
***Agrobacterium*-Mediated Genetic Transformation and Somatic Embryogenesis from Leaf Callus of *Hevea brasiliensis*: Effect of Silver Nitrate**

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Abstract

Somatic embryogenesis and plant regeneration systems developed in *Hevea brasiliensis* are being used in *Hevea* crop improvement through transgenic approaches. In the present study, the effect of silver nitrate in improving the efficiency of the somatic embryogenesis pathway from leaf explants as well as *Agrobacterium*-mediated genetic transformation was studied. Experiments were done to find the effect of silver nitrate in callus proliferation and embryogenic callus initiation. Silver nitrate (0–30.0 mg/l) was added in the medium used for second and third subcultures for callus proliferation and in the embryo induction medium. Experiments were also carried out to find the effect of silver nitrate in controlling bacterial overgrowth and improve callus texture when freshly proliferated callus induced from leaf explants in the above medium was used as target tissue for *Agrobacterium* infection. *Agrobacterium tumefaciens* strain EHA 101 carrying a gene-encoding isopentenyltransferase (*ipt*) from the Ti plasmid of *Agrobacterium tumefaciens* was used for the study. Silver nitrate (0–30 mg/l) was added to the *Agrobacterium* infection as well as the cocultivation and selection medium. Addition of 20.0 mg/l silver nitrate in the callus proliferation medium used for the second and third subcultures and 10 mg/l in the embryo induction medium helped in the texture improvement of the callus which in turn increased the efficiency of the system by aiding embryogenic callus initiation. It was also observed that inclusion of 10.0 mg/l silver nitrate in the infection and cocultivation medium and 20 mg/l in the selection medium significantly controlled bacterial overgrowth, reduced tissue damage and improved the texture of callus in newly emerged putatively transgenic cell lines. Proliferation of the transgenic callus and somatic embryogenesis were also achieved. From the results, it has become evident

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that silver nitrate could improve somatic embryogenesis as well as *Agrobacterium*-mediated genetic transformation when included in the respective media used in the plant regeneration system developed from leaf explants.

Keywords

Agrobacterium • Genetic transformation • *Hevea brasiliensis* • Somatic embryogenesis

Abbreviations

BA	6-benzyl aminopurine
2,4-D	2,4-dihydrophenoxyacetic acid
GA ₃	gibberellic acid
Kin	kinetin
MS	Murashige Skoog
NAA	naphthalene acetic acid

Introduction

Hevea brasiliensis, a cross-pollinated perennial tree species belonging to the family Euphorbiaceae, is the only commercially cultivated species as a source of natural rubber. In *H. brasiliensis*, plant regeneration through somatic embryogenesis has been obtained from immature anther, inner integument and immature inflorescence [1–5]. All the protocols developed earlier for somatic embryogenesis in *H. brasiliensis* were from floral-/fruit-derived explants. Since flowering in *H. brasiliensis* is seasonal and adverse environments during the season may hinder normal flowering, explant availability and viability is unpredictable. Recently, a system for somatic embryogenesis and plant regeneration was also developed from leaf explants, an easily available explant [6, 7]. Somatic embryogenesis is affected by numerous factors, notably ethylene. Ethylene is known to reduce somatic embryogenic competence in many plants. Silver nitrate (AgNO₃) has been reported to have influence in inducing embryogenic potential to fresh callus in several crops [8]. The use of silver nitrate, an ethylene action inhibitor, has been shown to

increase *in vitro* embryogenesis and regeneration rates [9]. Presence of silver nitrate in culture medium has shown to have important effects in tissue culture of cassava, such as improving somatic embryogenesis and plant regeneration [10]. However, the influence of ethylene on embryogenic response is genotype specific [11].

The success in generation of transgenic plants depends on two major factors, transgene integration and regeneration capacity of explants. Due to the simplicity of the transformation system and precise integration of transgenes, *Agrobacterium* Ti plasmid-based vectors continue to offer the best system for plant transformation [12]. The major requirements for the development of transgenic plants include the availability of reliable and reproducible *in vitro* regeneration systems and efficient transformation protocols [13]. The main challenge with genetic transformation of tree species is achievement of high transformation efficiency and efficient plant regeneration for desired clones or cultivars [14]. It can be hypothesised that successful transgenesis occurs when regeneration capacity of a cell coincides with its ability to accept a transgene. *H. brasiliensis* being a tree crop, conventional agriculture relying on selection and traditional breeding programmes needs more time to deploy new varieties with modified traits into the field.

