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Original Article

Determination of Sensitivity Level of Hypocotyl Explants to paromomycin Antibiotic and Optimization of Rooting in Transgenic Medicinal Plant *Papaver somniferum*

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Abstract

Poppy (*Papaver somniferum*) is the most important commercial source of analgesic drugs and of compounds related to them. Because of the excellent medicinal properties of poppy, finding genotypes with changed alkaloid content through breeding or by producing transgenic plants in tissue culture has attracted great interest. The present research was conducted to optimize rooting in somatic embryos of transgenic poppy plants. Sensitivity of hypocotyl explants to the antibiotic paromomycin was first determined in kill-curve experiments. Transformation of the hypocotyl explants was carried out using the GV3101 strain of *Agrobacterium tumefaciens* having the vector with the *NPT II* gene. The explants were then transferred to MS-based medium containing the 2, 4-D plant growth regulator (PGR) at 1 mg/L, 10 mM MES, and paromomycinat 15 mg/L. After the somatic embryos were produced, a factorial experiment was conducted in the rooting stage with two factors including medium (MS and 1/2 MS) and PGR (500 µM IBA PGR and no PGR). Also, confirmation of derived transgenic plantlets was confirmed by PCR technique. Results of ANOVA and comparison of the means indicated the highest rooting percentage happened in the 1/2 MS medium containing the IBA.

Keywords: Agrobacterium, *Papaver somniferum*, Somatic Embryosis, Paromomycin, Rooting

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Introduction

Production of abundant and reliable transgenic plants depends on employing efficient gene transfer and tissue culture methods. The family *Papaveraceae* is one of the most important families with the ability of producing important medicinal alkaloids. It has 23 genera and 200 species among which the *Papaver* genus and its species are of special importance. Poppy contains about 80 types of alkaloids and its medicinal properties are related to its ability to produce and biosynthesize a group of benzophenanthrene alkaloids of the sub-group benzylisoquinoline alkaloids [1]. Many of these alkaloids have significant medicinal properties and activities, and some of them are also used as drugs. These include the analgesics morphine and codeine, antibiotics such as sanguinarine that has antimicrobial and anti-inflammatory properties, noscapine with antitussive and antitumor properties, papaverine as a vasodilator, and tubocurarine as a muscle relaxant. Due to their complicated structures, chemical synthesis of these compounds is very difficult. Therefore, wild or domesticated plants are of interest as the only commercial source of these compounds [2]. The increasing information on the biosynthetic pathway of alkaloids and on metabolic engineering through somatic embryogenesis can create an unpredictable capacity for genetically engineering new plant varieties with the desired commercial properties and expand the borders of the legal poppy industry.

Plant tissue culture is a commonly technique for plant propagation in respect of commercial uses or basic scientific achievement. In plant tissue culture, many techniques are used such as somatic embryogenesis, microspore culture [3]. Many studies have been done on tissue culture and genetic engineering of *Papaver somniferum* plant [4-6]. Use of somatic embryogenesis to produce transgenic poppy is superior to direct organogenesis in which adventitious roots are formed on poppy shoot primordia. Moreover, fewer escape plants are produced in somatic embryogenesis compared to organogenesis [7]. Chimerism is eliminated in somatic embryogenesis because a single cell is the origin of the somatic embryo. Somatic embryogenesis in poppy is a multiple-stage process. One of the advantages of somatic embryogenesis over organogenesis as a regeneration system for poppy is that in somatic embryogenesis it is possible to use a selection agent in the stages of callus induction and proliferation [8]. Embryonic structures obtained from tissues or haploid or diploid cells without fusion of gametes are called vegetative (somatic) embryos. Regeneration of poppy plants from somatic embryos with various hormonal concentrations and different explants has been reported [9-11]. However, somatic embryos usually have lacked roots or have had few roots [12, 13]. In the present study, in order to determine the level of antibiotic paramomycin for transgenic tests, sensitivity of hypocotyl explants to antibiotic paramomycin was first determined in kill-curve experiments and then this research intended to optimize rooting in transgenic poppy plants obtained by somatic embryos through designing and applying rooting treatments.

Materials and Methods

Plant materials

Poppy seeds were surface-washed with agitation using 70% ethanol for 30 to 60 seconds and then disinfected for 20 minutes in sodium hypochlorite (1% w/v). They were then rinsed 3 to 4 times with double distilled water until no smell of bleach remained. The seeds were then placed on B5-based medium with the concentration of 3.16 g/L and sucrose at 20 g/L and agar at 8 g/L. Before adding the agar, the pH was adjusted to 5.6-5.8. The dishes in which the seeds were placed were sealed with parafilm and imbibed at 4˚C for 24-48 hours. Following that, they germinated at 24˚C in a 16 h light/8 h dark cycle. Seven to eight days after germination, the hypocotyls were excised from the plantlets and cut into 3-6 mm pieces and used in the transformation experiments.

