J Plant Biotechnol (2016) 43:240-247
DOI:http://dx.doi.org/10.5010/JPB.2016.43.2.240

ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

A novel method for high-frequency transgenic shoot regeneration via *Agrobacterium tumefaciens* in flax (*Linum usitatissimum* L.)

Ramazan Beyaz* · E. Selcen Darçın · Murat Aycan · Mustafa Kayan · Mustafa Yıldız

Received: 24 April 2016 / Revised: 24 April 2016 / Accepted: 25 April 2016

Abstract In this study, routinely used transformation method, which includes transferring explants onto co-cultivation medium after inoculating them with bacterial solution for a while, was compared with 3 different inoculation methods. In every 3 methods, hypocotyl explants excised from 7-day-old sterile flax seedlings having cotyledon leaves and no root system dried under air flow in sterile cabin for 35 min were inoculated with different volumes of bacterial solution at different inoculation periods. GV2260 line of *Agrobacterium tumefaciens* having 'pBIN 19' plasmid containing *npt II* (neomycin phosphotransferase II) gene and GUS reporter gene was used in transformation studies. After inoculation, hypocotyl segments of seedlings (0.5 cm in length) - were excised and left to co-cultivation for 2 days. Then, explants were transferred to regeneration medium supplemented with different antibiotics. The presence of *npt-II* and GUS genes in transformants was confirmed by PCR and GUS analysis. The highest results in all characters examined in all cultivars were obtained from the 2 inoculation method in which hypocotyls excised from seedlings inoculated with 500 µl of bacterial solution after drying in sterile cabin for 35 min were used.

R. Beyaz (✉)
Department of Soil Science and Plant Nutrition, Faculty of Agriculture, Ahi Evran University, Bagbası, Kırşehir, 40100, Turkey
e-mail: ramazanbeyaz@gmail.com

E. S. Darçın
Department of Field Crops, Faculty of Agriculture and Natural Sciences, Bilecik Şeyh Edebali University, Bilecik, Turkey

M. Aycan · M. Kayan
Department of Field Crops, Graduate School of Natural and Applied Sciences, Ankara University, Ankara, 06110 Diskapi, Turkey

M. Yıldız
Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, 06110 Diskapi, Turkey

Keywords *Agrobacterium tumefaciens*, Gene transfer, Inoculation method, Flax, Transgenic

Introduction

Agrobacterium tumefaciens-mediated transformation has been widely used to obtain transgenic plants having desired traits such as insect resistance and herbicide tolerance (Hooykaas and Schilperoot 1992). Two genetic components of bacteria, virulence genes (*vir*) and chromosomal genes (*chv*), are directly involved in the transfer of T-DNA from *Agrobacterium* to plant cells (Hooykaas and Schilperoot 1992). The molecular basis of *Agrobacterium*-mediated transformation is the transfer and stable integration of a DNA sequence (T-DNA) from Ti (tumor-inducing) plasmid of *Agrobacterium tumefaciens* into the plant genome (Bevan and Chilton 1982; Binns and Thomashow 1988). *Agrobacterium tumefaciens*-mediated transformation has some advantages compared with direct gene transfer methods such as integration of low copy number of T-DNA with plant genome, stable gene expression and transformation of large size DNA segments. However, production of transgenic plants is still not easy in most of plant species.

One of the main objectives of tissue culture studies is to obtain high-frequency shoot regeneration, which is also a prerequisite for an efficient transformation system. The introduction of foreign genes coding agronomically important traits into plant cells has no meaning unless transgenic plants are regenerated from the genetically modified cell(s). Adventitious shoot regeneration capacity of cells or tissues to be used in transformation studies affects the success of genetic transformation significantly (Jordan and Mc Hughen 1988; Dong and McHughen 1993).

Flax (*Linum usitatissimum* L.) is an annual plant species from *Linaceae* family. It is an important crop as source of natural fiber and industrial oil and has the potential of meeting edible oil and protein deficiency (Green and Marshall 1984;

Caillot et al. 2009). The products of flax are used in various industries including textiles, insulation materials, fibre-reinforced composites, pulp and paper, inks, paints, varnishes, etc (Caillot et al. 2009). Morover, due to some protection properties of lineseed oil, it is recommended in some diseases such as respiratory track disease, gastrointestinal track disease, skin disease, diabet and some cancer disease (Lorench-Kukula et al. 2005).

Moreover, it has been used as a model system for genetic manipulation studies due to its small nuclear genome (Millam et al. 1992). Although flax regenerates easily, transformation efficiency is low when inoculated hypocotyls are directly co-cultivated (Jordan and McHughen 1988). Transformation efficiency could be increased by modifying physical conditions of explant and preconditioning of explants (Dong and McHughen 1993; Yıldız and Er 2002b)

This study was aimed to increase the transformation efficiency in flax by modification of tissue's osmotic pressure together with different inoculation durations and bacterial density. In this study, together with routinely used inoculation method, three inoculation methods were compared with respect to transformation efficiency.

