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AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION IN LENTIL (*LENS CULINARIS* MEDIK) CROP OF PAKISTAN

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ABSTRACT

Lentil *(Lens culinaris* Medik*)* is ranked as sixth important pulse crop of the world. Conventional breeding in lentil is hampered due to inadequate genetic variability, high degree of self pollination and small flowers. Cotyledonary nodes (CN), epicotyls and hypocotyls of two varieties of lentil namely Masoor-85 and 2009 were used as explants source to establish a protocol for genetic transformation system using *Agrobacterium tumefaciens* strain (EHA105 and LBA4404) containing pGreen plasmid vector with GUS gene. Cotyledonary nodes (93%) and epicotyls (56%) of Masoor-2009 showed maximum growth on shooting media as compared with Masoor-85. Epicotyls of Masoor-2009 and Masoor-85 showed 56% and 45% regeneration with 2-3 shoots developed on each epicotyl. The performance of EHA105 and LBA4404 in GUS expression was 70% and 60% respectively. Stable integration of GUS gene was also confirmed through PCR.

Key point: Lentil, *Lens culinaris*, Transformation, PCR, GUS assay

INTRODUCTION

Lentil (*Lens culinaris* Medik.) is widely cultivated in the Middle East, West Asia, North Africa and Indo-Pakistan and it ranks the sixth most important pulse crop of the world (Neelam *et al*., 1988). In many developing countries of the world, pulses have achieved significant importance in view of the wide prevalence of protein malnutrition. Pulses are considered as the "meat of the poor" as well as the main source of protein for livestock feed and inland fish production. Lentil seeds are very nutritious having protein levels ranging 20 to 36% (Gulati *et al*., 2002). In addition to its nutritive value, it has the ability to fix atmospheric nitrogen in the soil (Duranti and Gius, 1997). Lentil is typically characterized by low yield potential due to several factors which include its susceptibility to pest and diseases, massive flower drop as well as post harvest loss. Conventional breeding methods including hybridization technique and selection are being carried out to develop improved varieties in a wide range of crops. However, in lentil the progress of such improvement is hampered due to the inadequate genetic variability caused by a predominantly high degree of self-pollination and crossing in microsperma lentil is tedious because of its small size of flowers. It is imperative to look for some other methods to induce genetic variability in lentil. Bio safe Genetic transformation can supplement traditional crop improvement procedures and this approach can be used in introducing desired traits which is not possible through conventional breeding alone (Gardner 1993). Thus it might be possible that, genetic transformation combined with traditional breeding techniques could aid in improving both the quality and yield of lentil. Recently limited information is available on lentil genetic transformation using *Agrobacterium tumefaciens* (Akcay *et al*., 2009). A few reports are also available on the *in vitro* regeneration of plants in lentil. These reports include the regeneration of plants using various explants of lentil such as shoot apices (Singh and Raghuvanshi, 1989), nodal explants (Ahmad *et al*., 1997), intact seedlings (Malik and Saxena 1992), cotyledonary nodes (Gulati *et al.*, 2001) and cotyledons (Tavallaie *et al*., 2011). A limited progress has been made both on *in vitro* regeneration and transformation of microsperma lentil varieties of BM-1, BM-2, BM-3 andBM-4 cultivated in Bangladesh (Hassan *et al.*, 2007). It has been possible to transfer marker gene into local microsperma lentil varieties but the recovery of transgenic plant is limited due to lack of effective *in vitro* root system (Sarker *et al.* 2003). The main objective of this study was to develop a protocol for *Agrobacterium* mediated genetic transformation for local microsperma varieties of lentil for their improvement. Moreover, attempts were also made to develop roots of lentil plant.

MATERIALS AND METHODS

Artificial Media and growth condition for lentil: Seeds of two lentil varieties (Masoor-85 and Massor-2009) were germinated on sterile filter paper and transferred on MS medium as described by Murashige and Skoog (1962).