Tissue culture media and conditions

B5 medium was used for planting the seeds and the MS medium (containing iron salts and the vitamins of the MS medium, sucrose at 30 g/L, and MES, at 2 g/L as the buffering agent for the medium) for the other culture media. The pH of the medium was about 5.6, and the gelling agent was 8 g/L Merck agar. All the media were autoclaved at 121˚C for 20 minutes. The MS-based medium lacked growth regulators and was used together with 10mM MES and sucrose at 30 g/L for keeping embryogenic calluses and for regeneration of shoot primordia and plantlets. The callus- inducing medium was the MS medium with sucrose at 30 g/L, the 2,4-D PGR at 1 mg/L, and 10 mM MES that were added to the medium before autoclaving. The antibiotic was filter sterilized and was poured into the medium after the medium was autoclaved and cooled to 55-60˚C. The explant and Type I calluses were kept at 24˚C and the Type II calluses and the somatic embryos at 18-21˚C.

Bacterial strains and vectors

The GV3101 strain of *Agrobacterium tumefaciens* was employed for transformation of poppy plants. It was stored in 20% glycerol at -80˚C and then cultured overnight at 28˚C in 10 ml of the LB broth without the antibiotic selection, was diluted until its OD600 reached 0.25, and was then used in the transformation experiments. The employed vector in this study was with the selection *NPT II* gene that is resistant to paromomycin.

Selective agent and its concentrations

Sensitivity of poppy explants to the antibiotic selection paromomycin was studied in kill-curve experiments. All these experiments were performed by inoculating the explants with the GV3101 strain of *Agrobacterium* that lacked the gene silencing vector.

After treatment with *Agrobacterium*, the explants were washed and placed on callus-inducing medium that contained the antibiotics paromomycin and cefotaxime. Cefotaxime was used at 100 mg/L, and paromomycin was tested in kill- curve experiments at 5, 10, 15, 20, 25, and 30 mg/L.

The transformation and embryogenesis method

The hypocotyls were excised from the plantlets and immediately immersed in the liquid *Agrobacterium* medium for 10 to 15 minutes. They were then directly put in the callus-inducing medium. Three to four days after the coculturation, the explants were washed twice in double distilled water (this was continued until the water was clear of evident *Agrobacterial* suspension) and blotted on filter paper. They were then transferred to the callus-inducing medium that contained the paromomycin and cefotaxime selection agents. Both in the control and in the transgenic experiments, the explants were transferred to fresh media once every three weeks. The explants first produced semi-translucent brownish calluses with large cells. These are the so-called Type I calluses. Following that, white, compact embryogenic calluses called Type II calluses were formed from Type I calluses. Type II calluses were transferred to MS-based media that lacked growth regulators and contained 10 mM MES, sucrose at 30 g/L, cefotaxime at 100 mg/L, and the paromomycin selection agent at 15 mg/L. After 1 or 2 culture periods, somatic embryos were formed in this medium.

Rooting of somatic embryos

In this stage, a factorial experiment with two factors (medium at the two levels: MS and 1/2 MS; and PGRat two levels: 500 mM IBA and no PGR) was conducted. The experimental design was completely randomized design with three replications. Some of the somatic embryos were transferred to the MS medium and some to the 1/2 MS medium. All media contain 30 g/L sucrose, 10 mM MES, and 8 g/L agar. In both media, the embryos in the cotyledonary stage were kept for 5 hours at 17˚C in 500 mM IBA and then, after the IBA PGR was removed, the embryos were put on fresh media with the same composition.

Analysis of transgenic plants

DNA extraction from the formed seedlings was carried out in the paromomycin selection medium. PCR was carried out with the amplification primers of the *NPT II* gene, and the extracted DNA was used as template. Table 1 lists the primers that were used for amplifying the *NPT II* gene.

Table 1. Primer sequences which were used for confirmation of transformation.

Primer name	Primer sequences (5′ to 3′)
nptII-F	AAGATGGATTGCACGCAGG
NptII-R	CAGAAGAACTCGTCAAGAAGG

Statistical analysis

Data on callus formation and on embryogenesis was recorded to determine the percentage of explants that produced calluses and also the percentage of embryogenic calluses. Statistical analysis of the final data and comparison of the means were carried out using MSTAT-C. Comparison of the means was based on Duncan's multiple-range test at the 0.05 level.

Results and Discussion

In experiment determining the level of antibiotic concentration of paromomycin, the kill-curve of the explants was drawn against the various concentration of paromomycin to select the suitable concentration of this antibiotic.