All the plant regeneration systems developed in *Hevea* were used for crop improvement through *Agrobacterium*-mediated genetic transformation. Transgenic plants incorporated with MnSOD gene were regenerated from anther

calli of *H. brasiliensis* [15, 16] through transformation technology. Leaf-derived embryogenic callus has also given good response as a suitable target tissue for *Agrobacterium*-mediated infection [17]. Silver nitrate has been found to suppress overgrowth of *Agrobacterium* facilitating plant cell recovery that resulted in increased transformation efficiency [18]. In apple, it has been reported that during *Agrobacterium*-mediated transformation, ethylene production was increased which resulted in reduced efficiency of gene transfer mechanism [19]. Ozden et al. [20] have reported that involvement of ethylene production leads to tissue browning which can be reduced in the presence of silver nitrate. Opabode [21] reported that silver nitrate is an anti-necrotic compound which can reduce oxidative burst during the interaction between plant tissue and *Agrobacterium*. The objective of this study was to evaluate the effects of AgNO_3 on somatic embryogenesis from leaf explants and *Agrobacterium*-mediated genetic transformation of *H. brasiliensis*.

Materials and Methods

Somatic Embryogenesis from Leaf Explants

A system for somatic embryogenesis and plant regeneration has been developed from leaf explants collected from glass house-grown bud-grafted plants of *H. brasiliensis* (clone RR1105) [6, 7]. The optimised medium for callus induction, embryo induction, embryo maturation and plant regeneration was used for the experiments (Table 36.1). Leaf explants collected from 6-month-old glass house-grown bud-grafted plants of *H. brasiliensis* were cultured for callus induction. Callus was proliferated by repeated subculture in medium where cytokinin/auxin ratio and sucrose concentration were increased (Table 36.1). The cultures were incubated at 25 ± 2 °C and kept in dark.

Effect of Silver Nitrate on Callus Friability and Embryogenic Callus Initiation

The effect of silver nitrate in improving callus friability and making the calli more embryogenic was examined. Silver nitrate stock solution (1.0 mg/ml) was prepared in water and the required quantity was filter sterilised and added to the autoclaved medium and was stored in the dark. Three different concentrations of silver nitrate (10, 20 and 30 mg/l) were added to the optimised proliferation medium during the second subculture. Embryogenic callus formation was tried in medium optimised earlier. In experiments on callus proliferation, silver nitrate has been found to have influence in changing the callus texture. Hence, different concentrations of silver nitrate (5, 10, 20 mg/l) were filter sterilised and added to the autoclaved medium, optimised for embryogenic callus formation.

Agrobacterium-Mediated Genetic Transformation

Bacterial Strain

Agrobacterium tumefaciens strain EHA 101 harbouring a binary plasmid vector carrying a gene-encoding isopentenyltransferase (*ipt*) from the Ti plasmid of *A. tumefaciens* was used. The binary vector also contains β -glucuronidase (GUS) gene as the reporter gene and *nptII* gene for kanamycin resistance as plant selection gene.

Agrobacterium Infection and Cocultivation: Influence of Silver Nitrate

50 μl of *Agrobacterium* suspension from glycerol stock was spread over semisolid AELB (Luria-Bertani) medium containing 50 mg/l kanamycin and 20 mg/l gentamicin and incubated at 28 °C for 2 days. *Agrobacterium* single colony was selected with sterile loop and suspended in 10 ml sterile liquid AELB medium containing the antibiotics 50 mg/l kanamycin and 20 mg/l gentamicin. The cultures were

Table 36.1 Media used for somatic embryogenesis and plant regeneration

Medium component	Callus induction	Callus proliferation	Embryogenic callus initiation	Embryo induction	Embryo maturation	Plant regeneration
Basal salts	MS + Ca(NO ₃) ₂ , 4H ₂ O -1,200 mg/l	MS + Ca(NO ₃) ₂ , 4H ₂ O -800 mg/l	MS + Ca(NO ₃) ₂ , 4H ₂ O -800 mg/l	MS + Ca(NO ₃) ₂ , 4H ₂ O -250 mg/l	MS modified 1/2x	MS modified 1/2x
Vitamins	B ₅	B ₅	B ₅	B ₅	B ₅	MS
Sucrose	20 g/l	40 g/l	60 g/l	60 g/l	60 g/l	30 g/l
Organic supplements	Casein hydrolysate (1 g/l), coconut water (5%)	Casein hydrolysate (1 g/l), coconut water (5%)	Casein hydrolysate (300 mg/l), coconut water (5%)	Casein hydrolysate (300 mg/l), coconut water (5%)	Casein hydrolysate (400 mg/l), coconut water (10%), malt extract (150 mg/l)	-
L-cysteine HCl	50 mg/l	50 mg/l	50 mg/l	50 mg/l	-	-
Proline	-	-	200 mg/l	200 mg/l	-	-
Glutamine	-	-	300 mg/l	300 mg/l	-	-
Arginine	-	40 mg/l	40 mg/l	40 mg/l	40 mg/l	-
Hormones	2,4-D (5.4 µM), BA (4.4 µM) and NAA (1.08 µM)	2,4-D (1.8 µM), BA (2.2 µM) and NAA (1.08 µM)	BA (2.2 µM), GA ₃ (2.9 µM), Kin (1.25 µM) and NAA (1.08 µM)	BA (2.2 µM), GA ₃ (2.9 µM), Kin (1.25 µM), ABA (0.75 µM), and NAA (0.54 µM)	BA (4.4 µM), Kin (1.3 µM), GA ₃ (4.4 µM) and IBA (0.49 µM)	-
pH	5.6	5.6	5.7	5.7	5.6	5.6

