Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Annals of Biological Research, 2012, 3 (5):2419-2427
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0976-1233
CODEN (USA): ABRNBW

Optimizing regeneration and reporter gene (*gus*) transformation of alfalfa (*Medicago sativa*)

Sobhanian, Neda[1,2], Habashy, Ali Akbar[1], Farshad Far[1], Ezatollah[2] and Tohid Far, Masoud[1]

[1]*Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj, Iran*
[2]*Department of crop production and plant breeding, International university of Razi, Kermanshah, Iran*

ABSTRACT

Medicago sativa is a pasture and a model plant for studying the molecular genetic. The objectives of this work were optimizing in vitro regeneration and Agrobacterium-mediated transformation of Iranian alfalfa cultivars (Gharghologh, Malek kanedi and Kozrah). For induction of multiple shoots, different explants (meristem and cotyledon) and various hormone concentrations in three varieties (Gharghologh, Malek kanedi and Kouzrah) were studied. Results showed that MS culture medium supplemented with 0.05 mgl^{-1} NAA + 1.5 mgl^{-1} TDZ was the most effective regeneration medium. To study gus gene transformation, Agrobacterium tumefaciens strains; LBA4404 and AGL1 containing pBI121 plasmid were used. The plasmid containing the neomycin phosphotransferas gene, as the selectable marker under control of the nopaline synthesis promotor and the β-glucuronidase gene was used in combination with the reporter gene under control of the CaMV35s promoter. Various factors including type of explants (meristem and cotyledon), preparation method of bacteria suspension (I & II) and time of cocultivation were investigated. Meristem and cotyledonary explants were inoculated with bacteria suspension I and II andcocultivated on the regeneration medium. The highest percentage of Kanamycin-resistant shoot (50%) and the most transformation frequencies of plantlets were obtained from AGL1 strain. Expression of gus in leaf and stem transgenic plants derived from suspension II was the result of successful transformation by gus gene. PCR analysis of putative transgenic plants showed the integration of at least one copy of gus and nptII genes into the alfalfa genome.

Key words: *Agrobacterium*, alfalfa, *gus*, regeneration, transformation and genetic engineering.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the word's most important forage legume and plays an important role in livestock feeding. This plant is a pasture legume and a model plant for studying the molecular genetics. Its contribution to the quality of pasture underpinning animal production is known through fixation of atmospheric nitrogen and its high protein content. Generally, cultivars of most forage legumes do not show high levels of plant regeneration [12]. The ability of alfalfa culture to regenerate plants is under genetic control and occurs with a frequency of 1 to 10 percent in most cultivars [5]. The variable response within a cultivar reflects the facts that alfalfa is an open-pollinated species and each cultivar is actually a heterogeneous mixture of genotypes [3]. Molecular breeding of these forage legumes can be enhanced through the application of genetic engineering. Strategies for the regeneration of pasture legumes include organogenesis and somatic embryogenesis using stolons, immature embryos, embryo cotyledons, callus, cell suspension cultures and protoplasts [15]. The media containing specific hormones and selection agents that supported proliferation of transformed cells, production of somatic embryos and regeneration into whole plants are also very important. There are two reports that state the important aspects of the existing method related to the

use of cytokinin gene-free binary vectors [2] and selectable marker genes [19]. One of the limiting factors in commercial applicability of the current methods is that they can only be applied to specific genotypes that possess little commercial value [21].

Since the first successful *Agrobacterium*-mediated transformation of alfalfa [22], [6]. there have been many published reports on different aspects of the procedure. Most of them showed that the efficiency of transformation depended on the genotype (Mariotti *et al*., 1984) and bacterial strain [7]. The efficiency of transformation also depends on the ability of selection procedure and the frequency of shoot regeneration [16].

An intriguing alternative method that was developed for the related plant species (*Medicago truncatula*) overcomes the complexity and genotype-specificity of hormone and selection-based transformation methods. The immersion of seedling or flowers in suspension containing *Agrobacterium*, allows plants to produce seed, and then selects progeny plants containing a transmitted T-DNA [24].

