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(RESEARCH ARTICLE)

Study of different factors affecting *Agrobacterium*- mediated genetic transformation in lentil (*Lens culinaris* Medik.)

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Abstract

The present work was aimed to study the various factors affecting *Agrobacterium tumefaciens* mediated genetic transformation of three BARI masur variety of lentil (*Lens culinaris* Medik.), namely, BM-4, BM-5 and BM-6. *Agrobacterium* strain LBA4404 containing plasmid pBI121 which carrying *gus* and *npt*II as screenable and selectable marker genes, respectively, was used for transformation. Factors affecting transformation efficiency, such as, type of explants, bacterial density, incubation time and co-cultivation period were studied. The result showed that decapitated embryo (DE) explants of BM-6 variety of lentil inoculated in *Agrobacterium* suspensions at density of 1.0 (OD_{600}) for 45 minutes and co-cultivated on MS medium with hormones in the dark for 3 days gave the highest transient expression of *gus* gene (86.90%). The lowest percentage (15.00%) of GUS +ve explants observed in cotyledonary node (CN) of BM-5 variety of lentil when they were inoculated in *Agrobacterium* suspensions at density of 0.5 (OD_{600}) for 15 minutes. Using this protocol agronomical and economically important trait could be transfer to the locally grown lentil varieties.

Keywords: Lentil; Bacterial density; Incubation; Co-cultivation; *Agrobacterium*; Transformation

1. Introduction

Lentils have been the part of the human diet since the aceramic (before pottery) Neolithic times. It is one of the first crops domesticated in the Near East and still the most important cool season annual pulse crops throughout the world. It is preferred over the other pulses by consumer's preference all over the world. This important grain legume gained worldwide economic importance as a source of protein for human and animal nutrition. In Bangladesh, people like eating only red cotyledon type of lentil, where it is often eaten as a product "Dhal" as a main dish, side dish, salads or with flat bread (Roti) or rice. Khichuri is another popular dish, which is made from a mixture of split lentil seeds and rice.

From the above discussion, it is evident that lentil is highly nutritious. The demand for this crop has been steadily increasing in the Indian subcontinent for its nutritional value, cooking quality and easy digestibility. On a global scale, lentil consumption is rising at a rate more than twice that of the human population growth. We expect that by 2030, world lentil consumption will be double.

Although lentil is considered as an important pulse crop for many parts of the world but its production in most countries is usually characterized by low yield potential. Several factors are supposed to be responsible for the lower production of this important crop which includes susceptibility to disease, pests, fungi, massive flower drop, post-harvest loss, and management problem. Besides this, several abiotic stresses such as cold, drought, heat, salinity, nutrient deficiency and nutrient toxicity adversely affect lentil yields worldwide [1-3].

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It is clear that there is a need to increase productivity, enhance the nutritional value and other desired agronomic quality of this crop. Cultivars resistant to biotic and abiotic stresses and better protein quality and quantity are required.

Conventional breeding methods including hybridization technique and selection are being carried out to develop improved varieties in a wide range of crops. But in lentil, the progress of improvement through conventional breeding is hampered due to lack of genetic variability which caused by a predominantly high degree of self-pollination and absence of resistance gene/s of interest in the existing germplasm. Moreover, hybridization between *microsperma* and *macrosperma* is hampered due to asynchronous flowering and small size of flowers. Besides these, it takes long time to incorporate agronomic traits using conventional breeding method.

However, now it is possible to introduce genes into crop plants in a relatively short period of time using the recent advances in genetic engineering of plants [4]. Thus genetic transformation combined with traditional breeding techniques, could aid in improving both the quality and yield of lentil [5-7].

To identify the integration of gene/s of interest into plant cell, a specific screenable marker gene and one or more selectable marker gene are requiredin all transformation experiments. In this case generally GUS (glucoronidase) gene and neomycin phosphotransfrerase termed as *npt*II (kanamycin resistant) gene have been used for screenable and selectable marker gene, respectively. This reporter gene can be recognized in plant tissue with the help of selectable marker agents, confirming transformation of the plant tissue (through histochemical GUS assay). In this way one can understand that the plant tissue subjected for transformation has really been transformed or not [8].

However transformation efficiency was depended on many factors such as type of *Agrobacterium* strain, genotype (host) compatibility and responsiveness of explants toward *Agrobacterium* infection, optical density (O.D.) of *Agrobacterium* suspensions, incubation and co-cultivation period were optimized in conducting transformation experiments. Moreover, the efficiency of transformation and transgenic plant production depends on the establishment of suitable protocols including the co-cultivation of host cell/tissue, regeneration and selection of transgenic plantlets.