incubated at 28 °C in a shaker with 200 rpm for 24 h to reach an optical density of 0.5 at 420 nm. Volume of bacterial suspension required for 10 ml infection medium was calculated, and bacteria were pelleted by centrifugation at 5,000 rpm for 10 min. The bacterial pellet was resuspended in the infection medium so as to get a bacterial density of 5×10^8 cells/ml. Murashige and Skoog (MS) [22] medium containing 100- μ m acetosyringone and 1.0 mM each of proline and betaine hydrochloride (Table 36.2) was used as the infection medium. pH of the medium was adjusted to 5.2 with 1 N KOH and filter sterilised. Infection of the bacterial culture with the target tissue was done after 4 h incubation in an incubator shaker at 28 °C.

100 mg of proliferated friable *H. brasiliensis* calli was transferred to a small sterile petri dish and air-dried for 10 min in the laminar flow hood. Bacterial solution (5 ml) was poured over the callus, and infection was done for 15 min. The callus in the bacterial solution was injured with a sterile scalpel blade to facilitate entry of the bacteria. After infection, the bacterial solution was drained out and the infected tissues were blotted dry by transferring them to sterile filter papers. For cocultivation, the infected tissues were spread over filter paper kept on the surface of a semisolid cocultivation medium. The infected tissues were incubated for 72 h in dark at 28 °C [15–17]. The tissues were transferred to the preselection medium containing the antibiotic carbenicillin to control bacterial overgrowth. After 2 weeks, the tissues were subcultured to selection medium containing both selection antibiotics carbenicillin (400 mg/l) and kanamycin (300 mg/l) (Table 36.2). Transformation efficiency was scored based on the number of callus lines that emerged from the infected tissues. After the second subculture, carbenicillin was avoided from the selection medium used for subculture of infected tissues devoid of bacterial overgrowth. After 1 month, the newly emerged lines were proliferated in selection medium containing only kanamycin

(200 mg/l). About 30 callus clumps were cultured in petri plates (90 × 15 mm) containing the selection medium.

Experiments were carried out to control overgrowth of bacteria by modifying the infection, cocultivation and selection media. The effect of silver nitrate in controlling bacterial overgrowth and improving transformation efficiency were studied. Different concentrations of silver nitrate were tried and the effects on percentage of proliferating lines with transient GUS expression were evaluated. Silver nitrate (10 mg/l) was supplemented in the infection and cocultivation medium. Selection medium was also supplemented with different concentrations of silver nitrate (0–30 mg/l) to find its effect on controlling bacterial growth and improvement in callus texture. pH of the infection medium was adjusted to 5.2 with 1 N KOH and the whole solution was filter sterilised before tissue infection. The pH of the cocultivation and selection media was adjusted to 5.7 with 1 N KOH before autoclaving at 121 °C for 10 min. Silver nitrate was added filter sterilised along with phytohormones and antibiotics to the autoclaved cocultivation and selection media. Each experiment was repeated thrice with five replications.

Somatic Embryogenesis of Transgenic Callus

The newly emerged lines were screened histochemically for GUS expression. Somatic embryogenesis was tried with all GUS positive lines. The proliferated transgenic calli were subcultured on embryo induction medium containing kanamycin (200 mg/l) for embryogenic callus initiation and subsequent embryogenesis. Embryogenic callus initiation and embryo induction were tried from proliferated callus in medium standardised earlier [7] which was modified MS basal medium ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ – 500 mg/l and KH_2PO_4 – 270 mg/l) containing Gamborg's B₅ vitamins [23], amino acids, organic supplements such as coconut water (5%), malt extract (50 mg/l), casein hydrolysate (300 mg/l), sucrose 60 g/l and phytohormones.