Hoffmann *et al*., (1997), described a superior *in vitro* regeneration and transformation method for the line R108-1. They reported 60-70% production of pre-embryogenic callus and up to 50% of the regenerated embryos into complete plantlets. The transformation efficiency of R108-1 was independent of the *Agrobacterium* strain [10]. The ability to transform alfalfa and to move desirable genes into the elite lines has opened new doors for alfalfa breeding. The genes used for transgenic research has focused mainly on traits dealing with agronomic performance, forage quality and producing industrial pharmaceutical proteins. Genetic engineering approaches require a good transformation system, desirable target genes and appropriate promoters for fine-tuning the expression pattern of the transgenes [26]. In the present investigation attempts were made to optimize *in vitro* regeneration and *Agrobacterium*-mediated transformation of Iranian alfalfa cultivars (Ghargholugh, Malek kanedi and Kozrah).

MATERIALS AND METHODS

2.1. Plant material and tissue culture media

Seeds of Iranian alfalfa cultivars (Ghargholugh, Malek kanedi and Kozrah) were obtained from seed and plant improvement institute of Iran. Mature seeds were immersed in 70% ethanol for 1 minute then were surface sterilized in 1.5% sodium hypochlorite. They were rinsed in sterile distilled water and imbibed overnight at 4ºC for two days. The media used in this study are listed in Table 1 and 2. Collected Data were statistically analyzed using factorial complete randomized design with three replications and the means were separated using Duncan's multiple range tests.

Root inducing medium contained MS media supplemented with one of the following auxins (0.5 mgl^{-1} IBA, 0.3 mgl^{-1} IBA, 0.2 mgl^{-1} IAA or 0.1 mgl^{-1} IAA).

The pH of all media was adjusted to 5.7 and 30 gr l^{-1} of sucrose and 0.8% plant agar for solidification were added before autoclaving.

Table 1: Media used for optimization of regeneration

Medium	Preparation Method
Medium A	Media MS + 0.1mgl^{-1} IAA+ 0.5 mgl^{-1} Zea
Medium B	Media MS + 0.2 mgl^{-1} IAA+ 1.5 mgl^{-1} BAP
Medium RMII	Media MS + 0.05 mgl^{-1} NAA+ 1 mgl^{-1} TDZ

Table 2: Media used for induction of multiple shoots

Medium	Preparation Method
Medium RMI	Media MS + 0.05 mgl^{-1} NAA+ 0.5 mgl^{-1} TDZ
Medium RMII	Media MS + 0.05 mgl^{-1} NAA+ 1 mgl^{-1} TDZ
Medium RMIII	Media MS + 0.05 mgl^{-1} NAA+ 1.5 mgl^{-1} TDZ

2.2. *Agrobacterium tumefaciens* strains and vectors for transformation

Agrobacterium strains AGL1 [13] and LB4404 were used. Bacterial cultures were selected with 50 mgl^{-1} kanamycin and 75 mgl^{-1} rifampicilin containing pBI121 (Fig 1).

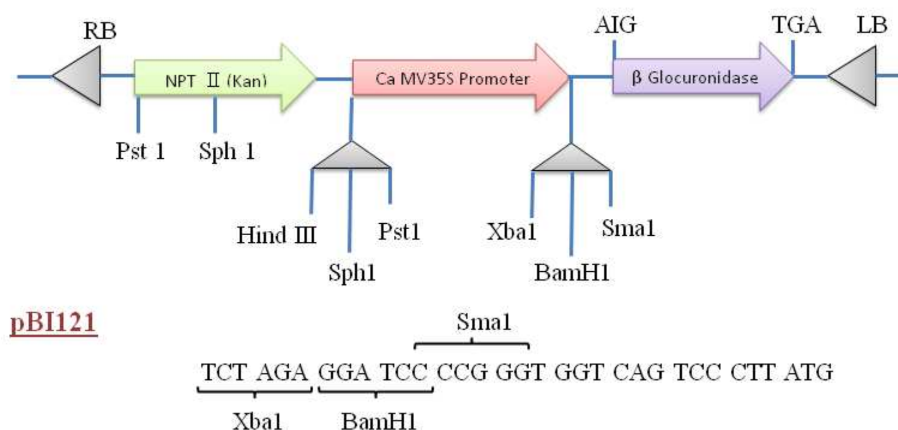


Fig 1) Plasmid map of the T-DNA carrying the chimeric *nptII* and *gus* gene

Agrobacterium suspension was obtained by inoculating 20ml of LB liquid medium (Sigma, St. Louis) containing the appropriate antibiotics at 28 ºC under constant rotation at 250 rpm incubated overnight. 200 µM of acetosyringone (AS) was added to the cultures four hours prior to infection, (suspension I). The cells were precipitated by centrifugation for 2 min at 2000 rpm. The bacterial pellet was dispensed in 15 ml of MS medium, (suspension II) to an OD_{600} of 0.6-0.7 (Table 3). Starter cultures were inoculated into 25 ml of LB medium supplemented with selective agents as before or explants were dipped in *Agrobacterium* suspension cells grown to the late log phase (OD_{600} =0.4) and incubated for the next three treatment (24, 48 and 72h).