Ding *et al.,* [9] obtained more than 100 putative transgenic after pre-culturing the explants for three days before infection with *Agrobacterium*. Zhang *et al.* [10] noted that in Chinese cabbage, co-cultivation for 2 days yielded the highest transformation frequency whereas in chickpea Krishnamurthy *et al*. [11] were able to obtain transgenic plants by using co-cultivation of explants for 3 days.

From the above background information of transformation the present study was conducted to see the effect of explants, bacterial density, incubation time, and co-cultivation period on genetic transformation of lentil.

2. Material and methods

Three microsperma varieties of lentil (*Lens culinaris* Medik) namely, BARI Masur-4 (BM-4), BARI Masur-5 (BM-5) and BARI Masur-6 (BM-6) cultivated in Bangladesh were used as the plant materials for this investigation. Seeds of these three 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 (CN), cotyledon attached decapitated embryo (CADE) and Decapitated embryo (DE) were used for genetic transformation of lentil. For seed germination, the seeds were first soaked in 70% ethanol for 1 minute and then they were surface sterilized with 0.1% $HgCl_2$ solution for 15 minutes. Seeds were then washed three - four 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 days old germinated seedlings following the removal of seed coats. The shoot and root tip portion were excised and discarded. CADE and DE explants were collected from overnight soaked sterilized seeds. The seed coats are removed and the seeds were split open and two cotyledons were separated. Before inoculation the shoot and root meristems from these embryos were excised. In cotyledon attached decapitated embryo explants, one part of cotyledon with embryos attached to it and sliced embryos were cultured. Explants were then placed on MS medium with or without supplemented with 0.5 mg/l BAP + 0.5 mg/l Kn + 0.1 mg/l GA_3 + 5.5 mg/l tyrosin for regeneration of shoots. The cultures were maintained under fluorescent illumination on a 16 h photoperiod at 25 ± 2°C.

Agrobacterium tumifaciens strain LBA4404 with the binary plasmid pBI121 (figure 1) was used for transformation experiments. The binary vector pBI121 has the background of pBIN19. It contains 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 [12]. Fifty ml of liquid YMB 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 following the method done by Hooykaas [13].

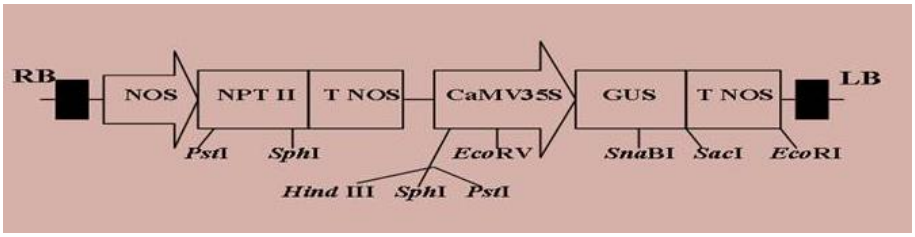

Figure 1 Diagrammatic representation of *Agrobacterium tumefaciens* strain LBA4404 containing plasmids pBI121 showing a part of T-DNA between left and right border

To prepare the *Agrobacterium* suspension overnight grown *Agrobacterium* culture taken from bacterial plate was centrifuged for 10 minutes at 5000 rpm and the pellet was re-suspended in liquid MS medium (pH 5.8). This *Agrobacterium* suspension was used for infection of explants. Before doing infection of the explants "Optical Density" or O.D. of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer (Shimadzu, Japan). To perform suitable and sufficient infection of the explants, lightly injured explants were dipped in bacterial suspension for different incubation periods. Following infection and incubation, the explants were soaked in filter papers for a short period of time to remove excess bacterial suspension before transferring them to co-cultivation medium. Then explants were maintained in co-culture medium for 2 - 4 days in dark condition on MS media with or without growth regulators.

Transformation ability of the explants was monitored by GUS histochemical assay [14, 15]. In this assay transformed explants shows insoluble indigo blue colour at the site of the cut end for GUS enzyme activity. Thus, it allows transformed tissues to be screened histochemically. For monitoring stable GUS expression tissues and shoots were kept under selection pressure of around two months.

2.1. Preparation of MS media

Commercially available dry powdered MS medium (Duchefa Biocheme, Netharlands) containing all the constituents of MS medium (inorganic salts, vitamins and amino acids) except sucrose and agar were used to prepare medium for *in vitro* regeneration of plants. Appropriate amount of MS powder is dissolved in distilled water (10% less than the final volume of the medium), and after adding sucrose (3%), the pH was adjusted to 5.8. To prepare agar solidified media, 0.8% agar was added.

