DEVELOPMENT OF FUNGAL DISEASE RESISTANCE IN LENTIL (*Lens culinaris* Medik) FOLLOWING AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION

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ABSTRACT

Attempts were made to integrate fungal disease resistance in lentil (*Lens culinaris* Medik) plants through *Agrobacterium*-mediated genetic transformation. As an integral part of this transformation protocol three different explants, namely, cotyledonary node, decapitated embryo and cotyledon attached decapitated embryo were employed to develop a transformation compatible regeneration system in lentil. Best regeneration of multiple shoots was achieved via direct organogenesis from cotyledonary node and cotyledon attached decapitated embryo on MS medium supplemented with 2.22 µM BA + 2.32 µM Kn + 0.29 µM GA3 + 30.35 µM tyrosin. However, these regenerated shoots failed to develop effective root system to produce complete plantlets. The techniques of *in vitro* flowering and seed formation on regenerated shoots were found to be effective to overcome the problem of rooting in obtaining complete plantlets. The best response regarding the development of *in vitro* flower and seed was obtained by culturing regenerated shoots on half strength of MS medium containing 98.4 µM IBA and 2.69 µM NAA. For genetic transformation *Agrobacterium* strain LBA4404 containing binary plasmid pBI121 conferring β-glucuronidase (GUS) and *nptII* gene resistant to kanamycin while another strain EHA105 harboring *bar* gene resistant to phosphinothricin and antifungal *chitinase* gene were used. Transformed shoots developed *in vitro* flowers following their subculture on half strength of MS medium containing 98.4 µM IBA and 2.69 µM NAA and 12-15 days were required to produce seeds from these *in vitro* grown flowers. Stable integration

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of *gus* gene as well as antifungal protein gene was monitored through PCR analysis. This technique of *in vitro* flowering and seed formation can be exploited to develop transformed seeds since *in vitro* root formation appears to be a major constraint in obtaining complete plantlet under *in vitro* condition in lentil.

Keywords: Lentil, In vitro flowering, Seed development, Genetic transformation, Fungal disease resistance.

INTRODUCTION

Grain legumes are one of the most important crops in agriculture all over the world. They are commonly known as pulses. The pulses are amongst the earlier food crops to be cultivated especially in Asia, Latin America and Africa. In many developing countries, grain legumes have gained much importance in view of the acute shortage in the production of animal proteins and wide prevalence of protein malnutrition.[1] Apart from this, the grain legumes are also considered as the main source of protein for livestock feed and inland fish production. Moreover, grain legumes have the unique ability to fix nitrogen symbiotically thereby improving the soil fertility.

Among the pulses lentil is the most popular one and ranks first in term of consumption in Indian subcontinent particularly in Bangladesh. The protein and nutritional quality of lentil have been reported to be superior to other pulse crops.[2-3] Thus lentil plays an important role in agricultural system of the South Asian countries with increasing annual demands for human consumption.

It is evident that the demand of lentil has been steadily increasing for its nutritional quality and acceptability. However, this crop is characterized by low yield potential. Several factors are believed to be responsible for the lower yield of due to susceptibility to pests and diseases, massive flower drop and post harvest loss.[4] Diseases of lentil at various stages of growth are caused by fungi, bacteria, viruses and nematodes.[5]

A number of attempts were made in the past to improve lentil varieties using conventional breeding techniques, but these efforts failed to achieve desired results due to the prevalence of narrow genetic base and non availability of suitable germplasms for lentil improvement. Under these circumstances plant genetic engineering and biotechnology can be a method of choice for the improvement of this crop. A number of studies have been carried out previously to develop a suitable in vitro regeneration system for lentil to explore the possibility of applying biotechnology in lentil improvement programme.[6-9]

Improvement of microsperma types of lentil through biotechnological methods has been proved to be difficult due to its recalcitrant nature particularly during the development of in

vitro effective root system. Previous reports indicated that, rooting in microsperma type of lentil was particularly difficult and appeared to be a major constraint in obtaining complete plantlet through in vitro techniques.[10-11] To overcome this problem of rooting in lentil micro-grafting had been applied earlier.[11] in obtaining in vitro derived plantlets. However, such micro-grafting in lentil was found to be difficult and complicated especially in case of microsperma type of lentil varieties. Under these circumstances in vitro flowering and fully developed seed formation have been considered to be an alternative approach in obtaining plant progenies using in vitro techniques. It is believed that in vitro flowering and seed development may significantly contribute towards the genetic improvement of lentil through biotechnology.

An alternative regeneration system was explored escaping in vitro root formation from regenerated shoots and to find out suitable protocol for in vitro flowering and seed formation in lentil to facilitate genetic transformation. Several reports are available on in vitro flowering in a number of plants including citrus,[12] cauliflower,[13] maize,[14] bamboo,[15-16] etc., as well as in vitro fruit development in tomato.[17]

In this study, a method for Agrobacterium-mediated genetic transformation of lentil is reported for local varieties of lentil (Barimasur) through in vitro flowering and seed formation.