Various factors including type of explants (meristem and cotyledon), preparation method of bacteria suspension and time of cocultivation were evaluated.

Table 3: Preparation Method of Bacteria Suspension

Suspension	Preparation Method
Suspension I:	Bacterial Colonie +L-brouth (LB) + Bacterial pellet +Liquid MS medium
Suspension II:	Bacterial Colonie +L-brouth (LB) +1ml grow Bacteria +5ml L-Brouth (LB)

2.3. Isolation and preparation of shoot apex and cotyledonary explants

Shoot apices were isolated from 3-5 day old seedlings with the aid of a dissecting stereomicroscope as described by Zapata *et al*., 1999 [27]. Each cotyledon was removed by pushing down on it until it snapped off to expose shoot apex. Shoot apices were excised from roots by cutting at the base of the apex. The unexpanded and primordial leaves were left in place to supply hormones and other growth factors. Shoot apices of two cultivars were pre-cultured for 2 days on MS medium supplemented with TDZ (1.5 mgl^{-1}) and NAA (0.05 mgl^{-1}), 30 grl^{-1} of sucrose and 0.8% plant agar.

After two days, isolated meristems were inoculated with 5 drops of *Agrobacterium tumefaciens* suspension. The plates were left open in a transfer hood until the drop had dried. The plates containing the shoot apices were sealed and incubated for 24, 48 and 72 hours in the dark at room temperature (25±2°C). Isolated cotyledonary explants were immersed in bacterial culture ($OD_{300-600}$ ~0.35) for 30 min at room temperature

The explants were transferred to media containing 50 mgl^{-1} kanamycin and 200 mgl^{-1} cefotaxime for two weeks. Explants were grown at 26ºC and 16h light/ 8h dark photoperiod under fluorescent lights intensify of 40- 50 µE m^{-2} s^{-1}.

2.4. Polymerase chain reaction analysis

Polymerase chain reaction (PCR) was carried out using specific primer pairs to amplify *nptII*, and *gus* transgenes from T0 (first generation) of transgenic alfalfa plants. Genomic DNA was extracted and purified from immature leaves following the protocol of Li *et al*., 2001[14]. PCR was performed in a total volume of 25 µl reaction mixtures consisting of 10x reaction buffer, 15ng DNA template, 15mM MgCl2, 10mM dNTPs, 60 ng of each primer and 0.5 unit Tag DNA polymerase (Cinagen Co., Tehran, Iran). PCR was carried out in a Touch gene (Model FTGO5TD) thermal cycler using the following conditions: initial denaturation at 94 ºC for 2 min followed by 35 cycles of denaturing at 94 ºC for 1 min, annealing at 55 ºC for *nptII* and annealing at 62 ºC for *gus* gene for 1 min, extension at 72 ºC for 3 min and final extension at 72 ºC for 5 min. The sequences of the primer pairs used in this assay were

as follows: *nptII* 1: 5′ -GAA CAA GAT GGA TTG CAC GC -3′ *nptII* 2: 5′ -GAA GAA CTC GTC AAG AAG GC -3′ and *gus* 1: 5′ -GGT GGG AAA GCG CGT TAC AAG -3′ *gus* 2: 5′ -TGG ATT CCG GCA TAG TTA AA -3′.

2.5. β-Glucuronidase (*GUS*) histochemical assay

Histochemical colorimetric assays were performed to localize *gus* gene activity [11]. The *gus* gene substrate buffer consisted of X-gluc. Staining was performed overnight at 37 °C and tissues were immersed in 70% ethanol to remove the chlorophyll. Stable *gus* gene activity was determined five weeks after *Agrobacterium* infection. β-Glucuronidase (*gus*) gene expression was assayed in tissue (T0) transgenic alfalfa.

