The Bioscan 11(3): 1637-1639, 2016 (Supplement on Agronomy)
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

AGROBACTERIUM TUMEFACIENS MEDIATED GENETIC TRANSFORMATION IN GREWIA OPTIVA

S. K. JHA[1*] R. N. SEHGAL[1] AND KAMLESH KANWAR[2]
[1]Department of Tree Improvement and Genetic Resources,
College of Forestry, UHF, Solan - 173 230, H.P., INDIA
[2]Department of Biotechnology, College of Horticulture, UHF, Solan - 173 230, H.P., INDIA
e-mail : sumanjha@nau.in

KEYWORDS
Grewia optiva
Genetic transformation
Agrobacterium
GUS

Received on :
16.01.2016

Accepted on :
26.07.2016

***Corresponding author**

ABSTRACT
An *Agrobacterium tumefaciens*-mediated transformation system for *Grewia optiva* was developed using callus and embryogenic clumps as explants. The explants were inoculated with a disarmed *A. tumefaciens* harboring the binary vector pBI121containing β-glucuronidase (GUS) and neomycin phosphotransferase (NPT II). Kanamycin sensitivity test of explants carried out at various concentrations of Kanamycin (10, 20, 30, 40, 50 mg l^{-1}). On the basis of sensitivity test, 50 mg l^{-1} kanamycin concentration further used for selection of putatively transformed callus however 30 mgl^{-1} kanamycin concentration for the embryogenic clumps. Various factors were found to influence the transfer-DNA delivery efficiency, such as preincubation duration and explant tissue co-cultivation duration. Maximum transformation frequency (6.68%) was observed for 48 hours pre-incubation followed by 48 hours co-cultivation for the callus. However, in the case of embryogenic clumps, maximum transformation frequency as observed in 48 hours co-cultivation irrespective of pre-incubation duration. Callus and somatic embryo showed positive GUS expression with X-Gluc staining.

INTRODUCTION

Grewia optiva Drummond belonging to family Tiliaceae is one of the most important fodder tree of north eastern and central Himalayas (Brandis *et al*., 1972). It provides green leaf fodder in lean periods especially during the winter season, when there is no other source of green fodder available. The leaves are highly nutritious and fairly rich in protein and mineral nutrients (Khosla *et al*., 1992; Negi, 1977). Its fodder can play an important role in improving the nutrition of livestock in the hills where the poverty of the farmers neither allows them to purchase concentrated feeds nor their small holdings allow them to resort to cultivation of nutritious leguminous fodder/ forage crops (Sharma *et al*., 2000; Swamy *et al*., 2002).

The long generation period of the tree is always a constraint for tree improver worldwide. Biotechnology provides tools to develop plants with desired traits which had been difficult to achieve using conventional breeding techniques (Bhat *et al*., 2013; Mishra *et al*., 2010). Genetic transformation is a technique enabling breeders to incorporate the desired gene quickly.Woody species tend to be difficult and often inefficiently transformed due to lack of proper regeneration system (Stevens and Pijut, 2014). Therefore, in present study different pathways are used to see the transformation frequency. Every regeneration system has its own advantages and disadvantages (Agarwal and Kanwar, 2007). Genetic transformation involving a callus phase may show increased shoot bud regeneration potential but result in chimeric shoots thus making selection of transformed tissues more extensive and difficult. Secondary somatic embryogenesis in particularly advantageous as they develop from a single cell on the surface of individual embryos (Agarwal and Kanwar, 2007). These cells can be infected by *Agrobacterium* and gave rise to transformed embryos that can, in turn, germinated to give transformed plants. Hence, the present investigation was carried out to observe the factor influencing efficient genetic transformation in *G. optiva* using different regeneration pathways.