MATERIALS AND METHODS

Two microsperma varieties of lentil (Lens culinaris Medik) namely, Bari Masur-4 (BM-4) and Bari Masur-5 (BM-5) were used as the plant materials for this investigation. Seeds of these two varieties of lentil were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur and maintained in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka. Three different explants namely, cotyledonary node, cotyledon attached decapitated embryo, and decapitated embryo were used for the development of in vitro shoots. To obtain such explants lentil seeds were germinated aseptically. For this purpose the seeds were first washed in 70% ethanol for 1 minute and then they were surface sterilized with 0.1% HgCl2 solution for 15 minutes. Seeds were then washed 3 - 4 times with sterilized distilled water. The surface sterilized seeds were then cultured on 0.3% water agar medium for germination.

Cotyledonary node explants were collected from three-day old germinated seedlings following the removal of seed coats. Cotyledon attached decapitated embryo and decapitated embryo explants were collected from overnight soaked sterilized seeds. The seed coats were removed and the seeds were split open and two cotyledons were separated. Before inoculation the shoot and root meristems from these embryos were excised. Explants were then placed on MS medium [18] supplemented with 2.22 µM BA + 2.32 µM Kn + 0.29 µM GA3 + 30.35 µM tyrosin for regeneration of shoots. The culture vessels were maintained under fluorescent illumination on a 16 h photoperiod at 25 ± 2°C.

For genetic transformation two strains of Agrobacterium tumifaciens were used in this study. Agrobacterium tumifaciens strain LBA4404 with the binary plasmid pBI121 had a scoreable reporter gene GUS (β-glucurunidase) driven by a CaMV35S promoter and NOS terminator and a selectable marker gene nptII fused between NOS promoter and NOS terminator encoding for the enzyme neomycin phosphotransferase conferring kanamycin resistance.[19] While another strain EHA105 (pSOUP-pGII-35S-N-CHIT) has bar gene resistant to phosphinothricin used as a selectable agent and an antifungal chitinase gene .

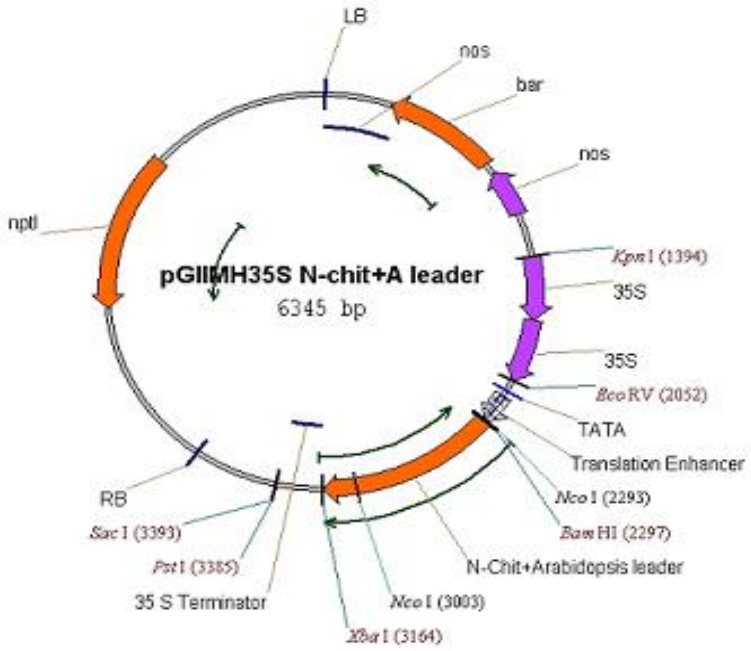

Fig.1. Diagrammatic representation of plasmid of *Agrobacterium tumifaciens* strain EHA105

For both the strains of Agrobacterium 50ml of liquid YMB[20] containing 50 mg/l kanamycin was inoculated with Agrobacterium from a fresh bacterial plate and grown at 180 rpm on a rotary shaker at 28°C for 16 h. The overnight grown Agrobacterium culture was centrifuged for 10 minutes at 5000 rpm and the pellet was resuspended in liquid MS medium (pH 5.8) to make the Agrobacterium suspension. This Agrobacterium suspension was used for infection and incubation. Prior to this O.D. (Optical Density) of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer (Shimadzu, Japan). Following the determination of density, seperated explants were dipped in bacterial

suspension for different incubation periods before transferring them to co-cultivation medium to get adequate infection of the explants.

Following infection and incubation, the explants were soaked in filter papers for a short period of time to remove excess bacterial suspension. All the explants were maintained in co-culture medium for 2-4 days in dark condition. Following co-culture the explants were washed with distilled water 3-4 times until no opaque suspension was seen then washed for 15 minutes with distilled water containing 300mg/l ticarcillin. Then explants were dried with a sterile Whatman filter paper and transferred to regeneration medium with 100 mg/l ticarcillin.