RESULTS AND DISCUSSION

3.1. Plant regeneration

Plants were regenerated from excised meristem and cotyledonary explants obtained from imbibed mature seeds of Iranian cultivars (Gharghologh, malek kanedi and Kouzrah). The essential step in the regeneration process, including proliferation of multiple shoots at the wound site of the cotyledonary and meristem largely through direct organogenesis are shown for kouzrah in Fig 2. Significant differences were observed between treatment A: (Zeatin 0.5 mgl^{-1} +IAA 0.1 mgl^{-1}), treatment RMII: (NAA 0.05 mgl^{-1}+TDZ 1 mgl^{-1}) and treatment B: (IAA 0.2 mgl^{-1}+BAP 1.5 mgl^{-1}). Explants in treatments RMII and B were swollen with translucent structures on callus. The analysis showed that variance between media were significant but means comparison showed that treatment RMII and B produced more multiple shoots in comparison to treatment A (Table 4).

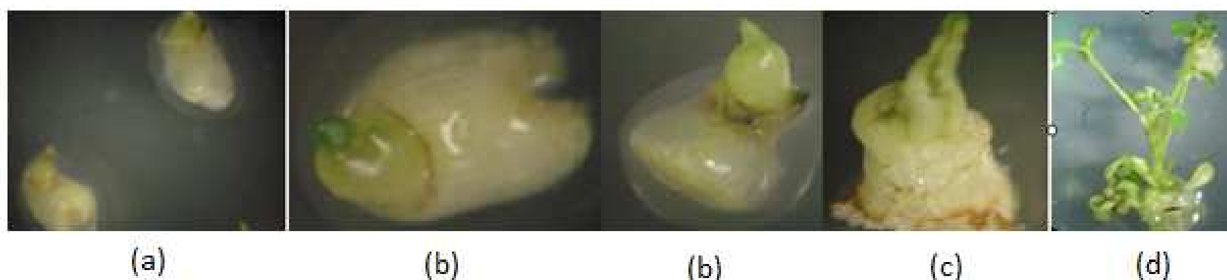


Fig 2: Plant regeneration from meristem explants a) meristem explant derived from imbibed mature subterranean alfalfa seed. b) meristem explant after 1 week. c) meristem explant after 2 week. d) regenerated shoots from meristem cultured on RMII medium.

In the present research, the combination of TDZ and NAA in the RMII medium and then BAP and NAA in the B medium was used as the appropriate treatment for induction of multiple shoots in Malek kandi then Gharghologh and Kouzrah respectively. It was also observed that induction of adventitious shoots from meristems were faster than that of cotyledon explants.

The findings of polisetty *et al*., 1997 confirmed the positive effect of BAP on induction of multiple shoots in alfalfa[18].

Table 4: Mean squares for the effect of different treatment on induction multiple shoots and vitrification

		Vitrification		Multiple shoots	
Source of variation	df	Mean squares (MS)	F	Mean squares (MS)	F
Explant type	1	4181.760	27.7652**	77.520	2.0932
variety	2	555.189	3.6862*	113.254	3.0580*
Explant *variety	2	855.104	5.6776**	26.962	0.7280
media	2	902.811	5.9943**	713.464	19.2646**
media* Explant	2	356.071	2.3642	32.141	0.8679
media * variety	4	32.579	0.2163	8.314	0.2245
Media * Explant *variety	4	56.555	0.3755	50.164	1.3545
Error	36	150.61		37.535	
total	53				

*significant at the 0.05 probability level **
*significant at the 0.01 probability level ***

Ding *et al*., 2003 used a combination of (NAA & TDZ) as the most efficient combination for induction of multiple shoots[8]. Adventitious shoot regeneration from hypocotyls, cotyledon, and stem and petiole explants of *A. cicer* was reported by Uranbey et al, 2003 using different concentration and combinations of BAP and NAA. However,

0.25, 0.5 and 1.0 mg/l cytokinin source for the first time were tested to induce shoot regeneration of *A.cicer*. TDZ gave reasonable shoot multiplication when compared to our previous study.

Various cultivars have different regeneration ability via multiple shoots. Perhaps, the main reason of this could be the response of various genotypes to different plant growth regulators.

In this study, treatment A (medium containing TDZ) produced shoots of an earlier stage compared with treatments B and RMII. Significant differences were observed between RMI treatment: (NAA 0.05 mgl^{-1}+TDZ 0.5 mgl^{-1}), RMII treatment: (NAA 0.05 mgl^{-1}+TDZ 1 mgl^{-1}) and RMIII treatment: (NAA 0.05 mgl^{-1}+TDZ 1.5 mgl^{-1}) at induction of multiple shoot stage.