Table 36.2 Media used for *Agrobacterium* infection and transformation

Medium component	Callus induction	Callus proliferation	<i>Agrobacterium</i> infection	Cocultivation	Preselection	Selection	Transgenic callus proliferation
MS basal	MS modified	MS modified	MS modified 1/2 x	MS modified 1/2 x	MS modified 1/2 x	MS modified 1/2 x	MS modified 1/2 x
Vitamins	B ₅	B ₅	B ₅	B ₅	B ₅	B ₅	B ₅
Sucrose	30 g/l	40 g/l	50 g/l	50 g/l	50 g/l	50 g/l	50 g/l
Organic supplements	Casein hydrolysate (1 g/l), coconut water (5%)	Casein hydrolysate (1 g/l), coconut water (5%)	-	-	-	-	-
Hormones	5.4 µM 2,4-D, 4.4 µM BA and 1.08 µM NAA	1.8 µM 2,4-D, 2.2 µM BA and 1.08 µM NAA	1.8 µM 2,4-D, 2.2 µM BA and 1.14 µM IAA	1.8 µM 2,4-D, 2.2 µM BA and 1.14 µM IAA	1.8 µM 2,4-D, 2.2 µM BA and 1.08 µM NAA	1.8 µM 2,4-D, 2.2 µM BA and 1.08 µM NAA	1.8 µM 2,4-D, 2.2 µM BA and 1.08 µM NAA
Acetosyringone	-	-	100 µM	100 µM	-	-	-
Proline	-	-	1.0 mM	1.0 mM	-	-	-
Betaine HCL	-	-	1.0 mM	1.0 mM	-	-	-
pH	5.7	5.7	5.2	5.6	5.7	5.7	5.7
Antibiotics	-	-	-	-	Carbenicillin (400 mg/l)	Carbenicillin (400 mg/l), kanamycin (300 mg/l)	Kanamycin (200 mg/l)

GUS Histochemical Assay

GUS staining was performed following the method reported by Jefferson [24]. Newly emerged lines from the infected tissues were immersed in X-Gluc solution (2 mM X-Gluc, 100 mM NaH₂PO₄, 0.5 mM potassium ferricyanide and 50 mM ferrocyanide). The tissues were incubated at room temperature. GUS expression frequency was examined and scored as blue spots.

PCR Amplification

DNA was isolated from transgenic as well as control calli of the *Hevea* clone RRII 105 following CTAB procedure [25]. PCR amplification was carried out using 20 ng of genomic DNA with isopentenyltransferase gene-specific primers. Gene primers were designed from the coding region sequence of isopentenyltransferase gene of *Agrobacterium tumefaciens*. The forward primer 5'-CTTGACAGGAAAGACGTCG-3' and reverse primer 5'-CGTAAGCGGCTGCG-3' were used for PCR amplification.

Amplifications were carried out in 20 µl reactions, which contained 1.0 µl template DNA (50 ng), 2.0 µl reaction buffer (Tris-HCl, pH 9–10, KCl 50 mM, MgCl₂ 15 mM), 2.0 µl dNTP mix (100 µM), 1.0 µl of each forward and reverse primer (250 nm each) and 0.5 U *Taq* DNA polymerase. Volume of the reaction mix was made up to 20 µl with sterile distilled water. The reaction mix was overlaid with a drop of mineral oil and amplification was carried out in MJ Research PTC 200 Peltier Thermal Cycler. The PCR conditions were 2-min initial denaturation at 94 °C followed by 36 cycles of denaturation (1 min, 94 °C), annealing (1 min, 55 °C) and extension (2 min, 72 °C). Final elongation was 10 min at 72 °C. The PCR products were analysed in 1.5% agarose gels.

Statistical Analysis

Data were subjected to square root transformation and analysed using one way ANOVA. All analyses were performed at 5% level.

Results and Discussion

Effect of Silver Nitrate on Callus Friability

Callus induction could be obtained within 40 days in the earlier standardised medium (Fig. 36.1a). Callus proliferation was obtained in the callus induction medium with reduced calcium nitrate (800 mg/l) and 2,4-D (1.8 µM) along with 40 g/l sucrose (Table 36.1). Proliferation of the callus was noticed after 3 weeks in this medium (Fig. 36.1b). The proliferated callus had a loose and friable texture. With 2–3 subcultures in proliferation medium, the texture of the callus could be improved further. When three different levels of silver nitrate (10, 20 and 30 mg/l) were tried in the proliferation medium during the second subculture, a concentration of 20 mg/l helped in improving the texture of the callus. Addition of 20 mg/l silver nitrate to the proliferation medium during the second and third subcultures made the calli yellow, more friable and nearly embryogenic compared with the control calli in which no silver nitrate was added (Fig. 36.1c).