For this purpose, paromomycin was used at 5, 10, 15, 20, 25, and 30 mg/L. At 5 mg/L, the explants entered the callus formation stage about one month and the embryo development stage after 45 days after transformation. At 10

mg/L, they entered the callus formation stage after 45 days and the embryo development stage after two months (on average) after transformation. At 15 and 20 mg/L, they remained in the swelling stage and did not enter into the callus formation and embryo development stages. Figure 1 presents callus formation responses of poppy explants to the selection agent paromomycin. Furthermore, figure 2 shows embryo development responses of poppy explants to this selection agent. In all of the experiments for determining kill-curves, the explants were inoculated with *Agrobacterium* containing no vector.

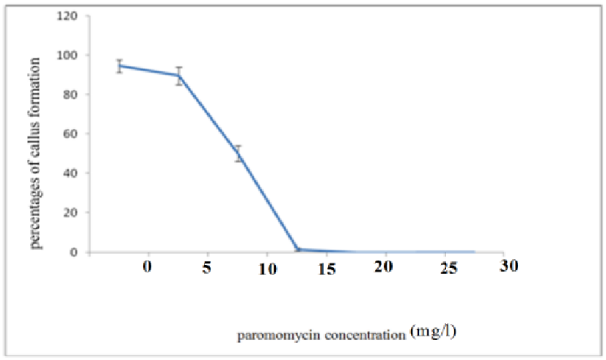

Figure 1. Diagram of hypocotyl explants response to paromomycin, with the curve showing percentages of callus formation at paromomycin concentrations of 0, 5, 10, 15, 20, 25, and 30 mg/L.

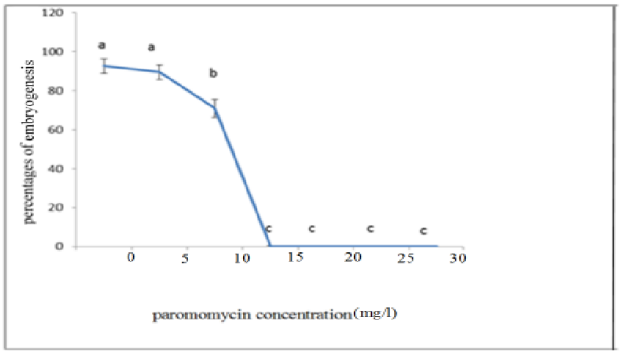

Figure 2. Diagram of hypocotyl explants response to paromomycin, with the curve showing percentages of embryo development at paromomycin concentrations of 0, 5, 10, 15, 20, 25, and 30 mg/L.

According to the kill-curve, callus formation and embryogenesis percentages at paromomycin concentration of 5 mg/l were very high (more than 90% and close to those of the control), at 10 mg/L these percentages were high, but at 20, 25, and 30 mg/L the explants did not enter into the callus formation and embryo production stages. It seems the 15 mg/L concentration is an ideal paromomycin concentration for producing transgenic poppy plants. Therefore, transgenic experiments were performed using paromomycin at 15 mg/L. The sensitivity of poppy hypocotyl explants to the selective in kill-curve experiments in other studies examined [14].

In the transgenic experiment in the medium containing 15 mg/L paromomycin, callus production was 60% and embryo production was 58%. In another study, different genotypes of poppy have been investigated and it is concluded that the concentration of 25 mg/L is the best paromomycin concentration in transgenic experiments which is different from the results of this study [14]. It seems that the sensitivity of the explants to the concentration of paramomycin is influenced by the genotype.

The results of rooting optimization experiment show that ten days after co-culture with *Agrobacterium* (on average), the explants swelled and entered the callus formation phase 40 days later. Two phenotypic types of calluses were observed: Type I calluses that formed on the explants from the beginning and Type II calluses that were in the form of white compact masses. In the control experiments, Type II calluses formed about 4 weeks after the explants were placed on the callus-inducing medium. However, Type II calluses were produced about 8 weeks after transformation of the explants with *Agrobacterium*, were transferred to the MS-based medium without the PGR, and somatic embryos were formed 6 weeks later. In the control experiments, somatic embryos were formed 3 weeks after Type II calluses were placed on medium without the PGR. Figure 3 shows growth stages of poppy in the tissue culture medium from the explants stage to the complete plant. Some of these embryos were able to produce plantlets.

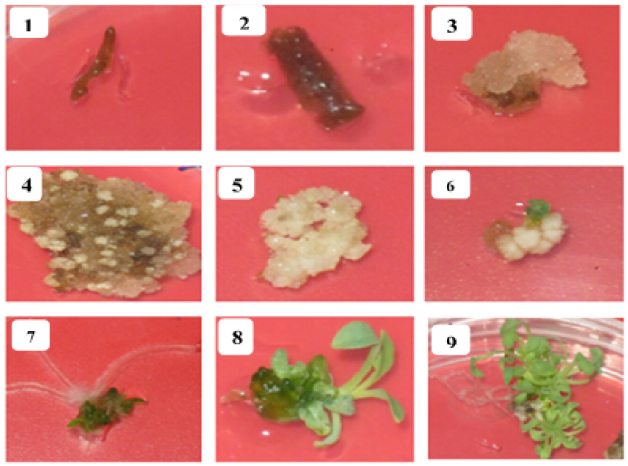

Figure 3. Stages in somatic embryo development in poppy from hypocotyl explants to somatic embryos that developed into root-bearing plantlets: hypocotyl explants excised from the mother plantlet (1), initial swelling in hypocotyl (2), initial callus formation (3), production of secondary calluses on the initial ones (4), somatic embryo development (5), embryo germination (6), root production and development (7), and formation of the complete plantlet.

