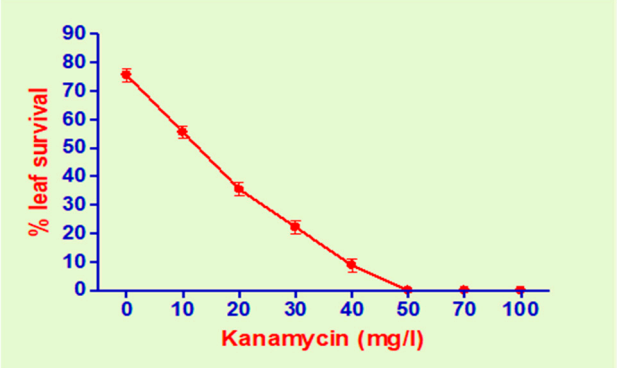

Figure 1. The effect of kanamycin in MS medium on the survival of leaf explants taken from microshoots of *E. alba*. Data were recorded after 4 weeks of culture. Values are the means of three experiments consisting of three replicates each (10 explants in each replicate).

The success of transformation is often dependent on the type of *A. tumefaciens* strain employed for the genetic transformation [28]. In this study, two *Agrobacterium tumefaciens* strains, namely LBA4404 and EHA105 (both harboring the vector pBI121), were employed for experimentation. The resulting frequencies of transient GUS activity were compared to determine the transformation efficiency produced by these strains. Upon infecting the explants with the LBA4404 strain, approximately 52.3% of the explants demonstrated transient GUS activity, while the EHA105 strain led to around 48.5% transient GUS activity (Table 1). The *A. tumefaciens* strain LBA4404 was found useful in numerous plant transformation efforts due to its tendency to be easily removed from plant tissues at low antibiotic concentrations [21]. Conversely, the strain EHA105 presents challenges in its complete removal from plant tissues [28]. Therefore, *A. tumefaciens* strain LBA4404 was utilized in subsequent experiments.

The effectiveness of T-DNA delivery into leaf explants was influenced by a variety of factors (as mentioned in Table 1), including pre-culture conditions, bacterial density, damage mechanisms, incubation parameters, the presence of acetosyringone and other relevant variables [29]. Therefore, special emphasis has been given in the present study to standardize various parameters for the genetic transformation of *E. alba* (Table 1). Certain plant species have exhibited improved transformation outcomes when optimal explant pre-culture conditions are employed before *Agrobacterium* infection, and this has emerged as a pivotal element contributing to the successful transformation of many plants [30]. In the current study, leaf explants that were pre-cultured (0–5 days) on MS medium + 1.0 µM BA + 15 µM NAA supplemented with 100 µM acetosyringone for a period of two days before infection with *Agrobacterium* demonstrated the highest transient GUS activity (56.8%; Table 1), compared with explants without pre culturing treatment (36.8%). This increased GUS activity is most likely due to the action of growth hormones and acetosyringone, which speeds up the cell and helps by changing the physiology of leaf explants and making them competent for transformation [31].

When actively developing cells are wounded, *Agrobacterium* recognizes the phenolics signal, which promotes the transformation of plant cells [31]. The method of tissue injury before bacterial infection was also observed to exert a notable influence on T-DNA delivery. Among the various methods of injury used, introducing small punctures in the tissue using a hypodermic needle resulted in a significant enhancement of transient GUS activity,

increasing from 36.7% (in undamaged explants) to 55.6% (Table 1). Pre-injuring the tissue prior to infection might help induce deeper penetration of bacteria into the tissue, thereby allowing improved accessibility of plant cells to *Agrobacterium* [31]. These factors are likely the primary contributors to the improved bacterial efficacy in T-DNA delivery [32].

Amalgamation of 150 µM acetosyringone in the co-cultivation medium increased transient GUS activity from 43.5% to 63.4% (Table 1). Previous research has demonstrated that acetosyringone enhances the efficiency of *Agrobacterium*-mediated genetic transformation in various plant species by triggering the activation of *vir* genes through the secretion of phenolic compounds [33,34].