MATERIALS AND METHODS

The genetic transformation experiments were performed using the already established regeneration systems through cotyledon callus culture and somatic embryogenesis. Disarmed Agrobacterium tumefaciens strain LBA4404 carrying a binary vector pBI121 (Jefferson *et al*., 1987) was used. The binary vector contains marker gene -glucuronidase (GUS) and neomycin phosphotransferase (NPT II), which provides kanamycin resistance. The coding sequence of GUS was connected to 35S promoter of cauliflower mosaic virus (CaMV 35S) and terminator from nopaline synthase gene (NOS). The coding sequence of NPT II was connected to nopaline synthase gene promoter and terminator sequences. The *Agrobactrium tumefaciens* strain LBA4404 was obtained from the Head, Department of Biotechnology, Dr. Y.S. Parmar University of Horticulture and Forestry, Solan. *Agrobacterium* strain maintained by subculturing the bacterial colonies on YMB medium containing filter sterilized 50 mgl^{-1} kanamycin (Alembic Chemicals Ltd. Vadodara). To achieve proper growth of Agrobacterium, the subcultured plates were incubated at

28°C under dark conditions for 3-4 days. For storage, the cultures were kept at low temperature (4 ± 2°C). Fresh cultures of *Agrobacterium* strains were prepared by inoculating a loopful of culture into 100 ml liquid YMB medium containing 50 mg l^{-1} kanamycin sulphate (filter sterilized). The cultures were incubated overnight at 28°C over an orbital shaker at 130 rpm.The dilution experiment was carried out by technique of serial dilution and optical density about 10^8 cells per ml (OD 0.520 at 540 nm) was used for further genetic transformation studied in all experiments.

To check the sensitivity of cultured tissues to kanamycin sulphate, different concentration of kanamycin (10, 20, 30, 40, 50 mg l^{-1}) were added to pre-sterilized molten MS medium by filter sterilization through 0.22 *i*m pore size membrane filter and the medium was poured in the petriplates. Pre-weighed explants (cotyledon for callus and embryogenic callus for somatic embryo) were inoculated on kanamycin supplemented medium (3.00 mg l^{-1} NAA + 1.00 mg l^{-1} BAP for callus and 3.00 mg 1^{-1} 2,4-D and 0.50 mg 1^{-1} BAP + 6.00 per cent sucrose for embryogenic clump) and observations were taken for fresh weight increase after 28 days.

For co-cultivation experiment, the fresh culture of *Agrobacterium* was centrifuged at 5000 rpm for 10 minutes, and the supernatant was discarded. The pellet was then re-suspended in 1-2 ml liquid MS medium to get a concentration of 108 cells per ml. This bacterial suspension and pre-conditioned explants were used for co-cultivation experiment. The co-cultivated explants were incubated at 25 ± 2 ºC under dark conditions for 24, 48 and 72 hours. Cotyledon and embryogenic clumps were incubated on medium supplemented with specific growth regulators according to the regeneration system. The cultures were preconditioned for 24, 48 and 72 hours followed by co-cultivation with *A. tumefaciens* strain for different durations (24, 48 and 72 hours). After co-cultivation, he explants were transferred to selective medium medium containing 50 mgl^{-1} kanamycin sulphate and 500 mg l^{-1} cefotaxime. To confirm the presence of transgene β-glucuronidase, the putative tissues were analyzed histochemically (Jefferson et al., 1987).

Using completely randomized design the data was subjected to analysis of variance (Gomez and Gomez, 1984). To improve normality an arc sine transformation was performed on percent data and counted data was transformed by taking the square root.

RESULTS AND DISCUSSION

This is the preliminary test to assay the resistance of non-transformed tissue to kanamycin. Typically the optimum concentration of selective agent should be one that prevents without regeneration without being toxic to the target explants (Agarwal and Kanwar, 2007; Agarwal *et al*., 2004; Han *et al*., 2000; Kanwar *et al*., 2003; Satyavathi *et al*., 2002). On control medium (free from kanamycin) for callus induction, the cotyledon explants were healthy and produced with the maximum increase in fresh weight after four weeks. With the increase in kanamycin concentration, the relative increase in fresh weight of callus decreased. At 50 mg/l kanamycin, the callus started dying. So the 50 mg l^{-1} kanamycin concentration was further used for selection of putatively transformed callus. However, up to 30 mgl^{-1} kanamycin concentration, the embryogenic clumps survived. At higher concentration than 30 mg l^{-1} the embryogenic clump started to die (Fig-1). Kanamycin concentration of 50 mg l^{-1} was found the optimum for selection of cotyledons producing callus and 30 mg l^{-1} for the embryogenic callus. The concentration above this resulted in necrosis of both the explants with negligible increase in the explant weight. A similar result was been reported by (Agarwal and Kanwar 2007; Agarwal *et al*., 2004; Bardar *et al*., 2016; Kanwar *et al*., 2003; Sivanandhan *et al*., 2016; Wang *et al*., 2016).