Explants (epicotyles, cotyledonary nodes and hypocotyles) were excised from aseptically newly grown seedlings with the help of sterilized blade. All explants were regenerated on four shooting medium SM01 (BAP 0.5 Kinetin 0.5, GA3 0.1 and Tyrocine 5.5 mg/L), SM02 (BAP 0.1 Kinetin 0.5, GA3 0.1 and Tyrocine 0.5mg/L), SM03 (BAP 0.2 Kinetin 0.5, GA3 0.1 and Tyrocine 5.5mg/L) and SM04 (BAP 0.1 Kinetin 0.25, GA3 0.1 and Tyrocine 5.5mg/L). Healthy shoots were transferred to rooting media (3% sucrose, 0.4% phytagel, pH 5.8 and IBA 0.25mg/L) with various auxin concentrations (1, 2, 3mg/ml). Culture jars and tubes were incubated at 25 ± 2ºC in plant growth chamber under light intensity (2000 lux). The rooted plants were transferred to sterile soil and grown under a photoperiod of light and dark at 24°C in a plant growth chamber for two weeks. Regeneration of explants is not successful without development of roots. Different experiments were conducted to obtain efficient rooting by using IBA and IAA in different concentration in rooting media (RM). Three replications were done for each media and data were taken after two months.

***Agrobacterium* mediated genetic transformation:** It was carried out according to the protocol described by Akcay *et al.,* (2009). Plasmid vector (pGreen) containing the target gene under the control of constitutive promoter and *Agrobacterium* (LBA4404 and EHA105) harboring used for transformation. A single colony of *Agrobacterium* was multiplied in liquid broth medium and bacteria were re-suspended in MS liquid medium and O.D was adjusted to 0.4-1.0. The pre-conditioned nodes were wounded and kept submerged in MS liquid media (200µM acetosyringone) containing bacteria. Shaking of flasks was done to make sure that explants remained in contact with medium. The infected explants were co-cultured on artificial media for three days at 24ºC in dark.

Regeneration and Selection of Transgenic Explants: Plants were first washed with sterilized water and then MS liquid containing different concentration of cefotaxime (250 and 500mg/L) for 20 min. Plants were dried and hygromycin (20, 50 and 80mg/L) was alter to monitor its effective concentration for cotyledonary node explant regeneration and after 2 weeks.

Histochemical GUS and PCR assay: Transformation ability of the lentil plants was monitored by GUS histochemical Assay (Jeffereson *et al.*, 1987) by submerging them in the presence of X-gluc (1.0mg/ml) and incubation was done at 37˚C for three days. Transferred lentil plants were washed with alcohol (70%) and scored for GUS expression with negative (uninfected lentil plants) and positive control (transgenic tobacco tissues). Tissues and shoots under selection pressure of around two months were monitored for stable GUS expression. PCR was also performed for confirmation of transmission.

RESULT

Seed sterilization and shooting media selection: Lentil plants showed best result on SM04 while minimum at SM01 and average response was at SM02. Seeds were germinated on MS medium and after two days of germination cotyledonary nodes, hypocotyls and epicotyls were taken from five days old Seedlings (Fig 1-a). The process of direct organogenesis involves differentiation of cells and tissues leading to shoot bud formation, shoot development and rooting of the shoots. Cotyledonary nodes, epicotyls and hypocotyls portions were taken as explants for regeneration of viable shoots and rooting. Epicotyl was taken as portion above cotyledons, hypocotyls as a portion below cotyledons, while cotyledonary node was obtained after cutting epicotyls, hypocotyls and cotyledon. The size of epicotyls and hypocotyls were about 3 mm. Epicotyl was second best while hypocotyls showed no regeneration on any type of media. In this study cotyledonary nodes developed multiple shoots with green leaves reaching a maximum length of 8 cm while epicotyl developed 2-3 shoots with a maximum length of 10 cm in three weeks. Regeneration started 2-3 days after transfer to regeneration media and data for development of good shoots were taken after three weeks (Fig. 1-b).

Genotype and rooting media selection: Cotyledonary nodes of Masoor-2009 had regeneration potential 93% and developed average 10 shoots per explants with maximum length of 8 cm while epicotyls showed 56% regeneration with 2-3 shoots development on each epicotyls and the maximum length of shoots was 10 cm. Epicotyls of Masoor-85 showed 45% regeneration with 2-3 shoots developed on each epicotyls and the maximum length of shoots was 10 cm. Cotyledonary nodes regeneration potential of Masoor-85 was 83% with average 7 shoots per explants and the maximum length of shoots was 8 cm. and hypocotyls of both varieties showed no regeneration.