After 7-10 days, the regenerated shoots were then sub-cultured in selection medium containing 50 mg/l kanamycin and 100 or 50 mg/l ticarcillin. Cultures were sub-cultured regularly at an interval of 12-15 days and the concentration of selective agents was gradually increased up to 200 mg/l in case kanamycin on selection medium. On the other hand phosphinothricin (0.5 to 2.5 mg/l) was used as selectable agent in the experiments where plasmid contained the bar gene. Shoots survived on selection medium were sub-cultured on ½ strength of MS medium containing 98.4 µM IBA and 2.69 µM NAA with 50 mg/l kanamycin for in vitro flowering and seed formation.[21]

Transformation ability of the explants was monitored by GUS histochemical assay [22] and produced an insoluble indigo blue colour at the site of GUS enzyme activity. Thus, it allowed transformed tissues to be screened histochemically. Tissues and shoots under selection pressure of around two months were monitored for stable GUS expression.

When required the presence of the GUS, nptII and chitinase genes in the lentil genomic DNA was analysed by polymerase chain reaction (PCR). DNA was isolated from nont-ransformed plant and transformants using the CTAB method.[23] For the detection of the nptII coding sequence, DNA was subjected to PCR using the following primers and conditions: forward 5'-TGA TTG AAC AAG ATG GAT TG-3´ and reverse 5´-CAT TTT CCA CCA TGA TAT TC-3´. For the GUS gene the primers were: forward 5´-CCT GTA GAA ACC CCA ACC CG-3´ and reverse 5´-TGG CTG TGA CGC ACA GTT CA-3´ (MGW-Biotech, AG, Germany). A separate set of primers was used for amplification of chitinase gene. All primers were used at a concentration of 100 pmol/µl. The plasmid pBI121 isolated from Agrobacterium tumefaciens was used as the positive control. PCR reaction mix of 25 µl contained 2.5 µl of 10× PCR buffer with 15 mM MgCl2 (Gene Craft, Germany), 1 µl of 5 mM of the dNTP mix, 1 µl of Red Taq polymerase (Natutech, Germany), 1 µl of each of the respective primers, and 1 µl (50 - 80 ng/µl) of the sample DNA and 17.5 µl ultra pure water. For PCR amplification of the GUS gene, DNA was denatured at 94°C for 3 min and then amplified in 30 cycles using 94°C for 1 min, 64°C for 1 min (annealing) and 72°C for 1 min followed by 5 min at 72°C. For nptII gene the cycling conditions were 3 min at 94° denaturtion and 30 amplification cycles using 94°C for 1 min, 55°C for 1 min (annealing) and 72°C for 1 min followed by 5 min at 72°C. The amplified DNA was run on 1.0% agarose gel and stained with ethidium bromide (0.05 µg/ml).

RESULT AND DISCUSSION

The present investigation was carried out to establish an efficient protocol for developing transgenic lentil (Lens culinaris Medik.) plants cultivating in Bangladesh through Agrobacterium-mediated genetic transformation. Among the different approaches, Agrobacterium-mediated genetic transformation has been considered as the most common and effective method used in various leguminous crops such as soyabean,[24-25] chickpea,[26-27] peanut,[28] lentil.[29] In this investigation attempts were also made to integrate fungal disease resistance through this transformation protocol in locally grow lentil plants following the techniques of in vitro flowering and seed formation.

In vitro regeneration of shoots: In vitro regeneration in lentil is not well established compared to the success obtained in other grain legumes. In vitro culture of lentil has proved to be difficult. However, over the last several years in vitro propagation of lentil has been progressively improved.[6-7, 11, 30] In this study, highest number of healthy multiple shoots were obtained from the explants of cotyledonary node and cotyledon attached decapitated embryo on MS medium supplemented with 2.22 µM BA + 2.32 µM Kn + 0.29 µM GA3 + 30.35 µM tyrosin (Fig. 2). This finding confirms the earlier reports in case of microsperma type of lentils cultivated in Bangladesh.[9]

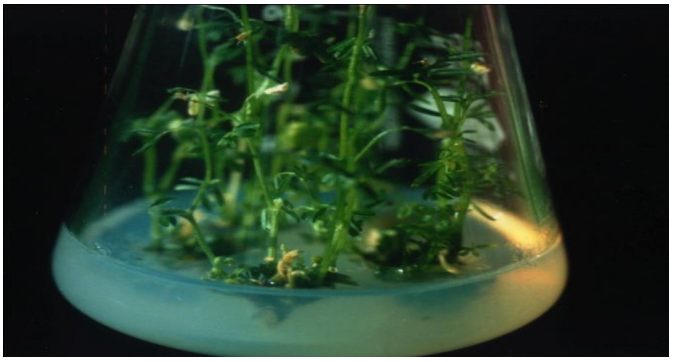

Fig. 2. *In vitro* regenerated shoots fron cotyledonary node explant of BM-4 on MS medium supplemented with 2.22 µM BA + 2.32 µM Kn + 0.29 µM GA3 + 30.35 µM tyrosin

In vitro root development: Shoots produced from different explants did not produce roots spontaneously. Therefore, for the induction of roots 2- to 4-cm long freshly regenerated individual shoots were excised and cultured on MS media containing different supplements of auxins. Several experiments were carried out to initiate rooting using various auxin supplemented medium. Root induction was observed when shoots were cultured on MS medium supplemented with 98.4 and 123.0 µM IBA. In these cases the number of roots varied from 3- to 8 per shoot. However, these roots did not develop from the base of the shoots rather at a level slightly higher than the cut ends (Fig. 3).