2.2. Preparation of *Agrobacterium* culture media

For the growth of *Agrobacterium tumefaciens*, YMB medium was used. This media was prepared in the following manner:

Table 1 Composition of YMB media

Components	Concentration of components in gram per 1000 ml medium
Yeast Extract	0.4
Mannitol	10.0
NaCl	0.1
$MgSO_4.7H_2O$	0.2
K_2HPO_4	0.5

All the above ingredients of the medium were added in 750 ml distilled water and mixed properly. Then the final volume was made up to 1000 ml by adding distilled water. The pH was adjusted at 7.0 - 7.2 before autoclaving the media.

To prepare working culture medium (to make bacterial suspension), liquid YMB medium was used whereas; maintenance medium was prepared by adding 7.5 g agar to the medium before autoclaving. Filter sterilized antibiotic kanamycin (100 mg/l), streptomycin (50 mg/l) and rifampicin (10 mg/l) was added to the autoclaved and cooled liquid medium (working) prior to bacterial inoculation. The medium was then poured into petri plates and these culture plates were stored at 4˚C. After solidification, the media were ready for bacterial culture.

2.3. Preparation of GUS assay solution

For the preparation of GUS staining solution following steps were followed-

Firstly few drops of DMSO (Dimethyle sulphoxide) were taken in a beaker and 0.9 gm of X-gluc was added. The beaker was kept in shaker and gently shaken until X-gluc was completely dissolve. Then 2 ml of Chloramphenical was added into the beaker. After that, 1 ml of 10% titron X was added to the X-gluc solution and Finally 20 ml methanol was added to the solution and gently mixed. The pH was adjusted to 7.15 by adding pH- 10 buffer solution.

3. Results and discussion

3.1. Optimization of different parameters influencing transformation efficiency of explants

Available reports on *Agrobacterium*-mediated genetic transformation indicate that, transformation efficiency is influenced by several factors such as optical density (O.D.) of *Agrobacterium* suspensions, incubation time, co-cultivation period and culture media for co-cultivation of explants etc. Optimizations of these conditions were done by monitoring transient expression of the GUS reporter gene after co-cultivation of explants in *Agrobacterium* strains LBA4404 containing binary plasmid pBI121.

3.2. Influence of optical density (O.D.) and incubation period of *Agrobacterium* suspension on transformation

A specific density and time period is required by *Agrobacterium* for its attachment and for transfer of its T- DNA to explants. Less incubation time period, of course produces low number of transformed explants whereas higher time period can also be lethal due to overgrowth of *Agrobacterium* that behaves as parasites with to explants and hence low frequency of transformation is occurred.

In this experiment the relationship between optical density and incubation period of *Agrobacterium* suspension as well as transformation efficiency of explants were studied. For this purpose optical density (O.D.) was measured at 600 nm and bacterial suspension with optical density of 0.5, 0.8, 1.0 and 1.2 having incubation period of 30, 45 and 60 and 75 minutes were used in these experiments. Transformation efficiency was found to increase with the increased optical density. Maximum percentage of transformation was observed at O.D of 1.0 and 1.2. At O.D of 1.2 the percentage of GUS positive explants was 80, whereas minimum transient GUS expression (15 %) was recorded at O.D. of 0.5 (table 2).

These experiments also exhibited that the percentage of GUS positive explants was increased with the increase of incubation period but increase of the percentage of GUS positive explants was not remarkable beyond 45 minutes of incubation. The maximum percentage of GUS positive explants was observed at 45 and 60 minutes of incubation at O.D of 1.2 (table 2). It was also observed that when the duration of incubation and optical density of the bacterial suspension was more than 60 minutes and 1.0 respectively then overgrowth of bacteria in the co-cultured plates was observed which hampered desired regeneration capability of the explants. In some cases overgrowth of bacteria was noticed following one or two subcultures when the explants were treated with the above mentioned conditions. This undesired overgrowth of bacteria can be checked when bacterial suspension had an O.D. of 1.0 with incubation period of 45 min. Therefore, the optimum incubation period for the explants was found to be 45 minutes with optical density of 1.0.

Jaiwal *et al.* [16] also reported such results where the highest transformation efficiency in *Vigna radiata* at O.D. of 1.0. A similar result has also been reported in many plant species, such as alfalfa [17], oil palm [18] and chickpea [11].