Materials and Methods

Plant material

Flax (*Linum usitatissimum* L.) seeds of 'Madaras', 'Clarck' and '1886 Sel.' cultivars obtained from Northern Crop Science Laboratories, in North Dakota, USA were used in the study. Seeds were surface sterilized with 40% commercial bleach containing 5% sodium hypochlorite at 10°C for 20 min with continuous stirring and then were washed three times with sterile water at the same temperature (Yildiz and Er 2002). Sterilized seeds were germinated on a basal medium containing the mineral salts and vitamins of (MS) (Murashige and Skoog 1962), 3% (w/v) sucrose and 0.7% (w/v) agar.

Culture conditions

All cultures were incubated at 24± 1°C under cool white fluorescent light (27 μmol m^{-2} s^{-1}) with a 16h light/8h dark photoperiod. The pH of the medium was adjusted to 5.8 prior to autoclaving. Hypocotyl segments (length) were excised from 7-day-old seedlings as reported by Yıldız et al. (2003).

Agrobacterium tumefaciens strain

Agrobacterium tumefaciens strain GV2260 harboring plasmid p35S GUS-INT was used for inoculation. The binary plasmid p35S GUS-INT contains neomycin phosphotransferase II (*npt-II*) gene driven by nopaline synthase (NOS) promoter and β-glucuronidase (GUS) gene controlled by cauliflower mosaic virus (CaMV35S) promoter. GV2260 strain carrying p35S GUS-INT strain was grown overnight in a liquid NB (Nutrient Broth) medium containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin at 28°C in a rotary shaker (180 rpm) (OD = 0.6) and used for transformation.

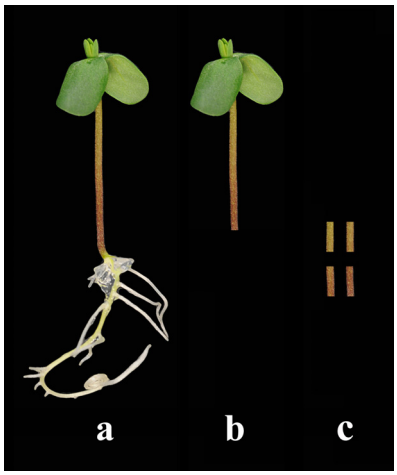

Fig. 1 Seven-day-old sterile flax seedling (a), Explant (7-day-old sterile flax seedling having cotyledon leaves and no root system used in transformation studies) used for inoculation in 2nd, 3rd and 4th inoculation methods (b), and explants used for inoculation in 1 inoculation (routinely used) method (c)

Transformation procedure

Four different inoculation methods were used for transformation to flax by *Agrobacterium tumefaciens*. Three of these methods were based on inoculation of 7-day-old sterile seedlings having cotyledon leaves and no root system that were dried under air flow in sterile cabin for 35 min. with different volumes of bacterial solution at different inoculation periods (Fig. 1). The reason why 7-day-old seedlings having cotyledon leaves and no root system were dried under air flow in sterile cabin was to enable seedlings to intake bacterial solution rapidly towards inner cells by increased osmotic pressure of seedlings in the stage of inoculation and by this way to increase the number of cells inoculated and consequently to increase the transformation efficiency. And these three inoculation methods were compared to the routinely used inoculation protocol as a control.

1st Inoculation (Routinely Used) method

Hypocotyl segments, 0.5-cm in length, excised from 7-day-old

sterile flax seedlings were immersed in a liquid regeneration medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA for 20 min. with a 500 µl of overgrown GV2260 p 35S GUS-INT strain.

2nd Inoculation method

7-day-old sterile flax seedlings having cotyledon leaves and no root system were inoculated with 500 µl bacterial solution added to liquid regeneration medium for 20 min after drying in sterile cabin for 35 min.

3rd Inoculation method

7-day-old sterile flax seedlings having cotyledon leaves and no root system were inoculated with 500 µl bacterial solution added to liquid regeneration medium for 40 min after drying in sterile cabin for 35 min.

4th Inoculation Method

7-day-old sterile flax seedlings having cotyledon leaves and no root system were inoculated with 1000 µl bacterial solution added to liquid regeneration medium for 40 min after drying in sterile cabin for 35 min.

After inoculation, hypocotyl explants were transferred to solid regeneration medium containing 1 mg L^{-1} BAP and 0.02 mg L^{-1} NAA for co-cultivation for 2 days in culture room at a temperature of 25± 1°C. After co-cultivation, explants were transferred to regeneration medium supplemented with 100 mg L^{-1} kanamycin and 500 mg L^{-1} augmentin.