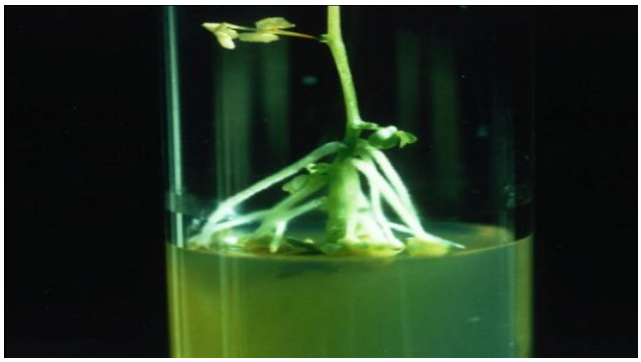

Fig. 3. Roots develop along the length of the shoots. These roots were not effective.

Therefore, these roots did not have an effective connection with the vascular system of the shoots. Moreover these freshly developed roots were found to produce callus when their tips were in contact with the medium. This result was found to be much identical to previous reports.[7-8] These observations indicated that rooting from the in vitro regenerated shoots in lentil is still a serious problem for the development of complete plantlets and their successful establishment in soil.

In vitro flowering and pod formation: As no effective root system was developed in this study, therefore attempts were made to initiate in vitro flowering and seed formation from the in vitro regenerated shoots to overcome the problems of in vitro effective root development in obtaining plantlets through in vitro techniques. Flowering is considered to be a complex mechanism regulated by a combination of several environmental and genetic factors.[31-33] Earlier reports indicated that in vitro flowering in ornamental and medicinal plants had been achieved on MS medium containing a wide range of supplements including BA and Fe+2,[34-35] GA3 and NAA,[36] BA and Kn,[37] BA and GA3.[38]

Following the above reports on in vitro flowering, in vitro derived shoots of lentil were transferred on MS medium containing several concentration and combinations of IAA, IBA and NAA on MS medium to induce in vitro flowering and seed setting. The results of these experiments demonstrated that MS medium supplemented with 98.4 µM IBA and 2.69 µM NAA exhibited the best response towards formation of in vitro flowers (Fig. 4) regarding the number of flower per shoot.

Fig. 4. Flower developed from *in vitro* raised shoot.

During this study in vitro flower buds initiated within 7 to 10 days of culture of the shoots. The flower bearing shoots were maintained in the same medium for in vitro pod formation. After 10 to 12 days pods were found to develop from healthy flowers (Fig. 5).

Fig. 5 Formation of *in vitro* pods from shoots on half strength of MS medium containing 98.4 µM IBA + 2.69 µM NAA.

Best response regarding the number of pod per shoot was obtained on half strength of MS medium supplemented with 98.4 µM IBA and 2.69 µM NAA. About 58% flowers produced seeds and the average number of pods per shoot was 4.0. The in vitro developed seeds fully matured within 20 to 30 days under in vitro condition and the seeds obtained were then harvested.

Genetic transformation: To date limited information is available on lentil genetic transformation using Agrobacterium.[8, 28] In these experiment transforming capability of

various explants, namely, cotyledonary node, decapited embryo, and cotyledon attached decapited embryo of BM-4 and BM-5 variety of lentil was evaluated using Agrobacterium strains LBA4404 harboring the binary plasmid pBI121 conferring β-glucoronidase (GUS) and nptII gene.

Agrobacterium-mediated genetic transformation procedure is believed to be influenced by several factors.[39] Factor that influence successful transformation such as optical density (O.D) of Agrobacterium suspensions, incubation and co-cultivation period were optimized during this study. During optimization of regulatory factors maximum number of explants found to be transformed with bacterial suspension having an O.D. of 1.0 with 45 minutes of incubation and 3 days of co-cultivation when LBA4404 bacterial strain was used.

Among all the explants used in this experiment, decapitated embryo explants showed the best response towards transformation with LBA4404 strain and in this case the percentage of GUS positive explants for BM-4 and BM-5 was 86.9 and 83.33 respectively (Table 1).

Table 1. Responses of various explants of BM-4 and BM-5 variety of lentil towards GUS histochemical assay following co-cultivation.