Table 2 Influence of optical density (measured at 600 nm) of *Agrobacterium* suspension and effect of different incubation periods on *Agrobacterium*-mediated transformation of various explants of BM - 5 variety of lentil analyzed through transient GUS histochemical assay

$O.D_{600}$	Incubation period (min)	Number of explants infected	Number of explants assayed for GUS	Number of GUS+ve explants	Percentage of GUS+ve explants (%)
0.5	15	50	20	3	15
	30	50	20	4	20
	45	50	20	5	25
	60	50	20	7	35
0.8	15	50	20	6	30
	30	50	20	6	30
	45	50	20	8	40
	60	50	20	9	45
1.0	15	50	20	6	30
	30	50	20	9	45
	45	50	20	16	80
	60	50	20	16	80
1.2	15	50	20	10	50
	30	50	20	13	65
	45	50	20	16	80
	60	50	20	16	80

3.3. Influence of co-cultivation period on transformation

Transformation frequency and duration of co-cultivation have been reported to be directly correlated as has been described in peas [20]. In most of the transformation experiments 2 to 3 days of co-cultivation period was used as has been reported in tobacco [21]. However, Arundhati [22] reported increased frequency (47.8%) of transformation when leaf discs explants of pigeon pea were co-cultured for a period of 4 days. These results revealed that duration of infection and co-cultivation are genotype specific and has great influence on transformation frequency.

In the present investigation four different co-cultivation periods ranging from 2 - 5 days were tried to find out the optimum co-cultivation period. It was found that, percentage of transformation could be increased with the increase of co-cultivation period at a constant optical density (1.0) of bacterial suspension and a constant incubation period of 45 minutes. Although percentage of GUS positive explants increased with the increase of co-cultivation period, it was also observed that a co-cultivation for more than 3 days sometimes promoted overgrowth of bacteria (figure 2). As a result good number of explants in co-culture media was found to suffer from poor health and became brown thus failed to regenerate. Therefore, 3 days of co-cultivation period was found to be optimum for transformation.

In chickpea Krishnamurthy *et al.* [11] incubated mature embryo explants for 20 minutes and then co-cultivated the explants for 3 days and were able to obtain transgenic plants. Tewari-Singh *et al.* [23] employed the same co-cultivation period of 3 days but incubated explants in bacterial suspension for 1- 2 hours and got transgenic plant. In the present study it was found that, such longer period of incubation (more than 45 minutes) and co-cultivation (more than 3 days) reduced survivability of explants and lead bacterial overgrowth in culture medium thus hampering the proper growth of infected explants. Warkentin and McHugen [24] reported that, they were able to observe transient GUS expression from inoculating lentil epicotyl explants incubated only for 10 - 15 minutes, but they did not mention the information on transformation frequency. In the same report they also mentioned that longer co-culture period was capable of enhancing the explants area for GUS expression. Although the inoculation period differs but their observation supports present results regarding the expression of GUS gene.

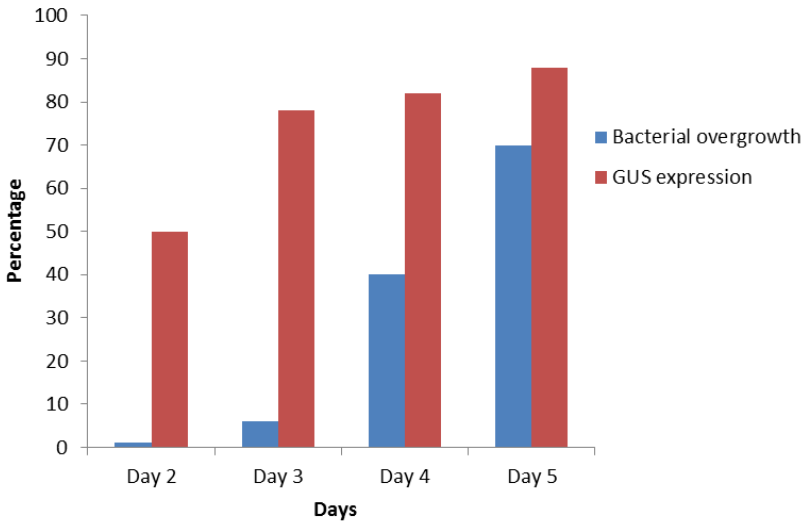

Figure 2 Interaction between bacterial overgrowth and GUS expression in context with co-culture period of explants in bacterial suspension

3.4. Type of explants

Transformation ability of various explants, namely, DE, CN and CADE of BM-4, BM-5 and BM-6 variety of lentil was monitored through the expression of the GUS gene following incubation for a period of 45 minutes with *Agrobacterium* strain LBA4404 having an O.D. of 1.0 and co-cultivation for 72 hrs. GUS expression was detected by histochemical assay and such expression was characterized by the formation of indigo blue colour within the transformed cells of the infected explants. Control explants were always maintained in each set of experiment and were subjected to GUS histochemical assay in order to understand the difference between transformed and non-transformed tissue. Results of these experiments are presented in table 3.