Table 5: Mean squares for the effect of different TDZ on induction multiple shoots

Source of variation	df	Mean squares (MS)	F
media	2	363.576	3.3889*
variety	2	40.237	0.3751
variety* media	4	77.974	0.7268
Explant	1	7.935	0.0740
Explant *media	2	43.891	0.4091
Explant * variety	2	15.585	0.1453
Explant* media* variety	4	130.306	1.2193
Error	36	107.283	
total	53		

*Significant at the 0.05 probability level **

The results showed that RMIII treatment produced more multiple shoots per explant in comparison with RMI and RMII treatments (Table 5).

Explants (meristem and cotyledonary), in the three treatments were swollen with translucent structures. The color of tissues changed to yellow, after 3 weeks of culture initiation (Fig 3)

Fig 3: Formation of multiple shoots from meristem explant

Germination and plantlet formation

Explants were transferred to the same medium for germination and plantletformation. The observation showed that germination and rooting occurred 8 and 10 weeks, respectively.

Vitrification

The results indicated that there was a significant difference between different treatments (A, RMII and B) on the rate of vitrification. The result showed that treatment RMII produced 23.7% vitrification whereas treatment B, produced 10.5% vitrification per explant. Treatment RMII caused vitrification after 8 weeks of culture initiation (Table 1).

Rooting plantlet

The use of auxins (IBA and IAA) in MS medium induced root formation in the Malek kanedi, Gharghologh and Kouzrah cultivars. The percentage of rooting was 90% in the medium containing 0.1 mgl^{-1} IAA whereas it was 22.5% , 40% and 5% in the media supplemented with 0.2 mgl^{-1} IAA , 0.3 mgl^{-1} IBA and whereas 0.5 mgl^{-1} IBA respectively.

These results disagree with the report by Amutha *et al*; 2008 who stated well-developed shoots when transferred to rooting medium containing IBA induced higher frequency of roots than when IAA was used.

Table 6: Effect of different auxin hormones on percentage of root induction

hormone	percentage of root induction
0.5mgl^{-1} IBA	5%
0.3mgl^{-1} IBA	40%
0.2 mgl^{-1} IAA	22.5%
0.1 mgl^{-1} IAA	90%

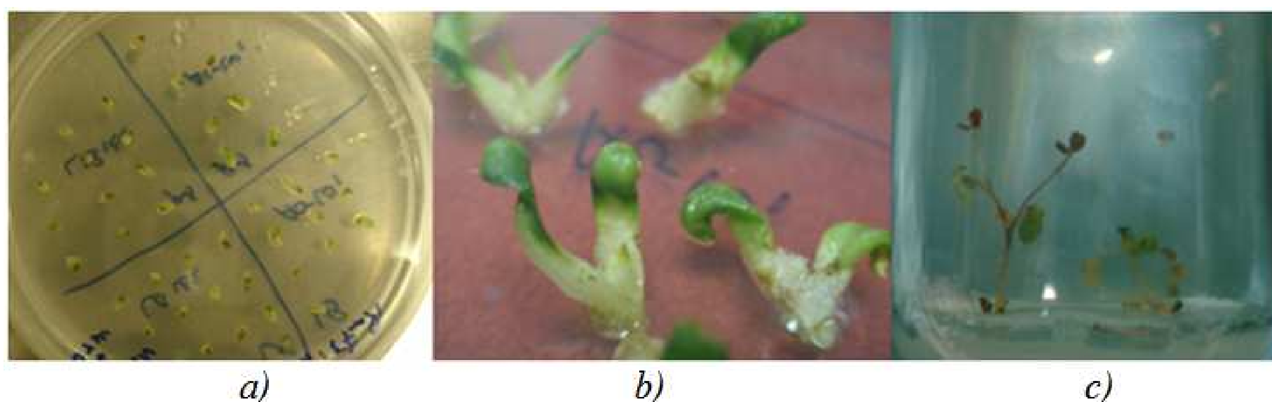
a) b)

Fig 4: a) Formation of multiple shoots on the RMII media b) formation of root

Analogous regeneration processes applied to all cultivars tested. Independent fully developed shoots were subcultured on fresh media for 2-3 weeks. *In vitro* root development generally occurred within another 2-3 weeks before plantlets were transferred to soil. This regeneration system was successfully applied to the three cultivars (Fig 4).