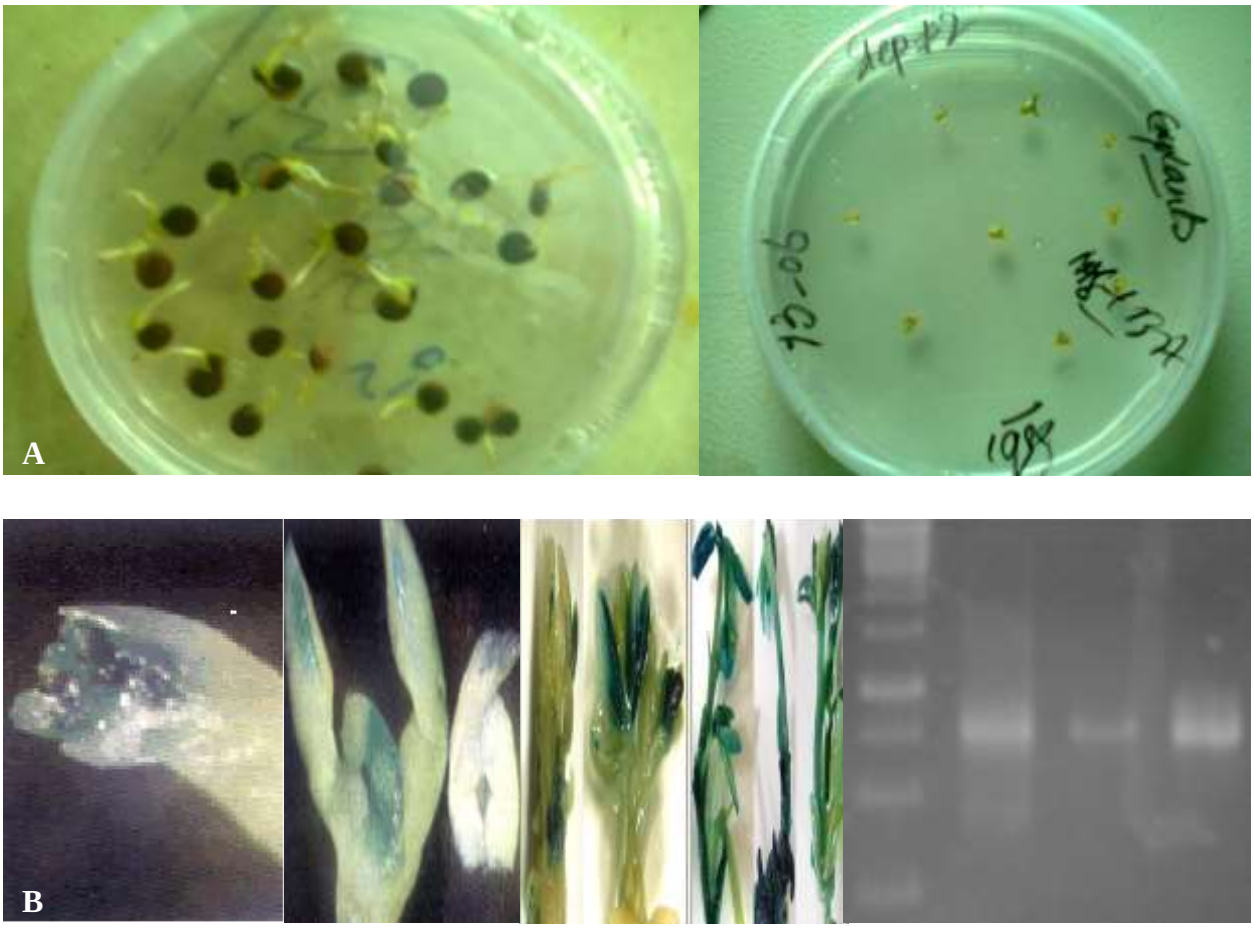

Fig 1. Seed germination, regeneration of lentil plants (a) and confirmation of transformation thorough GUS and PCR (b).

Table 1. Regeneration response of varieties and explants on different shooting media.

Variety	Explants	Shooting Media				
		SM01	SM02	SM03	SM04	Average
Masoor-2009	Cotyledonary Nodes	91	90	93	99	93
	Epicotyls	65	68	40	52	56
Masoor-85	Cotyledonary Nodes	92	95	66	80	83
	Epicotyls	54	65	25	36	45

Table 2. Effect of Auxin on root formation of plantlets of Masoor-2009.