Histochemical GUS assay

Histochemical GUS assay was carried out as reported previously (Jefferson, 1987). Samples were incubated at 37°C overnight in histochemical reagents consisting of 1 mM 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-Gluc), 10 mM EDTA, 0.1% Triton X-100 and 100 mM sodium phosphate at pH 7.0. The samples were then fixed in 70% ethanol. A sample was described as GUS positive if there was at least one discrete dark-blue region on the tissue.

Rooting of shoots and recovery of transgenic plantlets

Regenerated shoots were then transferred to rooting medium containing 3 mg L^{-1} indole-butyric acid (IBA) and 100 mg L^{-1} kanamycin in Magenta vessels (60 × 60 mm) with four replications (shoots from each petri dish were transferred to the separate Magenta vessels) and incubated for 3 weeks at 24± 1°C to induce root formation. Rooted shoots were transferred to pots with four replications again (plantlets from each Magenta vessel were transferred to pots) and grown at a room for 3 weeks where light (27 µmol m^{-2} s^{-1}), temperature (24± 1°C) and humidity were controlled. Humidity was decreased gradually from 100% to 40% during three weeks. Plants were irrigated with 50 ml water containing 100 mg L^{-1} kanamycin at 2 day-intervals during 14 days for further selection.

Genomic DNA extraction

Genomic DNA was extracted from transformed and untransformed (control) tissues according to via Genejet Plant Genomic DNA purification mini kit (#K0791 Thermo, Lithuanian).

Polymerase chain reaction (PCR)

PCR amplification of the *npt-II* genes was done with the following specific primers: 5′- GATTGCACGCAGGTTCT CCG -3′ and 5′- AGCCCCTGATGCTCTTCGTC -3′ for the *npt-II* gene. Standard PCR was performed in 2 µL containing 200 ng of DNA, 0.5 pmol of each primer, 0.25 µM dNTP, 1× PCR buffer, 2mM $MgCl_2$ and 0.625 U of DreamTaq DNA polymerase (#0702 Thermo). PCR was performed in a programmable thermocycler (MJ Research) with the following conditions: 5 min at 95°C; followed by 36 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C; and finally extension with 10 min at 72°C. Samples were then stored at 4°C. Amplified DNA was detected by using ultraviolet light after electrophoresis on 1% agarose/ethidium bromide gels using 1× Tris-borate-EDTA as running buffer.

Observations

Regeneration percentage, shoot number per explant, the highest shoot length, mean shoot number growing on selection medium per petri dish, total GUS (+) shoot percentage, mean shoot number rooted per Magenta vessel, total plant number growing in soil, total PCR (+) plant number and transformation efficiency were recorded 4 weeks after culture initiation.

Statistical analysis

Four replicates were tested. Petri dishes (100 × 10 mm) containing 25 explants, Magenta vessels containing shoots and pots containing rooted plantlets were considered the units of replication. One-way Analysis of Variance (ANOVA) was

used to test the effect of inoculation methods on transformation efficiency in each cultivar. All experiments were repeated two times. Data were statistically analyzed by "SPSS 15.0 for Windows" computer program. Duncan's multiple range test was used to compare the means. Data presented in percentages was subjected to arcsine ($\sqrt{X}$) transformation before statistical analysis (Snedecor and Cochran 1967).

Results

Routinely used inoculation method in which hypocotyl explants excised from 7-day-old sterile seedlings were directly inoculated with a 500 µl bacterial solution for 20 min., was ranked in second order with respect to all characters examined in all cultivars except shoot number per explant in cv. '1886 Sel.' (Fig. 2a, Table 1). After a 4-week-cultivation period on selection medium containing 100 mg L^{-1} kanamycin and 500 mg L^{-1} augmentin, the highest results of all characters examined in all cultivars were obtained from newly described inoculation

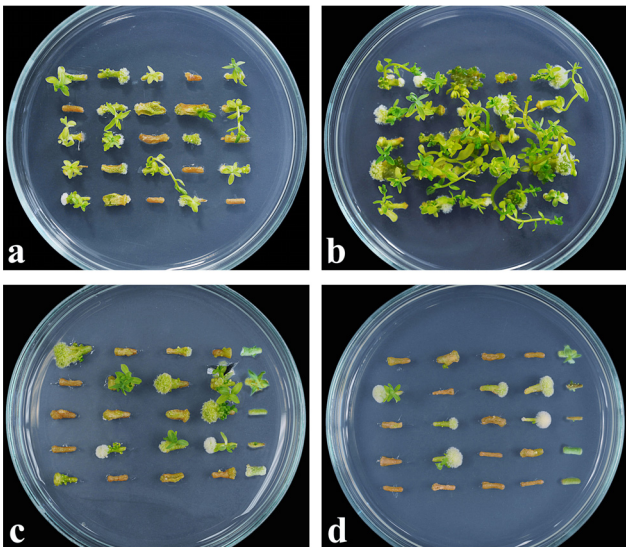

Fig. 2 Shoot regeneration from hypocotyl explants of flax cv. 'Madaras' inoculated by 4 different methods (a. the 1 inoculation method, b. the 2nd inoculation method, c. the 3rd inoculation method and d. the 4th inoculation method) on selection medium 4 weeks after culture initiation