Table 1 showed that the maximum transformation frequency (6.68%) was observed for 48 hours pre-incubation followed by 48 hours co-cultivation.How ever, in the case of

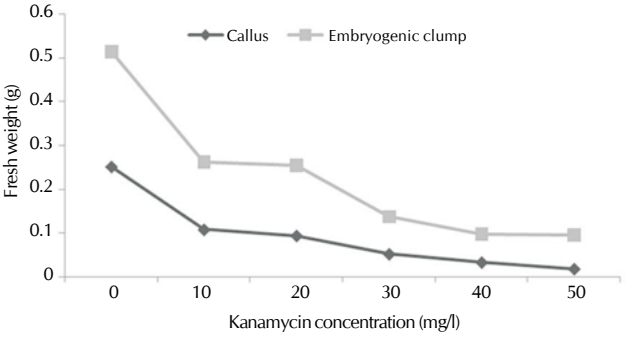

Figure 1: Effect of kanamycin concentration on fresh weight (g) of cotyledon producing callus and embrogenic clump after 28 days of culture

Table 1 : Effect of preculture and co-cultivation duration on transforamation efficiency in *G. optiva*

Pre-culture Days	Co-cultivation days	explants survived in selective medium* Callus	Embryogenic	Transformation efficiency Callus	Embryogenic clump
24	24	1	0	1.67	0
	48	4	1	5.01	4.76
	72	1	0	1.67	0
48	24	0	0	0	0
	48	3	1	6.68	4.76
	72	1	0	1.67	0
72	24	0	0	0	0
	48	1	1	1.67	4.76
	72	0	0	0	0

*for callus -60 and for embryogenic clump 21 explant per treatment

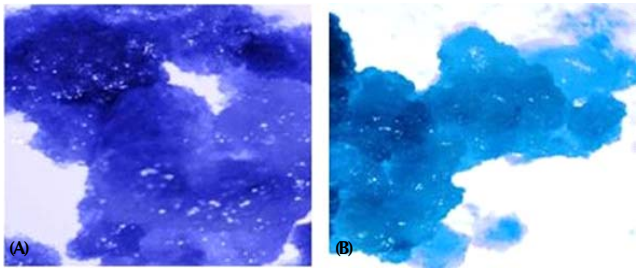

Figure 2: Putative transformed explants showing gus positive staining in *G. optiva* a) callus b) embryogenic clump

embryogenic clumps, maximum transformation frequency as observed in 48 hours co-cultivation irrespective of pre-incubation duration. Co-cultivation for 72 hours resulted in excessive bacterial growth hampering the callogenesis, whereas 24 hours co-cultivation did not produce any response. Regarding effect of pre-culturing duration although exact cause is still to be revealed, however it has been proposed that during pre-culture the explants undergo a physiological and developmental shift to enter for morphogenic competency, and when the T-DNA is inserted following this short period, the recipient cell have already entered the regeneration pathway (Agarwal and Kanwar, 2007). Similar significant effect of cultivation and pre-culture duration has been reported by several authors (Agarwal and Kanwar, 2007; Agarwal *et al*., 2004; Bardar *et al*., 2016; Kanwar *et al*., 2003; Sivanandhan *et al*., 2016). The explants growing on selective medium was randomly selected and subjected to X-Gluc staining. The transformed plants gave indigo blue staining (Fig-2). This kind of GUS positive staining has already been noticed by several authors (Agarwal and Kanwar, 2007; Agarwal *et al*., 2004; Kanwar *et al*., 2003; Prakash and Gurumurthi, 2009).

To the best of our knowledge, this is the first report on the genetic transformation of *Grewia optiva*. However, further research on confirmation of stable integration and copy number is required to be confirmed by molecular tools (blotting and RT-PCR). Present information will lead to initiation of biotechnological interventions for improvement of this important fodder tree.