Although *A. tumefaciens* typically thrives under neutral pH conditions [35], prior investigations into *Agrobacterium*-mediated transformation have revealed that lower pH promotes the ideal expression of the *vir* cascade [36]. Furthermore, enhanced *vir* gene induction has been reported under low pH conditions in the presence of acetosyringone, suggesting that the induction of the *vir* gene is favored under the acidic conditions [32]. Earlier studies also indicated that low pH during co-cultivation was favorable for *Agrobacterium*-mediated transformation [37,38]. Therefore, in this particular study, adjusting the co-cultivation medium's pH to 5.4 resulted in the most pronounced transient GUS expression (Table 1), highlighting the significance of maintaining the appropriate pH levels throughout the co-cultivation process.

The density of the *Agrobacterium* inoculum used for infecting plants cells is a crucial factor in *Agrobacterium*-mediated transformation systems. In the current work, leaf explants were infected with *Agrobacterium* at varied cell densities (Table 1). The maximum transient GUS activity was observed in explants treated with a bacterial suspension at an OD_{600} of 0.6. As bacterial density increased beyond this point, transient GUS activity gradually decreased. This decline in transient GUS activity with increasing bacterial density could be attributed to the likelihood of increased damage to plant tissue, necrosis, and an elevated production of toxic compounds when inoculated with higher *Agrobacterium* densities [39]. Recently, the influence of *Agrobacterium* density on the genetic transformation of *Solanum betaceum* was reported by Cordeiro et al. [40].

In addition, the study also looked at the effect of altering the duration of the co-cultivation time (0–5 days). When explants were subjected to a co-cultivation period of two days, a peak of 54.6% exhibited transient GUS activity. Along with a co-cultivation time of 5 days, an infection time of 15 min was found to be the optimum for the genetic transformation of *E. alba* (Table 1). Prolonged co-cultivation periods and infection time have been known to lead to explant mortality due to bacterial overgrowth, which can negatively impact the transformation process [41].

Finally, changing the light conditions during the co-cultivation phase was discovered to have a considerable influence on transient expression efficiency [42]. The highest number of explants (56.5%, Table 1) exhibited GUS activity when exposed to a light cycle of 16/8-h light/dark ratio. Light conditions have been demonstrated to enhance *Agrobacterium-mediated* T-DNA transfer to plants as plant transformation is reported to be regulated by light signaling pathways mediated by photoreceptors, which regulate some of the crucial processes involved in genetic transformation [43,44].

After optimizing the abovementioned parameters for genetic transformation of *E. alba*, experiments were conducted for the recovery of transgenic shoots. Transgenic shoots were recovered on MS medium supplemented with 1.0 µM of BA, 15 µM of NAA, along with 50 mg/l kanamycin and 500 mg/L cefotaxime (selection medium). The cultures were subcultured each time with a 20-day gap in the same condition. A transformation efficiency of 4–5% was achieved on the selection medium using the abovementioned parameters. After 8–10 weeks of incubation on the selection medium, transgenic shoots of *E. alba* showing positive GUS activity were recovered (Figure 2).