Table 1 The development of shoots regenerated and transformed form flax hypocotyl explants inoculated with *Agrobacterium tumefaciens* on selection medium 4 weeks after culture initiation

Cultivars	Inoculation Method	Shoot regeneration (%)	Shoot number per explant	The highest shoot length (cm)	Mean shoot number growing on selection medium per petri dish	GUS+ shoot (%)	Mean shoot number rooted per Magenta vessel	Total plant number growing in soil[1] (Over 4 replications)	Total PCR+ plant number[2] (Over 4 replications)	Transformation efficiency (%) (2/1 × 100)
'Madaras'	1	41.00±3.24b	1.09±0.07b	1.75±0.31b	11.25±3.33b	56.25±3.85b	8.50±2.89b	25.00±2.20b	8.00±0.87b	30.76
	2	71.25±2.93a	1.65±0.32a	3.50±0.35a	30.00±6.40a	90.00±5.00a	28.75±5.86a	104.00±5.32a	88.00±1.32a	84.61
	3	17.50±3.06c	0.21±0.04c	1.13±0.29bc	0.97±0.28c	4.13±1.63c	0.83±0.21c	3.00±0.22c	1.00±0.13c	3.00
	4	23.75±4.59c	0.25±0.06c	0.63±0.15c	1.62±0.59c	8.00±2.92c	1.43±0.50c	5.00±0.36c	0.00±0.00c	0.00
'Clarck'	1	42.50±4.62b	1.03±0.04b	1.25±0.31b	10.50±1.00b	51.25±2.93b	8.25±1.05b	18.00±0.61b	6.00±0.35b	33.33
	2	73.75±2.93a	1.65±0.23a	2.50±0.61a	33.25±4.22a	88.75±8.05a	28.75±3.44a	103.00±3.25a	82.00±2.31a	79.61
	3	10.00±0.00c	0.16±0.05c	0.88±0.15b	0.40±0.11c	2.00±0.54c	0.25±0.00c	0.00±0.00c	0.00±0.00c	0.00
	4	5.00±4.33d	0.05±0.04c	0.75±0.18b	0.17±0.18c	0.84±0.76c	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00
'1886 Sel.'	1	20.00±4.94b	1.15±0.62b	1.25±0.31b	6.00±1.94b	30.00±9.21b	4.00±1.41b	11.00±0.92b	3.00±0.35b	27.27
	2	67.50±3.90a	1.46±0.62a	3.00±0.00a	25.25±4.27a	90.00±5.83a	23.50±4.80a	86.00±4.40a	76.00±1.32a	88.37
	3	28.75±7.77b	0.28±0.61c	1.00±0.00b	2.33±0.76b	11.63±4.70b	1.50±0.35b	3.00±0.18b	1.00±0.13c	3.00
	4	0.00±0.00c	0.00±0.00d	0.00±0.00c	0.00±0.00b	0.00±0.00c	0.00±0.00b	0.00±0.00b	0.00±0.00c	0.00

Each value is the mean of 4 replications containing 25 explants per replication. All experiments were repeated 2 times.
Values within a column for each cultivar followed by different letters are significantly different at the 0.01 level

1. Hypocotyl explants isolated from 7-day-old seedlings were inoculated with 500 µl bacterial solution for 20 min.
2. Seven-day-old sterile seedlings having cotyledon leaves were dried in sterile cabin for 35 min. Then, they were inoculated with 500 µl bacterial solution for 20 min. Finally, hypocotyl explants excised from inoculated seedlings were placed on co-cultivation medium.
3. Seven-day-old sterile seedlings having cotyledon leaves were dried in sterile cabin for 35 min. Then, they were inoculated with 500 µl bacterial solution for 40 min. Finally, hypocotyl explants excised from inoculated seedlings were placed on co-cultivation medium.
4. Seven-day-old sterile seedlings having cotyledon leaves were dried in sterile cabin for 35 min. Then, they were inoculated with 1000 µl bacterial solution for 40 min. Finally, hypocotyl explants excised from inoculated seedlings were placed on co-cultivation medium.