Variety	Explants	No. of explants assayed for GUS	No. of GUS+ve explants	% of GUS+ve explants
BM-4	Cotyledonary node	150	58	38.60
	Decapitated embryo	138	120	86.90
	Cotyledon attached decapitated embryo	110	81	73.60
BM-5	Cotyledonary node	146	47	32.19
	Decapitated embryo	126	105	83.33
	Cotyledon attached decapitated embryo	120	86	71.60

Next to decapitated embryo explants cotyledon attached decapitated embryo showed better responses towards transformation and percentage of GUS positive explants for BM-4 and BM-5 was 73.6 and 71.6 respectively. In case of cotyledonary node explants percentage of GUS positive explant for BM - 4 and BM - 5 variety of lentil was 38.16 and 32.19 respectively which was lowest among the three explants studied. In spite of maximum transformation efficiency in decapitated embryo further transformation experiments were not carried out for decapitated embryo as regeneration ability of this explant was lowest.

So, further transformation experiments were done with cotyledonary node and cotyledon attached decapitated embryo explants.

Considerable number of explants which co-cultured with Agrobacterium strain LBA4404 showed positive to GUS staining. GUS positive regions were visualized at the peripheral area of the cut surfaces as well as within the internal tissues of various explants. Prominent blue coloured (GUS +ve) zones within co-cultured explants were visualized following stereomicroscopic observation (Fig. 6).

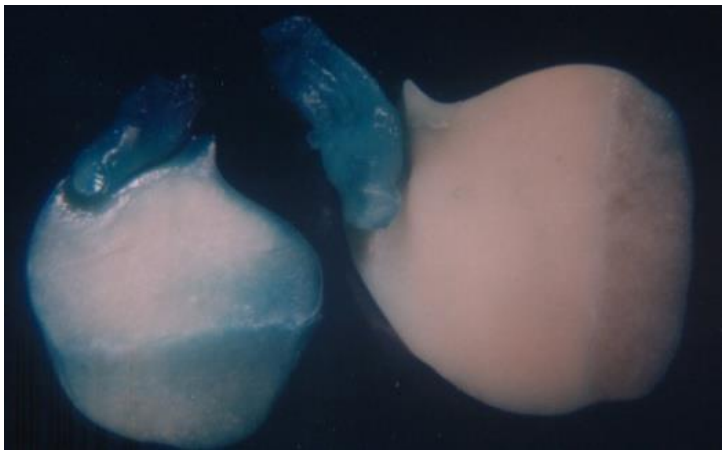

Fig. 6 Presence of blue colour on cotyledone attached decapitated embryo explants due to GUS activity.

Following transient GUS assay the remaining explants after co-cultivation were soaked with 300 mg/l ticarcillin for 15 minutes and then transferred to previously developed [(9)] regeneration medium comprising MS medium 2.22 µM BA + 2.32 µM Kn + 0.29 µM GA3 + 30.35 µM tyrosin with 100 mg/l ticarcillin for shoot multiplication. When the regenerating shoots attained a height of 2-3 cm, the shoots were sub-cultured in the same media but with selectable agents. In each set of experiments, regenerated control explants were also maintained to perform various comparative studies between transformed and non transformed tissues. The Agrobacterium strain used in these experiments (LBA4404/ pBI121) contains nptII gene conferring kanamycine resistance of the transformed cells. Therefore, selections of the transformants were carried out using various concentration of kanamycin.

For successful transformation experiments towards the development of transformed shoots, at first infected shoots were transferred to regeneration medium with 100 mg/l ticarcillin and 50 mg/l kanamycin. After 14 days, only green and healthy shoots were subcultured in fresh medium with 100 mg/l kanamycin and 100 mg/l ticarcillin. To select transformed

shoots kanamycin concentration was raised up to 150 mg/l in the third subculture and then 200 mg/l in the fourth subculture.

Finally those shoots that survive in this selection medium containing 200 mg/l kanamycin for 15 days and remained green and healthy were selected as possible transformed shoots

Fig. 7. Putative transformed shoots of BM – 4 survived on the selection medium containing 200 mg/l kanamycin

It was observed that, none of the cotyledonary node derived shoots were capable to continue their growth in presence of higher concentration (200 mg/l) of kanamycin. Whereas a few cotyledone attached decapited embryo-derived shoots were recovered after such selection. A total of 31 Kanamycin resistance shoots out of approximately 3072 infected shoots in case of BM-4 and 12 of 1125 (approximately) in case of BM-5 were recovered in final selection medium with LBA4404 strain of Agrobacterium containing binary plasmid pBI121. Therefore the frequency of recovery of putative transformed shoots was about 1.009 in case of BM-4 and 1.06 in case of BM-5.

Histochemical GUS assay (Jefferson 1987) was used to detect the expression of GUS gene in developing shoots and leaves at the time of each subculture in selection medium. Such assay demonstrated the presence of blue coloured zones on few selected shoots and leaves (Fig. 8).