Effect of Silver Nitrate on Embryogenic Callus Initiation

Embryogenic calli originated from more than one region of each primary callus clump as a small yellow lump which further proliferated within a month to form a mass of friable golden yellow callus (Fig. 36.1d). The frequency of embryogenic callus formation was 38% in the control. Embryogenic calli could be obtained from the primary callus clump when subcultured in fresh medium. It was observed that in the medium containing 10 mg/l silver nitrate, proliferating callus became more friable. At 5.0 and 10.0 mg/l concentrations of silver nitrate, significant improvement in the frequency of embryogenic callus formation was noted. Embryogenic callus formation could be obtained with improved frequency (43%) from the proliferated leaf callus in medium when 10 mg/l silver nitrate was added (Table 36.3). No significant difference was observed in the

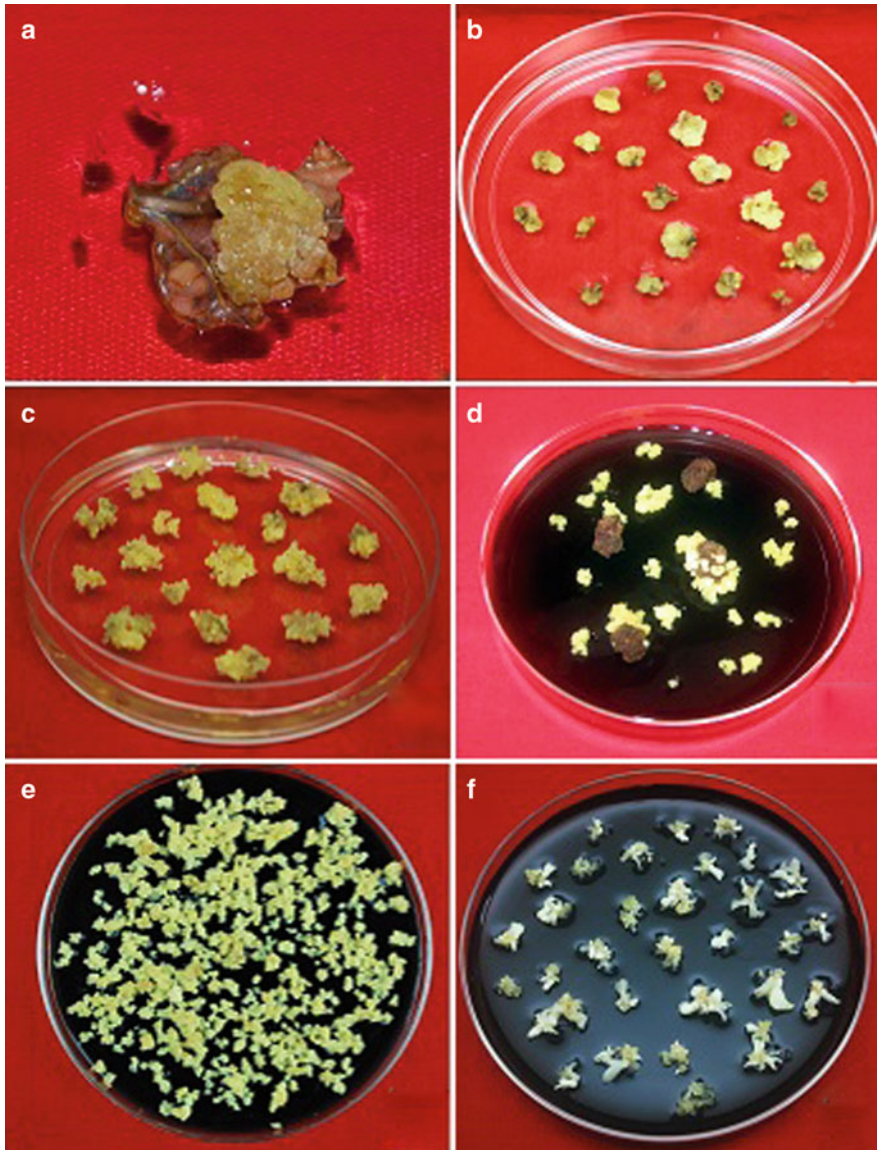


Fig. 36.1 (a–f) Different stages of somatic embryogenesis. (a) Callus induction from leaf explants. (b) Callus proliferation in medium without AgNO_3 (c) Callus proliferation

in medium supplemented with AgNO_3 . (d) Embryogenic callus initiation. (e) Proliferated embryogenic callus. (f) Somatic embryo induction

frequency of embryogenic callus formation at higher levels of silver nitrate. Somatic embryogenesis could be obtained from the proliferated embryogenic callus in medium standardised earlier (Table 36.1) (Fig. 36.1e and f).