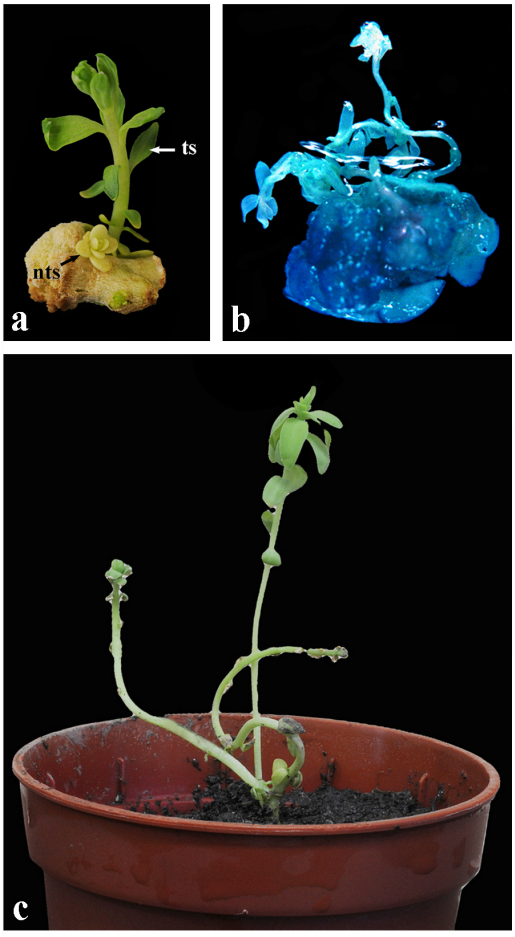

Fig. 3 Kanamycin-resistant shoot formation of flax cv. 'Madaras'. Transgenic shoot (ts) and non-transgenic shoot (nts) of cv. 'Madaras' on selection medium 4 weeks after culture initiation (a), Transient GUS expression in callus and leaves of Flax cv. 'Madaras' (b), PCR-confirmed transgenic plants growing in soil (c)

method in which 7-day-old sterile flax seedlings having cotyledon leaves, and no root system were inoculated with 500 µl bacterial solution for 20 min after drying in sterile cabin for 35 min (Fig. 2b, Table 1). The results of all characters decreased dramatically in the other inoculation methods (Fig. 2c, Fig. 2d, Table 1).

The highest shoot regeneration percentage was recorded as 71.25%, 73.75% and 67.50% in cvs. 'Madaras', 'Clarck' and '1886 Sel.' in the 2nd inoculation method, respectively. The difference in results between two inoculation methods indicated more than two times increase in shoot regeneration percentage on selection medium. In the same way, the highest shoot number per explant was observed as 1.65, 1.65 and 1.46 in cvs. 'Madaras', 'Clarck' and '1886 Sel.' in the 2nd inoculation method, respectively. After culturing explants on selection medium for 4 weeks, it was observed that kanamycin resistant shoots which were putative transgenic, grew normally while non-transgenic ones died after a while (Fig. 3a). As an indicator of shoot health and the success in rooting stage, the highest shoot length was recorded as 3.50 cm, 2.50 cm and 3.00 cm in cvs. 'Madaras', 'Clarck' and '1886 Sel.' the in 2nd inoculation method while it was noted as 1.75 cm, 1.25 and 1.25 cm in the 1st inoculation method, respectively. Mean shoot number growing on selection medium per petri dish was recorded as 30.00, 33.25 and 25.25 in cvs. 'Madaras', 'Clarck' and '1886 Sel.' as the highest in the 2nd inoculation method, respectively. This figure was only 11.25, 10.50 and 6.00 in cvs. 'Madaras', 'Clarck' and '1886 Sel.' in the 1st inoculation method, respectively. These figures showed that by the protocol decribed in this study, putative transgenic shoot frequency increased more than a rate of 90.00%, 88.75% and 90.00% out of putative transgenic shoots (30.00, 33.25 and 25.25) in cvs. 'Madaras', 'Clarck' and '1886 Sel.' in the 2nd inoculation method was found to be GUS positive which indicated a very big success in increasing transgenic shoot frequency, respectively (Fig. 3b, Table 1).

After 4-week-cultivation on selection medium, shoots were transferred to rooting medium. From the 2nd inoculation method, 28.75, 28.75 and 23.50 shoots transferred to Magenta vessels in cvs. 'Madaras', 'Clarck' and '1886 Sel.', respectively, rooted successfully. Totaly 104.00, 103.00 and 86.00 putative transgenic plantlets over 4 replications grew in soil, reached to maturity, and no morphological abnormalities were observed in cvs. 'Madaras', 'Clarck' and '1886 Sel.' the in 2nd inoculation method, respectively (Fig. 3c). In the 1st inoculation method, only 11.25, 10.50 and 6.00 shoots were regenerated on selection medium in cvs. 'Madaras', 'Clarck' and '1886 Sel.', respectively. Out of this, 8.50, 8.25 and 4.00 shoots rooted, transferred to soil and reached to maturity in cvs. 'Madaras', 'Clarck' and '1886 Sel.', respectively (Table 1). Totaly 25.00, 18.00 and 11.00 putative transgenic plantlets over 4 replication were obtained in cvs. 'Madaras', 'Clarck' and '1886 Sel.', respectively, in the 1st inoculation method (Table 1).