3.2. *Agrobacterium*- mediated transformation and molecular analysis of transgenic plants

Four meristem explants with 72h infection and two cotyledonary explants with 48h infection were suspension I. *Gus* assay was observed only in one plants from meristem explant. In five plants tested no detectable constitutive *gus* expression was observed. The response of the suspension I was compared to the response of suspension II. Six plants from meristem explants with 72h infection and three plants from cotyledonary explants with 48h infection were similarly regenerated from suspension II (Fig 5).



a) b) c)

Fig 5: a) Cotyledonary explants from isolated cotyledon pairs arranged in grid-pattern after 14 days of co-cultivation with *A. tumefaciens* on RMIII medium. b) Meristem explants after 6 days of co-cultivation with *A. tumefaciens* on RMIII medium. c) kanamycin-resistant shoots derived from transformed meristem explants growing on RMIII medium supplemented with 50 mg/l of kanamycin.

Gus activity was observed in leaf and stem tissue. Figure 6 shows that results from *gus* assay. The expression of *gus* gene was confirmed in three plants. The plants showed positive results by PCR for the presence of the transgene.

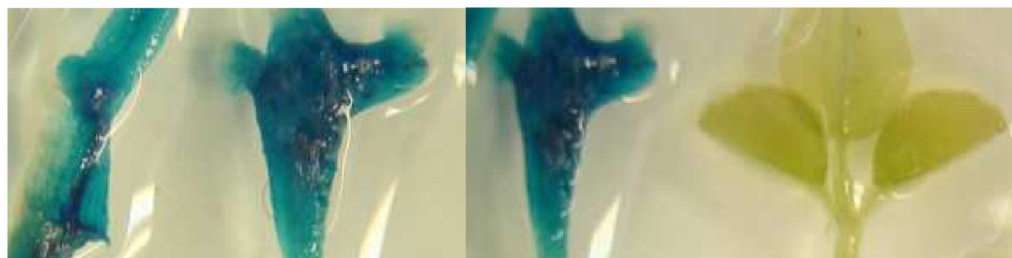


Fig 6: Expression of *gus* gene in leaf and stem

Decapitated meristem selected as the best explant for gene transformation and co-cultivation for 72 hour in suspension II showed the best result. On the basis of *gus* gene expression and analysis 2 putative transformed shoot obtained from 450 meristem explant and 1 putative transformed shoot obtained from 270 cotyledon explant. Frequency of transformation for meristem and cotyledon were 0.44% and 0.37%, respectively. The reason for the low frequency of Gus-positive plants may be that even in very young leaves, the activity of the cell division cycle gene promoters was already too low to be detected by the histochemical method [23].

PCR amplification has been undertaken to screen for the presence of *gus* gene in the transformation process with AGL1 and LBA4404. LBA4404 has been the most frequently used *Agrobacterium* strain in transformation of *M. sativa* [9] and [22]. In the investigation described here, the transformation frequency obtained with AGL1 was higher than with LBA4404. Leaf samples were collected from putative transformants immediately after regeneration. of the plants that showed positive results by PCR analysis (presence of an amplified product of~400 bp) also survived on RMIII whereas plants scored as negative by PCR analysis were unable to survive, thus confirming the practical authenticity of PCR and the kanamycin selection (Fig 7).

Shao *et al*., (2000) and Desgagnes, et al., (1995) reported that kanamycin resistance has proved to be a powerful tool for transgenicity screening in alfalfa [23],[7]. In some cases, kanamycin affected callus development and regeneration of alfalfa plants [7] ,[17]. Trinh *et al*., (1998) reported that kanamycin selection is not efficient in *M. sativa* ssp. 80% of regenerated plants were transformed using a construct containing hygromycin, and only 2% using the same construct containing kanamycin resistance. Hygromycin apparently is a more reliable selective agent than kanamycin [25].

PCR amplification of the *nptII* gene in genomic DNA samples of plasmid pBI121 and alfalfa transformants indicated a successful transformation event (Fig 8).