Culture medium compounds such as silver nitrate [26] have also been reported to modify callus texture in *Zea mays*. Addition of silver nitrate to the culture medium greatly improved

the regeneration of both dicot and monocot plants in tissue culture. Silver ions in the form of nitrate, such as AgNO_3 , play a major role in influencing somatic embryogenesis, shoot formation and efficient root formation which are the prerequisites for successful genetic transformation [27]. In the present work, good callus proliferation with improved texture was obtained in medium containing 20 mg/l silver nitrate. In *Coffea canephora*

Table 36.3 Effect of silver nitrate on embryogenic callus formation

Silver nitrate (mg/l)	*Embryogenic callus (mean %)
0	38.33
5	40.00
10	43.33
20	43.33
CD	1.41

*Each value is the mean % of 20 replicate samples repeated thrice

Pierre genotypes, it has been reported that AgNO_3 concentrations between 30 and 60 μM improved embryo yield during somatic embryogenesis [11]. In *Hevea* also, addition of 10 mg/l silver nitrate to the embryogenic callus induction medium favoured embryogenesis. In recent years, AgNO_3 has been employed in tissue culture studies for inhibiting ethylene action because of its water solubility and lack of phytotoxicity at effective concentrations. In carrot, AgNO_3 at concentrations of 10–20 μM caused a twofold increase in the number of somatic embryos without causing adverse effects on cell survival [28].

Influence of Silver Nitrate on Genetic Transformation

When transformation experiments were carried out using proliferated fresh leaf callus as the target tissue for *Agrobacterium* infection, following the earlier developed protocol [17], a transformation frequency of 9% was obtained with *Agrobacterium* strain EHA 101. Earlier bacterial overgrowth in the infected tissue was common causing hindrance to transgenic tissue regeneration after *Agrobacterium* infection. In the present study, when silver nitrate was supplemented in the infection, cocultivation and selection media, it was found that overgrowth of bacteria could be reduced without compromising the transformation frequency (Fig. 36.2a).

Infection medium containing silver nitrate (10 mg/l) was used for tissue infection. The infected tissues were transferred to cocultivation medium which was also supplemented with silver

nitrate (10 mg/l). After 72 h of cocultivation, the tissues were again subcultured in selection medium containing the antibiotic carbenicillin (400 mg/l) for controlling bacterial overgrowth. When different concentrations of silver nitrate (0–30 mg/l) was supplemented in the selection medium, it was observed that with increasing concentration of silver nitrate up to 20 mg/l the bacterial overgrowth could be reduced significantly (Table 36.4). In the control medium, when no silver nitrate was provided, overgrowth of bacteria was persistent and sometimes 50% or more of the cultures had to be discarded. Bacterial overgrowth was reduced significantly at silver nitrate concentrations starting from 5.0 mg/l onwards. At concentrations above this, significant difference was noted in control of overgrowth. Addition of silver nitrate (10 mg/l) in the infection, cocultivation and selection media controlled bacterial overgrowth by 80% (Table 36.4). Maximum control was obtained at a silver nitrate concentration of 20 mg/l. Increasing the silver nitrate concentration above this did not give significant difference. The optimum concentration of silver nitrate to be used in the different steps of transformation was identified as 10.0 mg/l in infection and cocultivation media and 20 mg/l in the selection medium. By supplementing silver nitrate in the selection medium, the texture of the putatively transgenic callus lines was also found to be improved, with most of them friable, yellow and showing good proliferation (Fig. 36.2b). The tissue recovered free of bacterial overgrowth was subcultured every 3 weeks to fresh medium.