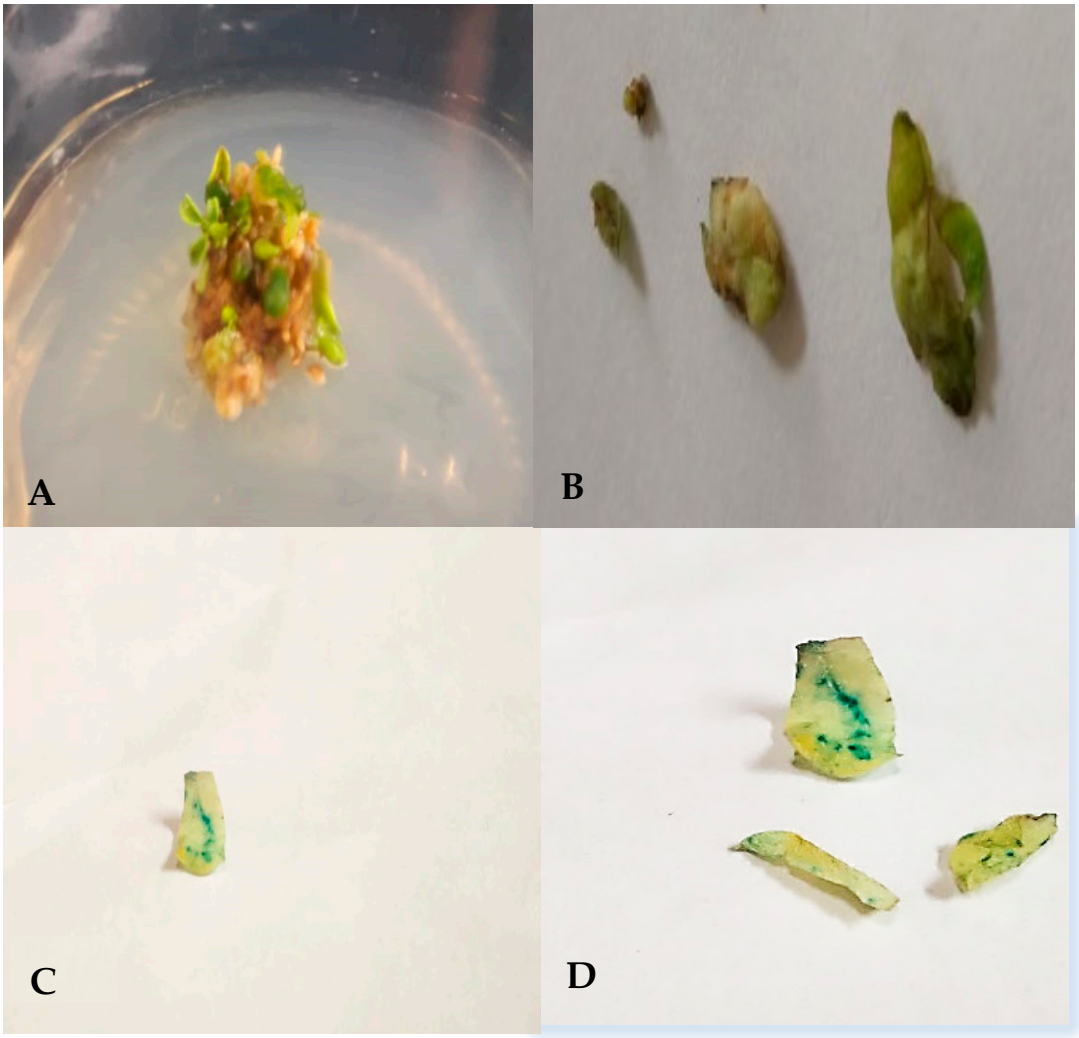

Figure 2. *Agrobacterium*-mediated genetic transformation of *E. alba* (**A**). Regeneration of putative transformed *E. alba* shoots on selection medium containing kanamycin (**B**). Leaves from the control plant showing no GUS activity (**C**,**D**). Transformed *E. alba* leaves showing GUS.

To rule out the possibility of false transient GUS expression due to the presence of residual *Agrobacterium* strains in the leaves of putative transgenic plants, 16S rRNA was carried out. No amplification of the DNA fragment specific to the 16S rRNA locus indicated the complete elimination of bacteria from these tissues (Figure 3A). Previously, such analysis has been used by researchers in other plants [21,25]. To further confirm the expression of the *nptII* and *uidA* genes in transgenic shoots, PCR amplification of these two genes was carried out; amplification yielded a 720 bp fragment specific to the *nptII* gene (Figure 3B) and a 1500 bp fragment specific to the *uidA* gene (Figure 3C) from transgenic shoots, confirming the integration of both genes in the transgenic lines.