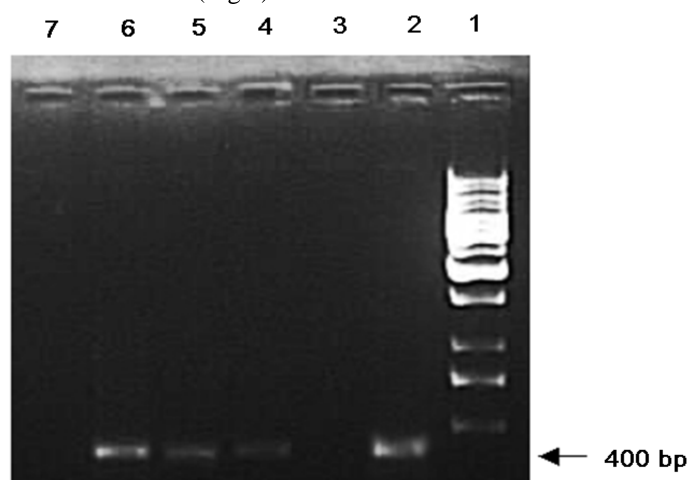


Fig 7: Agarose gel electrophoresis of PCR amplification of a 400 bp fragment of the *gus* gene from transformed plant genomic DNA. Upper bands in lanes 4, 5 and 6 refer to *gus* transformed plants. Lane 2 refers to partial DNA digested of pBI121 plasmid. Lane 3 used as a negative control and lane 7 for non-transgenic alfalfa.

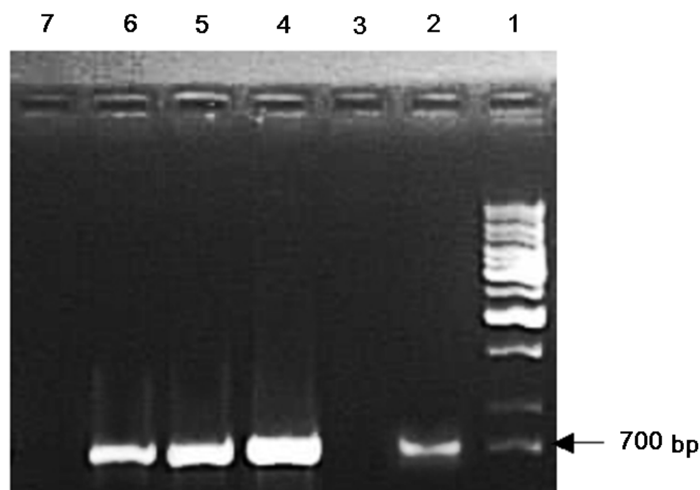


Fig 8: Agarose gel electrophoresis of PCR amplification of a 702 bp fragment of the *nptII* gene from transformed plant genomic DNA. Upper bands in lanes2, 5 and 6 refer to *nptII* transformed plants. Lane 4 refers to partial DNA digested of pBI121 plasmid. Lane 3 used as a negative control and lane 7 PCR analyses for non-transgenic alfalfa

Genetic engineering is used in breeding new varieties of alfalfa. Transgenic traits of interest include those that improve efficiency of forage or seed production. Yield enhancement, herbicide tolerance, insect resistance and stress tolerance are examples of transgenic "input" traits. Output traits are those that affect the quality of the crop product.

The major shortcoming of protocols for regeneration and transformation of species alfalfa published to date is the low rate of regeneration frequency, lack of reproducibility and genotype dependence [1], [20]. Using cotyledonary and meristem explants freshly dissected from imbibed mature seed, we were able to overcome this major drawback of earlier protocols.

The meristem explants provide several advantages over conventional methods. First, it limited the time, materials and resources required for complex *in vitro* manipulations while also eliminating the risk of somaclonal variation that is associated with both hormone treatment and callus formation [4]. Second, this explant substantially reduces the amount of time from transformation to plant set. Third, the plant transformation method was successfully applied to a commercial variety whereas the conventional method requires very specific highly regenerable genotypes such as RegenSY that have *little commercial value [21]. Fourth*, we believe that the meristem explants are not anticipated for issue transformation patents and therefore may provide a new path for commercialization. In conclusion present investigation showed that meristem explants are suitable for *Agrobacterium*-mediated transformation of alfalfa. Antibiotic selection based on the use of the marker gene Kanamycin-resistant was successfully employed in alfalfa transformation.