Fig. 8. Transformed shoots showing the presence of blue colour on the developing shoots due to GUS activity

But it was found that, although a number of randomly selected shoots from initial selection medium showed positive response towards GUS expression, but very few of them could

survive the final selection pressure. Chimeric expression of GUS gene was also observed in a number of transformed shoots during the present study.

Thus following such selection pressure of kanamycin, shoots that survived were allowed to develop in vitro flowers in the flowering medium (half the strength of MS with 98.4 µM IBA and 2.69 µM NAA) containing 50 mg/l kanamycin. A number of experiments were carried out to select the suitable flowering media using MS and half-strength MS media supplemented with different concentration of IAA or IBA and NAA (data not shown). From these experiments it is revealed that half-strength MS media supplemented with 98.4 µM IBA and 2.69 µM NAA showed best response towards in vitro flowering and seed formation. It was observed that in higher concentration of kanamycin (100 mg/l) about 50% of flower bud failed to open and in 200 mg/l of kanamycin no flower bud formation was observed. For these, shoots that survived in the selection medium were separated and transferred to half strength of MS medium containing 98.4 µM IBA and 2.69 µM NAA and 50 mg/l kanamycin. After 2-3 weeks in vitro flower formation was observed in the healthy shoots recovered through kanamycin selection (Fig. 9).

Fig. 9. *In vitro* flower developed from the transformed shoots of BM-4,

It was found that out of 41 transformed shoots only 11 shoots responded to flowering and the maximum number of flower per shoot was 3. It was also found that after 12-15 days 5 out of these 11 flowering shoots produced viable and healthy seeds (Fig. 10) under in vitro condition but only 1-2 pods were found to develop per shoot.

Fig. 10. Fully developed pod from the transformed shoots of BM-4.

In the present investigation the transgenic nature of the transformed plantlets were confirmed through the application of specific molecular techniques like polymerase chain reaction (PCR) analysis. The DNA isolated from shoots of both transformed and non-transformed lentil was subjected to PCR analysis for the amplification of gus gene and such PCR amplified DNA was visualized through agarose gel electrophoresis. Fig. 11 is presented to show the results obtained following PCR analysis for GUS expression.

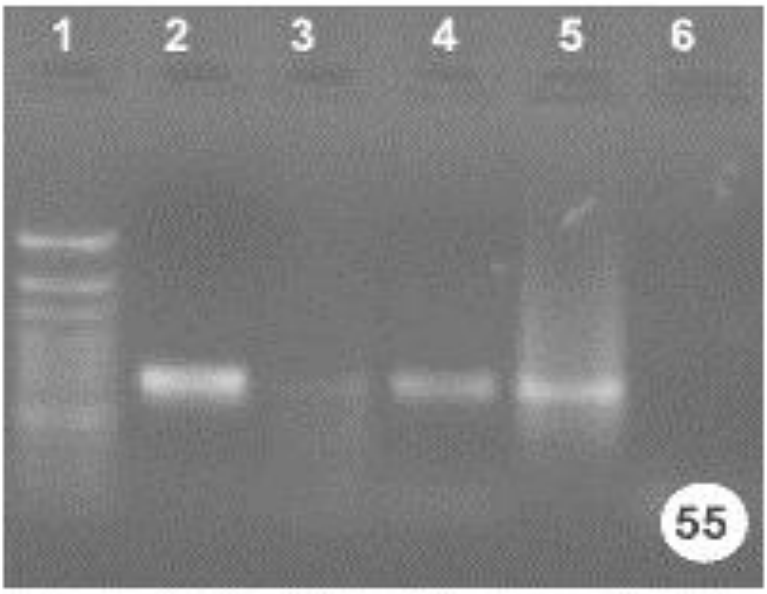

Fig. 11. PCR amplification of *gus* gene in two transformed plants (lanes 4, 5), lane 2 positive control. No signal from negative control (lane 3) and water control (lane 6).

Identical genetic transformation experiment was carried out using another Agrobacterium tumifaciens strain EHA105 (pSOUP-pGII-35S-N-CHIT) containing bar gene resistant to phosphinothricin used as a selectable agent and an antifungal chitinase gene. This gene construct was used for the integration of fungal disease resistant gene in lentil. In this set of experiments selection of the transformed shoots was performed using phosphinothricin. A series of experiments were conducted to initiate in vitro flowering and seed formation using putatively transformed shoots for the recovery of the transformed plantlets. Genomic DNA

isolated from the putatively transformed shoots was also subjected to PCR analysis. Results of such study are presented in Fig. 12. The results of this study demonstrated that antifungal chitinase gene had been successfully integrated in the genomic DNA of lentil plantlets.