Genomic DNAs of putative transgenic plants of all cultivars extracted from the upper parts of the plants were used in PCR analysis. 458 bp of the *npt-II* DNA fragment was amplified on the gel (Fig. 4). From the plants grown in soil, only 8 plants out of 25, 6 plants out of 18 and 3 plants out of 11 were found PCR-positive in cvs. 'Madaras', 'Clarck' and '1886 Sel.', respectively, in the 1st inoculation method. In this inoculation method, transformation efficiency was calculated as 30.76%, 33.33% and 27.27% in cvs. 'Madaras', 'Clarck' and '1886 Sel.', respectively (Fig. 4A1, Table 1). In the 2nd inoculation method, we described, 88 plants out of 104, 82 plants out of 103 and 76 plants out of 86 were found PCR-positive in cvs. 'Madaras', 'Clarck' and '1886 Sel.', respectively. And the

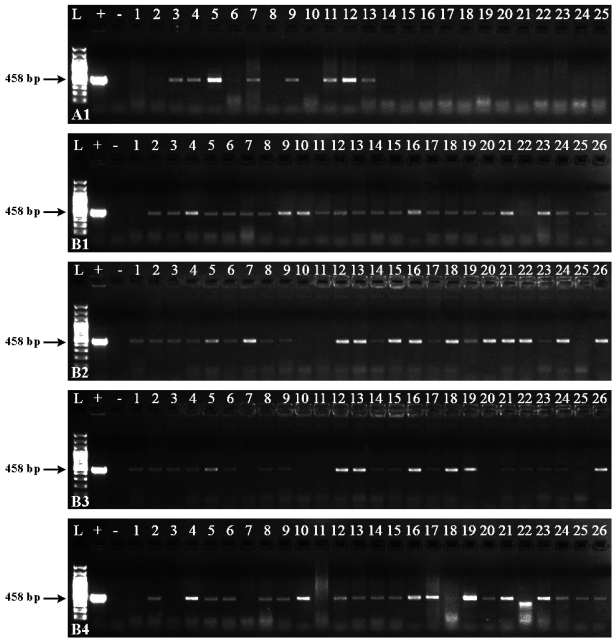

Fig. 4 PCR analysis of genomic DNA from transgenic and non-transgenic plants cv. 'Madaras' for amplification of 458 bp *npt-II* gene in the 1st inoculation method (A1) and in the 2nd inoculation method (B1-B4). *L* DNA ladder 100 bp, + Plasmid P1019 as a psitive control, - Water as a negative control; *Lanes 1-3* Putative transgenic plants

transformation efficiency for the 2nd inoculation method was calculated as 84.61%, 79.61% and 88.37% in cvs. 'Madaras', 'Clarck' and '1886 Sel.', respectively (Fig. 4B1-B4, Table 1).

Discussion

Agrobacterium *tumefaciens* is a soil phytopathogen commonly used for genetic transformation in plants (Zupan et al. 2000; Joubert et al. 2002). It has been reported that manipulation of the plant or bacteria might increase the transformation efficiency (Chakrabarty et al. 2002; Henzi et al. 2000). Pre-culturing of explants before inoculation has been increased the transformation efficiency (Amoah et al. 2001; Chakrabarty et al. 2002). Moreover, temperature (Chakrabarty et al. 2002; De Clercq et al. 2002), medium pH (De Clercq et al. 2002; Mondal et al. 2001) and chemicals such as acetosyringone (Chakrabarty et al. 2002; De Clercq et al. 2002; Henzi et al. 2000; Le et al. 2001; Lopez et al. 2004; Somleva et al. 2002) and have been studied to increase the virulence of bacteria.

Modification in some other factors such as bacterial density, co-cultivation period, vacuum infiltration and light density have also been reported to increase transformation efficiency (Amoah et al. 2001; Lopez et al. 2004; Mondal et al. 2001; Caillot et al. 2009).

Vacuum infiltration, which has been successfully used in *Agrobacterium*-mediated transformation, is based on forming negative atmospheric pressure that allows the penetration of bacteria into the inner cell spaces and consequently increases the transformation efficiency (Amoah et al. 2001; Haq 2004; Leelavathi et al. 2004; Mahmoudian et al. 2002; Spokevicius et al. 2005; Wang et al. 2003). In the method we described, negative atmospheric pressure was achieved in the explant tissue by drying 7-day-old seedlings with cotyledon leaves under air flow in sterile cabin for 35 min and this enables tissues to absorb the bacterial solution much more efficiently. The reason why we kept the cotyledon leaves of the seedlings was to form a negative pressure occurs by losing water from leaves. This negative atmospheric pressure will then facilitate water and solutes to pull up much more rapidly and easily.