REFERENCES

Agarwal, S. and Kanwar, K. 2007. Comparison of genetic transformation in *Morus alba* L. via different regeneration systems. *Plant cell reports*. **26:**177-185

Agarwal, S., Kanwar, K., Saini, N. and Jain, R. 2004. *Agrobacterium tumefaciens* mediated genetic transformation and regeneration of *Morus alba* L. *Scientia horticulturae*. **100:**183-191

Bardar, S., Kothari, S. and Kachhwaha, S. 2016. Optimization of Factors Influencing *Agrobacterium* mediated Genetic Transformation in *Eclipta alba* (L.) Hassk. *Plant Tissue Culture and Biotechnology*. **25:**155-164

Bhat, S., Jadhav, A., Pawar, B., Kale, A., Chimote, V. and Pawar, S. 2013. *In vitro* shoot organogenesis and plantlet regeneration in brinjal (*Solanum melongena* L.). *The Bioscan*. **8:** 821-824

Brandis, D., Stewart, J. L. and Fitch, W. H. 1972. *Illustrations of the forest flora of North-West and Central India*. Bishen Singh Mahendra Pal Singh,

Gomez, K. A. and Gomez, A. A. 1984. *Statistical procedures for agricultural research. John Wiley and Sons,*

Han, K. H., Meilan, R., Ma, C. and Strauss, S. 2000. An *Agrobacterium tumefaciens* transformation protocol effective on a variety of cottonwood hybrids (genus *Populus*). *Plant cell reports*. **19:**315-320

Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO J*. **6:**3901

Kanwar, K., Bhardwaj, A., Agarwal, S. and Sharma, D. 2003. Genetic transformation of *Robinia pseudoacacia* by Agrobacterium tumefaciens. *Indian J. experimental biology*. **41:**149-153

Khosla, P., Toky, O., Bisht, R. and Hamidullah, S. 1992. Leaf dynamics and protein content of six important fodder trees of the western Himalaya.*Agroforestry systems*. **19:**109-118

Mishra, S. P., Bhat, S. G. and Singh, S. 2010. Genetic manipulation of tomato plant with annexin gene through *Agrobacterium* mediated transformation. *Bioscan*. **5:**331-334

Negi, S. 1977. Fodder trees in Himachal Pradesh. *Indian Forester*. **103:**616-622

Prakash, M. and Gurumurthi, K. 2009. Genetic transformation and regeneration of transgenic plants from precultured cotyledon and hypocotyl explants of *Eucalyptus tereticornis* Sm. using Agrobacterium tumefaciens. *In Vitro Cellular and Developmental Biology-Plant*. **45:**429-434

Satyavathi, V., Prasad, V., Lakshmi, B. G. and Sita, G. L. 2002. High efficiency transformation protocol for three Indian cotton varieties via *Agrobacterium tumefaciens*. *Plant science*. **162:**215-223

Sharma, R., Singh, B. and Bhat, T. 2000. Nitrogen solubility, protein fractions, tannins and in sacco dry matter digestibility of tree fodders of Shiwalik range. *Indian J. Animal Nutrition*. **17:**1-7

Sivanandhan, G. *et al.* 2016. Factors affecting *Agrobacterium*-mediated transformation in *Hybanthus enneaspermus* (L.) F. Muell. *Plant Biotechnology Reports*. **10:** 49-60

Stevens, M. E. and Pijut, P. M. 2014. *Agrobacterium*-mediated genetic transformation and plant regeneration of the hardwood tree species *Fraxinus profunda*. *Plant cell reports*. **33:**861-870

Swamy, S., Puri, S. and Singh, A. 2002. Effect of auxins (IBA and NAA) and season on rooting of juvenile and mature hardwood cuttings of *Robinia pseudoacacia* and *Grewia optiva*. New Forests. **23:**143-157

Wang, J., Nie, J., Pattanaik, S. and Yuan, L. 2016. Efficient *Agrobacterium*-mediated transformation of *Artemisia annua* L. using young inflorescence. *In Vitro Cellular and Developmental Biology-Plant*. **52:** 204-211.