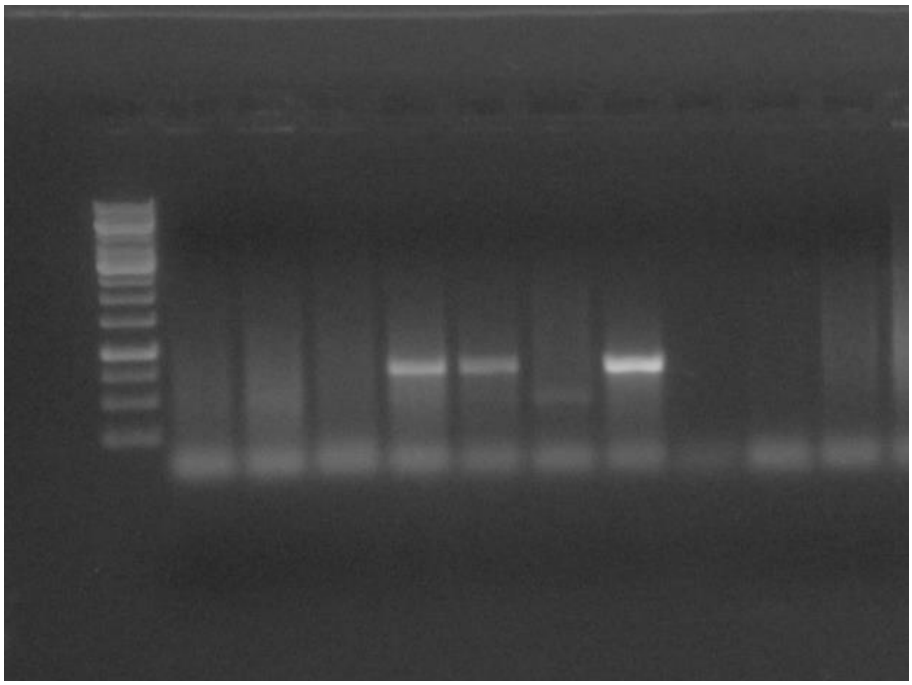

Fig. 12. PCR amplification of chitinase gene in transformed plants (lanes 5 & 6), positive control (lane 8). No signal from negative control (lane 11), water control (lane 10) and non transformed plants (lanes 2, 3, 4,7 & 9).

As rooting in lentil is extremely difficult, developed of in vitro flower and seed formation using the selected shoots has found to be effective in recovering transgenic lentil plantlets. Using this protocol, future study can be conducted to transfer useful genes conferring disease, insect and pest resistance in lentil varieties of Bangladesh.

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REFERENCES

1. Bressani R. Legumes in human diet and how they might be improved. PAG of United Nations System. 197; 15-42.

2. Singh S, Singh HD, Sikha KC. Distribution of nutrients in the anatomical parts of common Indian pulses. Cereal Chem. 1968. 45:13.

3. Bhatty RS. Composition and quality of lentil (Lens culinaris Medik.): A Review. Can. Inst. Food. Sci. Technol. J. 1988. 21(2):144-160.

4. Erskine W. Evaluation and utilization of lentil germplasm in an international breeding program. In: Witcombe, J.R., Erskine, W., eds. Genetic resources and their exploitation chickpeas, faba and lentils. The Hague: Martinus Nijhoff. 1984.

5. Gowda CL, Kaul AK. Pulses in Bangladesh. Joint publication of Bangladesh Agric. Res. Inst. and FAO. 1982.

6. Saxena PK, King J. Morphogenesis in lentil: plant regeneration from callus cultures of Lens culinaris Medik. via somatic embryogenesis. Plant Sci. Irish Republic. 1987. 52(3):223-227.

7. Polanco MC, Pelaez MI, Ruiz ML. Factor affecting callus and shoot formation from in vitro cultures of Lens culinaris Medik. Plant Cell, Tissue and Organ Cult. 1988. 15(2):175-182.

8. Warkentin TD, McHughen A. Regeneration from lentil cotyledonary nodes and potential of this explant for transformation by Agrobacterium tumefaciens. Lens Newsletter. 1993. 20(1): 26-28.

9. Sarker RH, Mustafa BM, Biswas A, Mahbub S, Nahar M, Hashem R, Hoque MI. In vitro regeneration in lentil (Lens culinaris Medik.). Plant Tissue Cult. 2003. 13(2):155-163.

10. Khanam R, Sarker RH, Hoque MI, Haque MM. In vitro root morphogenesis in lentil (Lens culinaris Medik.) Plant Tissue Cult. 1995. 5(1): 351-41.

11. Gulati A, Schryer P, McHughen A. Regeneration and micrografting of lentil shoots. In Vitro Cell and Dev. Biol. - Plant. 2001. 37(6):798-802.

12. Moss GI. Influence of temperature and photoperiod of flower induction and inflorescence development in sweet orange (Citrus sinensis L. Osbeck). Journal of Horticultural Science. 1969. 44: 311-320.

13. Vandana AK, Kumar A, Kumar J. In vitro flowering and pod formation in cauliflower (B. oleracea var. botrytis). Current Science. 1995. 69:25.