Flax has a good regeneration system (Gamborg and Shyluk 1976; Mathews and Narayanaswamy 1976). Flax regenerates more easily from hypocotyl explants *in vitro* (Dong and McHughen 1993; Friedt 1990; Gamborg and Shyluk 1976; Millam et al. 1992) and the medium containing 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA have been successfully used for shoot regeneration (Dong and McHughen 1993; Jordan and Mc Hughen 1988; Yildiz and Özgen 2004). However, the production of transformed flax plants from transformed cells was very low (Dong and McHughen 1993; Beranova et al. 2008). When hypocotyl segments were directly inoculated, although explants had transformed cells after a 2 day-co-cultivation period, recovery of transgenic shoots was extremely difficult (Jordan and McHughen 1988). This could be due to the fact that plant cells perceive *Agrobacterium* infection as a pathogenic attack, and regeneration capacity of explant after inoculation with *Agrobacterium* decreases significantly due to plant defense mechanism.

In the current study aiming to increase the transformation efficiency in three flax cultivars ('Madaras', 'Clarck' and '1886 Sel.'), four different inoculation methods were compared with regard to shoot regeneration percentage, shoot number per explant, the highest shoot length, mean shoot number growing on selection medium per petri dish, GUS (+) shoot percentage, mean shoot number rooted per Magenta vessel, total plant number growing in soil over 4 replications, total PCR (+) plant number over 4 replications and transformation efficiency. The highest results in all characters examined in 3 cultivars were obtained from the 2nd inoculation method which was firstly revealed in the current study. Totally, 88, 82 and 76 PCR (+) plants were harvested in cvs. 'Madaras', 'Clarck' and '1886 Sel.', respectively (Table 1). Whereas, Dong and McHughen (1993) obtained only 37 transgenic flax plants via the same *Agrobacterium tumefaciens* strain (GV2260 harboring plasmid

p35S GUS-INT) as ours. Mlynarova et al. (1994) reported that 19 out of 47 putative transgenic flax plants were found PCR (+).

In the current study, an efficient inoculation method was described which was based on enabling seedlings to intake bacterial solution rapidly towards inner cells by increased osmotic pressure of explants in the stage of inoculation with bacteria to increase the number of cells inoculated and consequently to increase the transgenic shoot frequency. To our knowledge, this is the first report describing a new protocol for high-frequency transgenic shoot regeneration and for high-frequency transgenic plants in flax and the procedure given in this paper can be easily used for the transformation of other plants via *Agrobacterium tumefaciens*.

References

Amoah BK, Wu H, Sparks C, Jones HD (2001) Factors influencing *Agrobacterium*-mediated transient expression of *uid*A in wheat inflorescence tissue. J Exp Bot 52:1135-1142

Beranova M, Rakousky S, Vavrova Z, Skalicky T (2008) Sonication assisted *Agrobacterium*-mediated transformation enhances the transformation efficiency in flax (*Linum usitatissimum* L.). Plant Cell Tiss Organ Cult 94:253-259

Bevan M, Chilton MD (1982) Multiple transcripts of T-DNA detected in nopaline crown gall tumors. J Mol Appl Genet 1: 539

Binns AN, Thomashow MF (1988) Cell biology of *Agrobacterium* infection and transformation of plants. Annu Rev Microbiol 42:575-606

Caillot S, Rosiau E, Laplace C, Thomasset B (2009) Influence of light intensity and selection scheme on regeneration time of transgenic flax plants. Plant Cell Rep 28:359-371

Chakrabarty R, Viswakarma N, Bhat SR, Kirti PB, Singh BD, Chopra VL (2002) *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. J Biosci 27:495-502

De Clercq J, Zambre M, Van Montagu M, Dillen W, Angenon G (2002) An optimized *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius* A. Gray. Plant Cell Rep 21:333-34

Dong J, McHughen A (1993) An improved procedure for production of transgenic flax plants using *Agrobacterium tumefaciens*. Plant Science 88:61-71

Friedt W (1990) Biotechnology in breeding of industrial oil crops: the present status and future prospects. Fat Sci Technol 90:51-55

Gamborg OL, Shyluk JP (1976) Tissue culture, protoplasts, and morphogenesis in flax. Bot Gaz 137:301-306

Green AG, Marshall DR (1984) Isolation of induced mutants in linseed (*Linum usitatissimum*) having reduced linolenic acid content. Euphytica 33:321-328

Haq IU (2004) *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum* L.) via vacuum infiltration. Plant Mol Biol Rep 22:279-288

Henzi MX, Christey MC, McNeil DL (2000) Factors that influence *Agrobacterium rhizogenes*mediated transformation of broccoli (*Brassica oleracea* L. var. italica). Plant Cell Rep 19:994-999