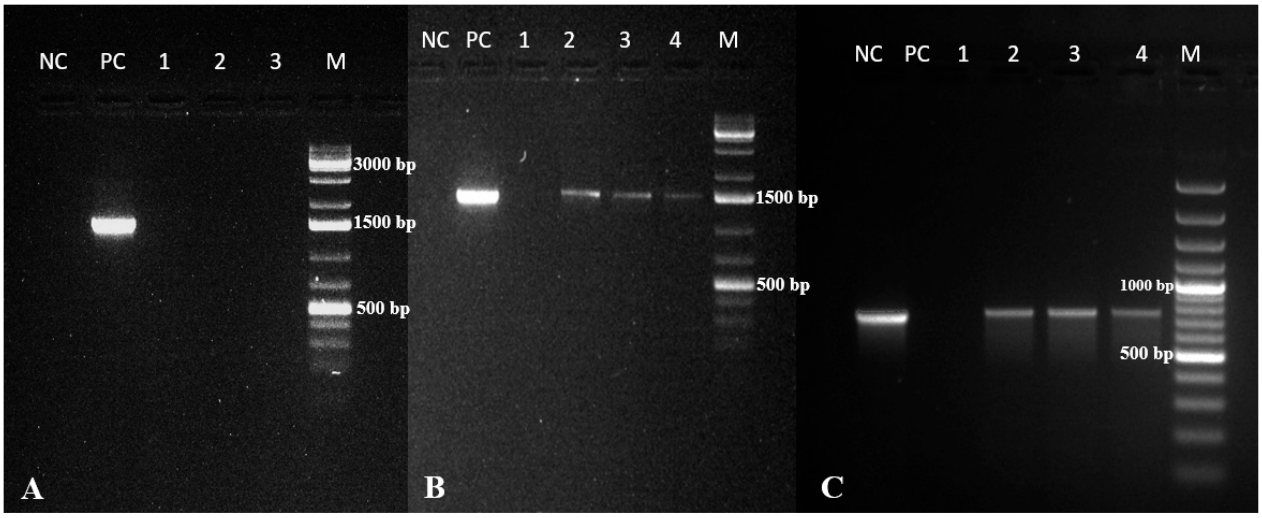

Figure 3. PCR-based analysis of the transformed shoots of *E. alba* (**A**). 16S rRNA analysis (~1500 bp) of transformed shoots. Lane-NC: DNA from the control plant, Lane-PC: Positive control bacterial genomic DNA, Lane-1–3: DNA from the transformed plants, Lane M: 1 KB ladder (**B**). Amplification of the *uidA* gene (~1500 bp) from the genomic DNA of the transformed tissue, Lane-NC: DNA from the untransformed plant, Lane-PC: Positive control (amplification from pBI121), Lane-1–4: Amplification from the DNA of the transformed shoot, Lane M: 1 KB ladder (**C**). Amplification of the *nptll gene* (~760 bp) from genomic DNA of the transformed tissue, Lane-NC: DNA from the untransformed plant, Lane-PC: Positive control (amplification from pBI121), Lane-1–4: Amplification from DNA of the transformed shoot, Lane M: 100 bp ladder.

4. Conclusions

In conclusion, an efficient protocol for *Agrobacterium*-mediated genetic transformation of *E. alba* was established. Various factors such as the *Agrobacterium* strain, OD value, co-cultivation period, method of injury, use of acetosyringone, and the pH of the co-cultivation medium. were efficiently applied and found to have influence on the genetic transformation efficiency of leaf explants of *E. alba*. The presence of the transgenes, i.e., *nptII* and *uidA* genes, in transformed shoots were confirmed using PCR analysis in comparison to control plants. Further, 16srRNA analysis was carried out to rule out the possibility of false GUS positive signals in transformed plant tissue due to residual *Agrobacterium* contamination. Finally, this developed protocol can be used for plant gene functional analysis, as well as for the genetic improvement of this important medicinal plant.

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