In previous research, it was observed that many initial shoot primordia were very brittle and had an abnormal glassy appearance which was caused by the relatively high humidity in culture shelves [15]. To prevent this problem from happening, the embryogenic calluses were removed from tissue culture rooms at 24˚C and put in those at 16˚C in the present research. This reduction in temperature increased embryo development and somatic embryo germination. The benefit of lowering temperature during somatic

embryogenesis was reported [14]. To control pH values of media, 10 mM MES was added to them. This compound directly or indirectly influenced gene transfer by *Agrobacterium*, callus production, and embryogenesis and embryo development because previous researches has shown that pH is important in T-DNA transfer from *Agrobacterium* [14, 16].

The produced embryos were transferred to a rooting medium. After collecting data concerning the transgenic experiments, statistical data analysis was performed. Results of ANOVA are presented in Table 2 and those related to comparison of the mean rooting percentages in Table 3. To confirm of transformation in putative transgenic, some of the obtained plantlets in the medium containing paromomycin were selected, and PCR was carried out with the primers of the *NPT II* gene. Results are presented in figure 4 and PCR amplification on DNA extracted from plants transformed with the construct containing *nptII* gene revealed a 784 bp product, which corresponded to the predicted size of the *nptII* gene with the above primers.

Table 2. Results of analysis of variance of the treatments in rooting

Change source	Degree of freedom	Mean squares
Media	1	88.167^{ns}
Hormone	1	1014**
Media × Hormone	1	4760.167**
Error	20	55.750
Total	24	

ns: non-significant; ** show significant differences at 1% probability levels.

Table 3. The results of comparison of mean rooting percentage in the examined treatments.

Media	Hormone	Percentage of rooted seedlings
MS	With IBA	20.83c
MS	Without IBA	36.00b
1/2MS	With IBA	52.83a
1/2MS	Without IBA	11.67d

The same letters show lack of statistical significance of the means differences.

Results of ANOVA showed that the mean squares of the interaction effects of medium and PGR were significant. According to the table related to comparison of the means, the use of 1/2 MS medium with the IBA PGR yielded the highest and the 1/2 MS medium showed the lowest rooting percentage. In previous research, IBA was used for rooting of poppy plants [8]. Moreover, use of 1/2 MS medium for inducing root production in poppy plants has been reported [17]. Results of the present research indicated that use of the 1/2 MS medium together with IBA was able to increase rooting percentage in poppy plants. Previous researches in other plants has shown that the best rooting treatment was observed on half strength MS medium supplemented with IBA, IAA and NAA [18]. In regenerated shoots of *Jatropha curcas*, induction of root formation was occurred on 1/2 MS medium with 0.1 mg/L IBA, and survival percentage reached above 90% [18]. This study is in agreement with our findings.

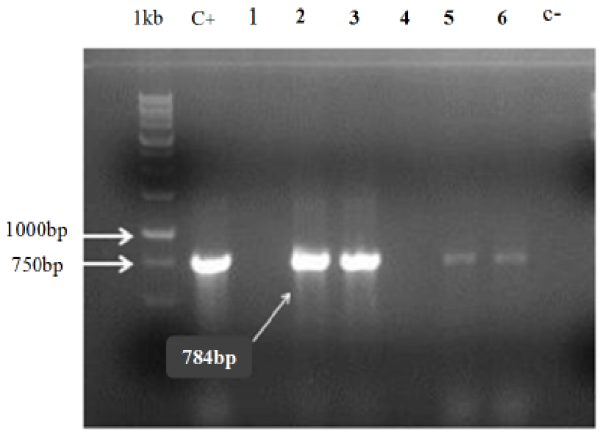

Figure 4. Transform confirmation of the regenerated plants in the medium containing paromomycin. The 1 kb marker is on the right side; c^+ is the positive control; Lane 1, 2, 3, 4, 5, and 6 are related to the selected plantlets; and c^- represents the negative control of the PCR.

Conclusion

The results of determining the level of antibiotic concentration of paromomycin showed that the 15 mg/L concentration is an ideal paromomycin concentration for producing transgenic poppy plants. The results of rooting optimization experiment indicated that use of the 1/2 MS medium supplemented with IBA was able to increase rooting percentage in poppy plants.

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