Auxin	Concentration	Rep. 1	Rep. 2	Rep. 3	Average
IAA	1 mg/L (RM 1)	49	44	48	47
	2 mg/L (RM 2)	19	26	29	24
	4 mg/L (RM 3)	24	13.5	20	19
IBA	0.25 mg/L (RM 4)	44	15.5	20	26

Each experiment was replicated thrice and data for good shoot formation was taken after three weeks. Regeneration from cotyledonary nodes and epicotyl of Masoor-2009 on artificial media was 99% and 68% respectively. In Masoor-85 cotyledonary node and epicotyl gave higher regeneration 95% and 65% respectively. However owing to large variations in response, there was no significant difference in media combination used in this

experiment. There was no single media which support all types of explants and all varieties (Table 1). Auxins are reported to induce root initiation and development along with other function as development of the embryo, leaf formation, apical dominance, fruit development. The localized accumulation of auxins in epidermal cells of the root initiates the formation of lateral or secondary roots (Daphne *et al*., 2005). The rooting response of lentil plants was 47%, 26% and 19% on RM1, RM4 and RM3 respectively (Table 2). Rooting on IAA took 7 weeks while on IBA it took only 4 weeks. Rooting was only observed in cotyledonary nodes of Masoor-2009 and none of the Masoor-85 plantlets developed roots. Low concentration of IAA supported root formation while higher concentration inhibited root formation. Data indicated that RM1 were different from RM3 in a significant manner but not from RM2 and RM4.

Transformation and confirmation of GUS gene in lentil plants: Cotyledonary nodes of Masoor-2009 were used for transformation experiment because cotyledonary nodes were observed to be the most regenerated explants and more responsive lentil variety used during tissue culture experiments. Cotyledonary nodes of five days old seedlings were taken and shifted to SM04 for two days. Forty five Explants were wounded four times and infected with both strains of Agrobacterium separately. Infected lentil plants were shifted on regeneration medium having 200µl acetosyringone and finally transferred to selection media after washing with cefotaxime. pGreen construct was electroporated into competent cells of Agrobacterium and bacterial clones were picked on the basis of antibiotic selection. Plasmid DNA was extracted from transformed clones and confirmed by using GUS primers. The primers successfully amplified 0.7kb fragment of GUS gene (Fig 1-b). Cotyledonary nodes were screened against different doses of hygromycin (20, 50 and 80 mg/L) and cefotaxime (250 mg/L and 500 mg/L) its effects of antibiotic was recorded after 4 weeks. Plantlets showed yellowing and very slow regeneration at 20 mg/L hygromycine, while higher concentration of hygromycin was more detrimental. No growth was observed at 80 mg/L hygromycine. Concentrations of cefotaxime had no affect on growth and regeneration of plantlets. The transient GUS expression was scored on the basis of blue spot and color developed by GUS staining. EHA105 showed 70% while LBA4404 showed 60% GUS expression (Fig 1-b).

DISCUSSION

Advances in molecular genetics and biotechnology have made plant transformation and regeneration an available and powerful tool for crop improvement. Regeneration of shoots from cotyledonary node or from other meristematic explants after Agrobacterium infection is emerging as a rapid and relatively efficient method in a number of legume species (Sommers *et al.,* 2003). The rooting to develop whole plant is a major problem in regenerated plantlets of lentil (Ford *et al*., 2007). The present investigation was undertaken to establish an efficient transformation protocol for lentil varieties and *Agrobacterium* mediated genetic transformation has been considered as the most common and successful method used in various leguminous crop (Krishnamurthy *et al*. 2000). A class of plant growth regulators cytokines (BAP and Kinetin) was used in this study that promote cell division, cell growth, differentiation and other physiological processes (Kieber, 2002). Gibberellin or GAs include a large range of chemicals that are produced naturally within plants, responsible for growth of new cells while tyrosine plays an important role in signal transduction (Grennan, 2006). Khawar and Ozcan (2002) reported 25% rooting with 0.25 mg/L (RM4) concentration of IBA. Transformation theoretically expands the sources of genes for plant improvement to all organisms, far beyond the gene pool accessible via sexual hybridization.

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