In apple, it has been reported that during *Agrobacterium*-mediated transformation, ethylene production was increased which resulted in reduced efficiency of gene transfer mechanism [18]. Ozden et al. [20] have reported that involvement of ethylene production leads to tissue browning which can be reduced in the presence of silver nitrate. Inclusion of silver nitrate in coculture medium has been proven for its anti-ethylene activity which is common with *in vitro* plant cultures. Opabode [21] reported that silver nitrate is an anti-necrotic compound which can reduce oxidative burst during the interaction between plant

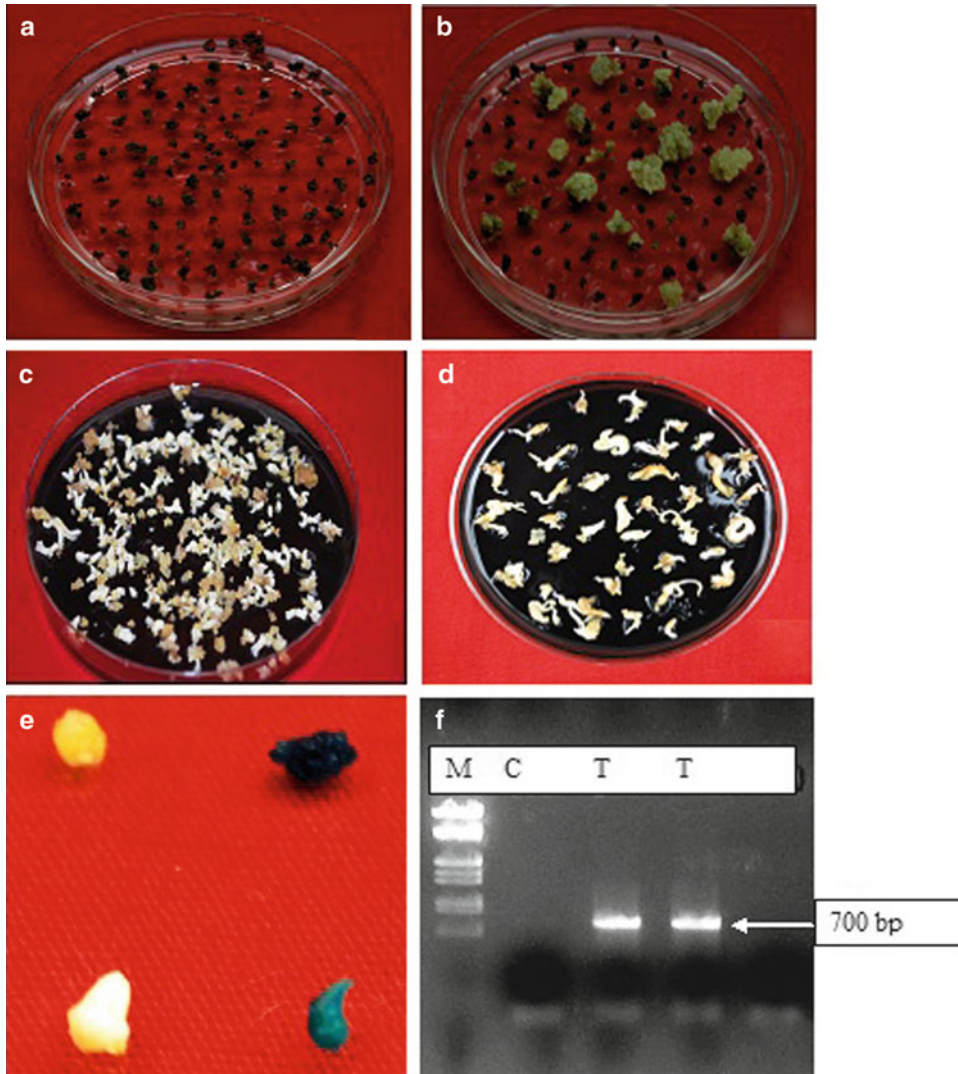


Fig. 36.2 (a–f) Different stages of *Agrobacterium* infection and somatic embryogenesis. (a) Infected callus in presence of AgNO_3 without bacterial overgrowth. (b) Infected tissues showing transgenic callus emergence in AgNO_3 -containing medium. (c) Somatic

embryogenesis from transgenic callus. (d) Transgenic somatic embryos. (e) GUS expression (control and transgenic calli and embryos). (f) PCR amplification (Lane, *M* Marker, *C* Control, *T1* Transgenic callus, *T2* Transgenic embryos)

tissue and *Agrobacterium*. Addition of silver nitrate in the coculture medium has shown to enhance stable gene transfer in maize [26, 32], carrot [28] and Fuji apple [29]. The suppressed *Agrobacterium* growth on target explants could facilitate plant cell recovery that resulted in increased efficiency of transformation. Orlikowska reported that addition of silver nitrate stimulated

direct shoot regeneration in leaves taken from *in vitro* cultures and infected with *Agrobacterium* [30]. It also inhibited bacterial growth after cocultivation with the explants. In the present study, presence of silver nitrate in the selection medium (20 mg/l) prevented overgrowth of bacteria for about 3 weeks. Similar observation was also made by Orlikowska [30] where presence of silver