Hooykaas PJJ, Schilperoort RA (1992) *Agrobacterium* and plant genetic engineering. Plant Mol Biol 19:15-38

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387-405

Jordan MC, Mc Hughen A (1988) Transformed calllus does not necessarily regenerate transformed shoots. Plant Cell Rep 7:285-287

Joubert P, Beaupe`re D, Lelie`vre P, Wadouachi A, Sangwan RS, Sangwan-Norreel BS (2002) Effects of phenolic compounds on Agrobacterium vir genes and gene transfer induction. A plausible molecular mechanism of phenol binding protein activation. Plant Sci 162:733-743

Le VQ, Belles-Isles J, Dusabenyagasani M, Tremblay FM (2001) An improved procedure for production of white spruce (*Picea glauca*) transgenic plants using *Agrobacterium tumefaciens*. J Exp Bot 52:2089.2095

Leelavathi S, Sunnichan VG, Kumria R, Vijaykanth GP, Bhatnagar RK, Reddy VS (2004) A simple and rapid Agrobacterium-mediated transformation protocol for cotton (*Gossypium hirsutum* L.): Embryogenic calli as a source to generate large numbers of transgenic plants. Plant Cell Rep 22:465-470

Lopez SJ, Kumar RR, Pius PK, Muraleedharan N (2004) *Agrobacterium tumefaciens*mediated genetic transformation in tea (*Camellia sinensis* [L.] O. Kuntze). Plant Mol Biol Rep 22: 201a-201j

Lorenc-Kukula K, Amarowıcz R, Skı JO, Doermann P, Starzyckı M, Skala J, Uk MZ, Kulma A, Szopa J (2005). Pleiotropic Effect of Phenolic Compounds Content Increases in Transgenic Flax Plant. J. Agric. Food Chem. 53:3685-3692

Mahmoudian M, Yucel M, Öktem HA (2002) Transformation of lentil (*Lens culinaris* M.) cotyledonary nodes by vacuum infiltration of *Agrobacterium tumefaciens*. Plant Mol Biol Rep 20:251-257

Mathews VH, Narayanaswamy S. (1976) Phytohormone control of regeneration in cultured tissues of flax. Z. Pflanzenphysiol 80: 436-442

Millam S, Davidson D, Powell W (1992) The use of flax (*Linum usitatissimum*) as a model system for studies on organogenesis *in vitro*: the effect of different carbonhydrates. Plant Cell Tiss Org 28:163-166

Mlynarova L, Bauer M, Nap J-P, Pretova A (1994) High efficiency *Agrobacterium*-mediated gene traansfer to flax. Plant Cell Rep 13:282-285

Mondal TK, Bhattacharya A, Ahuja PS, Chand PK (2001) Transgenic tea (*Camellia sinensis* [L.] O. Kuntze cv. Kangra Jat) plants obtained by *Agrobacterium*-mediated transformation of somatic embryos. Plant Cell Rep 20:712-720

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-479

Snedecor GW, Cochran WG (1967) Statistical Methods. Iowa State University Press, Ames, IA

Somleva MN, Tomaszewski Z, Conger BV (2002) *Agrobacterium*-mediated transformation of switchgrass. Crop Sci 42:2080-2087

Spokevicius AV, Van Beveren KS, Bossinger G (2005) *Agrobacterium*-mediated in vitro transformation of wood-producing stem segments in eucalypts. Plant Cell Rep 23:617-624

Wang WC, Menon G, Hansen G (2003) Development of a novel *Agrobacterium*-mediated transformation method to recover transgenic *Brassica napus* plants. Plant Cell Rep 22:274-281

Yildiz M, Er C (2002a) The effect of sodium hypochlorite solutions on *in vitro* seedling growth and shoot regeneration of flax (*Linum usitatissimum*). Naturwissenschaften 89:259-261

Yıldız, M., Er C (2002b) Increasing the injured area on hypocotyl explants of flax (*Linum usitatissimum* L.) leads to high frequency callus-based shoot regeneration. Turk J Biol 26:95-98

Yıldız M, Ulukan H, Özbay A (2003) The effect of different medium, gelling agents and explant ages on shoot regeneration from hypocotyl explants of flax (*Linum usitatissimum* L.). 13th Biotechnology Congress. pp. 137-144, Canakkale, Turkey

Yildiz M, Özgen M (2004) The effect of a submersion pretreatment on in vitro explant growth and shoot regeneration from hypocotyls of flax (*Linum usitatissimum*). Plant Cell Tiss Org 77:111-115

Zupan J, Muth TR, Draper O, Zambryski P (2000) The transfer of DNA from Agrobacterium tumefaciens into plants: a feast of fundamental insights. Plant J 23:11-28