14. Mandal AP, Maiti A, Elanchezhian R. In vitro flowering in maize (Zea mays L.) Asia Pacific Journal of Molecular Biology and Biotechnology. 2000. 8:81-83.

15. Nadgauda RS, Parasharami VA, Mascarenhas AF. Precocious flowering and seedling behaviour in tissue-cultured bamboos. Nature. 1990. 344: 335-336.

16. Singh M, Jaiswal U, Jaiswal VS. Thidiazuron induced in vitro flowering in Dendroclamus strictus Nees. Current Science. 2000. 79:1529-1530.

17. Sheeja TE, Mandal BA. In vitro flowering and fruiting in tomato (Lycopersicon esculentum Mill.). Asia pacific journal of Molecular Biology and Biotechnology. 2003. 11(1): 37-42.

18. Murashige T, Skoog FA. Revised medium for rapid growth and bioassays with tobacco tissue culture. Plant physiol. 1962. 15: 473-497.

19. Herrera-Estrella L, Depicker A, Van Montagu M, Schell J. Expression of chimeric genes transferred into plant cells using a Ti-plasmid derived vector. Nature. 1983. 303:209.

20. Hooykaas PJJ. Agrobacterium molecular genetics. Plant Molecular Biology Manual A. 1988. 4:1-13.

21. Sarker RH, Das SK, Hoque MI. In vitro flowering and seed formation in lentil (Lens culinaris Medik). In Vitro Cell. Dev. Biol. – Plant. 2012.48(5): 446-452.

22. Jefferson RA, Kavanagh TA, Bevan MW. GUS fusion: Beta-glucoronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 1987. 6: 3901-3907.

23. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. Focus. 1990. 12: 13-15.

24. Hinchee MAW, Connor-Ward DV, Newell CA, McDonell RE, Sato SJ, Gasser CS, Fischhoff DA, Re DR, Fraley RT, Horsch RB. Production of transgenic soyabean plants using Agrobacterium-mediated DNA transfer. Biotechnology. 1988. 6: 915-922.

25. Meurer CA, Dinkins RD, Collins GB. Factors affecting soyabean cotyledonary node transformation. Plant Cell Rep. 1998. 18: 180-186.

26. Fontana GS, Santini L, Caretto S, Frugis G, Mariotti D. Genetic transformation in the grain legume of Cicer arietinum L. Plant cell Rep. 1993. 21: 194-198.

27. Kar S, Johanson TM, Nayak P, Sen SK. Efficient transgenic plant regeneration through Agrobacterium-mediated transformation of chickpea. Plant Cell. Rep. 1996. 16: 32-37.

28. McKently AH, Moore GA, Doostdar H, Niedz RP. Agrobacterium-mediated genetic transformation of peanut (Arachis hypogea L.) embryo axes and the development of transgenic plants. Plant Cell Rep. 1995. 14: 699-703.

29. Sarker RH, Mustafa BM, Biswas A, Mahbub S, Hoque MI. Agrobacterium–mediated transformation of lentil (Lens culinaris Medik.). Plant Tissue Cult. 2003. 13(1): 1-12.

30. Williams DJ, McHughen A. Plant regeneration of the legume Lens culinaris Medik. In vitro Plant Cell. Tissue and Organ Cult. 1986. 7:149-153.

31. Bernier G. The control of floral evocation and morphogenesis. Annu Rev. Plant Physiol. Plant Mol. Biol. 1988. 39:175-219.

32. Weigel D. The genetics of flower development: from floral induction to ovule morphogenesis. Annu. Rev. Genet. 1995. 29:19-39.

33. Weller JT, Reid JB, Tayler SA, Murfet IC. The genetic control of flowering in pea. Trends Plant Sci. 1997. 2:412-418.

34. Wang S, Tang L, Chen F. In vitro flowering of bitter melon. Plant Cell Rep. 2001. 20:393-397.

35. Jumin HB, Ahmad M. High-frequency in vitro flowering of Murraya paniculata (L.) Jack. Plant Cell Rep.1999. 18: 764-768.

36. Tang W. High-frequency plant regeneration via somatic embryogenesis and organogenesis and in vitro flowering of regenerated plantlets in Panax ginseng. Plant Cell Rep. 2000. 19:727-732.

37. Amutha R, Jawahar M, Ravi Paul S. Plant regeneration and in vitro flowering from shoot tip of Basilicum polystachyon (L.) Moench – An important medicinal plant. Journal of Agricultural Technology. 2008. 4(2): 117-123.

38. Kanchanapoom K, Posayapisit N, Kanchanapoom K. In vitro flowering from cultured nodal explants of rose (Rosa hybrida L.) Not. Bot. Hort. Agrobot. Cluj. 2009. 37(2): 261-263.

39. Mansur EA, Lacortee C, DeFreitus VG, DeOliviera DE, Timmerman B, Cordeiro AR. Regulation of transformation efficiency of peanut (Arachis hypogaea L.) explants by Agrobacterium tumefaciens. Plant Sci. 1993. 89: 93-99.