Table 36.4 Effect of silver nitrate in controlling bacterial overgrowth

Silver nitrate (mg/l) (selection medium)	Overgrowth (mean %)
0	50.0 (3.87)
5	30.0 (2.59)
10	22.0 (1.72)
15	22.5 (1.72)
20	20.2 (1.57)
30	20.4 (1.57)
CD	0.3

Data from 30 replicate samples, experiment repeated thrice
Values in parenthesis indicate transformed values

nitrate (100 mg/l) in the rose regeneration medium completely retarded bacterial overgrowth for about 1 month. Thus, by the addition of silver nitrate, they were able to replace or reduce the concentration of antibiotics such as cefotaxime or carbenicillin used for controlling bacterial growth, which are often phytotoxic to tissues. Tissue damage by *A. tumefaciens* infection has been reported earlier also and seems to be one of the major obstacles for *Agrobacterium*-mediated transformation. In control experiments, the texture of the callus in the putatively transgenic lines that emerged from the infected tissues also varied, with most of the calli being hard and showing no proliferation. In the present study when silver nitrate was supplemented in the medium, the texture of the putatively transgenic callus lines that emerged from the infected tissue was found to be improved, with most of them friable, yellow and showing good proliferation. The use of silver nitrate has shown to have other important effects in plant tissue culture of cassava, such as improving somatic embryogenesis. It is also reported to have significant influence on micropropagation in many other species [10].

GUS Histochemical Assay

Approximately 2-mg callus from each emerging line was used for GUS histochemical staining. β -Glucuronidase catalyses the hydrolysis of the substrate liberating indoxyl and indolyl groups which gets dimerised to form insoluble

indigo causing blue colouration. Newly emerged putatively transgenic calli were antibiotic resistant and showed proliferation in selection medium and were found to be GUS positive (Fig. 36.2e).

Somatic Embryogenesis from Transgenic Callus

Proliferation of the putatively transgenic callus, embryogenic callus initiation and further embryo induction were obtained in medium standardised earlier for leaf explants [7]. Kanamycin (200 mg/l) was also added to all the media used for the sub-culture of transgenic callus. Callus proliferation could be obtained in modified MS medium with the addition of calcium nitrate (800 mg/l) and containing Gamborg's B₅ vitamins, sucrose (40 g/l) and growth regulators 2,4-D (1.8 μ M), BA (4.4 μ M) and NAA (1.08 μ M). Embryogenic callus initiation and embryo induction were obtained from proliferated callus in modified MS basal medium solidified with 0.5% phytagel. Rate of embryo induction from the transformed callus was low (40%) compared to untransformed controls (60%). Embryos obtained had a different appearance, with most of them having a shooty nature (Fig. 36.2c and d). Maturation of the embryos could be obtained in medium standardised earlier containing basal salts of Woody Plant Medium [31]. Plant regeneration from these embryos is being attempted.

PCR Amplification of *ipt* Gene

The genomic DNA isolated from the control and transgenic calli was used for PCR amplification using *ipt* gene-specific primers. When PCR amplification was carried out with gene-specific primers for isopentenyltransferase gene, an amplified band of approximately 700 bp was obtained. Amplification of the gene was obtained in the transgenic callus showing gene integration. No amplification could be obtained in the primer controls and in untransformed callus (Fig. 36.2f).

Summary and Conclusion

Efficient plant regeneration and transformation systems using easily available explants are indispensable for genetic modification of important crop plants. The present study reports the effect of silver nitrate in improving efficiency of the somatic embryogenesis system from leaf explants. Supplementing silver nitrate in the medium during the second and third subcultures made the calli more friable. Presence of silver nitrate in the embryo induction medium helped in embryogenic callus initiation, thereby increasing the efficiency of the system. The influence of silver nitrate on transformation efficiency by improving callus texture in the newly emerging transgenic cell lines and control of bacterial overgrowth in infected tissues were also studied. It was observed that addition of silver nitrate in the infection (10.0 mg/l), cocultivation (10.0 mg/l) and selection (20.0 mg/l) medium significantly suppressed bacterial overgrowth and improved the texture of callus in newly emerged lines. The transgenic callus obtained could be proliferated and somatic embryo induction obtained. Hence, silver nitrate was found to be a suitable medium supplement in improving somatic embryogenesis from leaf explants and in *Agrobacterium*-mediated genetic transformation using proliferated leaf callus in *Hevea brasiliensis*.

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