REFERENCES

[1] Bagga S, Sutton D, Kemp J.D, Sengupta-Gopalan C. *Plant Mol. Biol* **1992**; 19: 951-958.
[2] Barton K.A, Binns A.N, Chilton M.M, Matzke AJM. Regeneration of plants containing genetically engineered T-DNA, United States patent 6051757 2000.
[3] Cerasela P, Nedelea G. *Romanian Biotechnological Letters* **2009**; Vol:14. 6:4882-4886.
[4] Cheng X.Y, M.W Gao, Liang Z.Q, Liu G.Z, Hu T.C. **1992**. *Euphytica* 64: 1-10.
[5] Crea F,. Bellucci M,. Damiani F, Arcioni S. *Euphytica* **1995**; 151-155.
[6] Deak M, Kiss G.B, Koncz C, Dudits D. *Plant Cell Rep* **1986**; 5:97-100.
[7] Desgagnes, R, Laberge S, Allard G, Khoudi H, Castonguaz Z, Lapointe J, Michaud R, Vezina L. *Plant Cell, Tissue and Organ Culture* **1995**; 42: 129-140.
[8] Ding, Y.L, Guillermo A.H, Ludlow E, Drayton M, Lin Y.H, Nage J l, Dupal M, Zhao G, Pallaghy C, Kalla R, Emmerling M, Spangenberg G. *Plant Science* **2003**; 165:1419-1427.
[9] Hill K, Jarvis-Eagan N, Halk E, Krahn K, Liao L, Mathewson R, Merlo D, Nelson S, Rashka K, Loesch-Fries L. *Bio/Tech* **1991**; 9: 373-378.
[10] Hoffmann B, Trinh T.H, Leung J, Kondorosi A, Kondorosi E. *Mol Plant Micobe Interact* **1997**; 10: 307-315.
[11] Jefferson R.A, Kavanagh A.T, Bevan W.M. *EMBO Journal* **1987**; 6: 3901-3907.
[12] Khan M.R.I, Heath L.C, Spencer D, Higgins T.J.V. *Plant Physiol* **1994**; 105: 81-88.
[13] Lazo G.R, Stein P.A, Ludwig R.A. *Bio/Technology* **1991**; 9:963-967.
[14] Li H, Luo J, Hemphili J.K, Wang J.T, Gould J.H. *Plant Molecular Biology Reporter*; **2001**. 19:1-5.

[15] McKersie B.D, Brown D.C.W. Biotechnology and the improvement of forage legumes, CAB International, Wallingford, Oxon, UK **1997**; P.444.
[16] Ninkovic S, Miljus-Djukic J, Vinterhalter B, Neskovic M. *Acta biologica cracoviensia series botanica* **2004**; 46:139-143.
[17] Pezzotti M, Pupilli F, Damiani F, Arcioni S. *Plant Breeding* **1991**; 106: 39-46.
[18] Polisetty R, Paul V, Deveshwar J.J, Khetarpal S, Suresh K, Chandra R. *Plant Cell Rep*. **1997**; 16(8):565-571.
[19] Rogers S.G, Fraley R.T. Chimeric genes suitable for expression in plant cells, United States patent 6174724 2001.
[20] Samac D. *Plant Cell Tiss. Org* **1995**; Cult. 43: 271-277.
[21] Samac D.A, Austin-Phillips S. Alfalfa (*Medicago sativa* L.), In: Wang K (eds) Methods mol boil *Agrobacterium* protocols, vol. 343, 2nd edn. Humana Press, Totowa, NJ 2006; pp: 301-311.
[22] Shahin E, Spielmann A, Sukhapinda K, Simpson R, Yashar M. *Crop Sci* **1986**; 26: 1235-1239.
[23] Shao C-Y, Russinova E, Iantcheva A, Atanassov A, McCormac A, Chen D-F, Elliott M.C, Slator A. *Plant Growth Regulation* **2000**; 31: 155-166.
[24] Tried A.T, Burleigh SH, Kardailsky I.V, Maldonado-Mendoza I.E Versaw W.K, Blaylock L.A, Shin H, Chiou T.J, Katagi H, Dewbre G.R, Weigel D, Harrison M.J. *Plant J* **2000**; 22: 531-541.
[25] Trinh TH, Ratet P, Kondorsi E, Durand P, Kamate K, Bauer P, Kondorsi A. *Plant Cell Reports* **1998**; 17: 345-355.
[26] White D.W.R, Greenwood D. *Plant Mol Biol* **1987**; 8: 461-469.
[27] Zapata C, Park S.H, El-Zik K.M, Smith R.H. *Theor Appl Genet* **1999**; 98:252-256