Table 3 Responses of various explants of BM - 4 variety of lentil towards GUS histochemical assay following co-cultivation

Variety	Explants	Number of explants assayed for GUS	Number of GUS+ve explants	Percentage of GUS+ve explants (%)
BM-4	CN	95	53	55.79
	CADE	85	72	77.70
	DE	90	70	84.70
BM-5	CN	146	47	32.19
	CADE	126	105	75.38
	DE	120	91	83.33
BM-6	CN	150	58	38.60
	CADE	138	120	78.18
	DE	110	86	86.90

*CN= Cotyledonary node, CADE= Cotyledon attached decapitated embryo, DE= decapitated embryo

A good number of co-cultured explants showed positive to GUS staining. GUS positive regions were visualized mostly at the peripheral area of the cut surfaces as well as within the internal tissues of various explants (fig. 3A- D).

Prominent blue coloured (GUS+ve) zones within co-cultured explants were visualized under stereomicroscope. In some cases the whole explant appeared to be blue in colour (fig. 3E) but in other cases this blue colour was localized in some

specific areas of the explant (fig. 3F). It was evident from table 3 that, DE explants showed the best response towards transformation with LBA4404 strain and the percentage of GUS positive DE explants was 86.9 in case of BM-6 variety of lentil. Next to DE explants CADE explants showed better responses towards transformation and in this case the percentage of GUS positive explants was found to be 78.18. In case of CN explants highest percentage of GUS positive explant were 58 in case of BM-6 which appeared to be the lowest among the three explants studied. This result is almost similar to the earlier results reported by Sarker *et al.* [25], Hassan *et al.*[26] and Das *et al*. [27] in lentil.

In some cases, much greater areas of explants exhibited GUS expression while in other cases only a small portion of the wounded tissue were competent for transformation. Similar results regarding the expression of the GUS gene in lentil tissue have been reported by Warkentin and McHughen [24] in lentil.

3.5. Effect of growth regulators in the co-cultivation media

The composition of co-culture media particularly the hormonal supplements is also influence transformation efficiency of *Agrobacterium*. In the present study it was observed that presence of growth regulator in co-culture media enhance the induction of adventitious shoots and found to improve the health of regenerated shoots. Schroeder *et al.* [28] reported that in case of *Pisum sativa* presence of growth regulators in the co-cultivation media enhanced recovery of putative transgenic plants. In chickpea, Kar *et al*. [29] reported that absence of growth regulators in co-cultivation media greatly reduced transformation efficiency and recovery of transgenic plants.

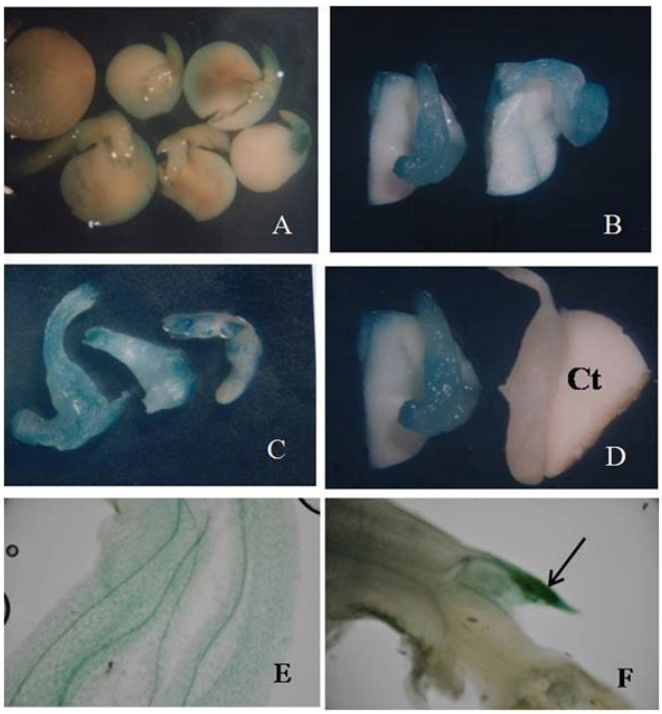

Figure 3 GUS activity in explants. A-Histochemical localization of GUS activity in explants of CN ×20. B- Same as fig. A but in case of CADE explants ×20. C- Same as fig. A but in case of DE explants ×20. D- Same as fig. B, but showing the absence of GUS activity in control (Ct) explants ×20. E- Magnified view of internal tissue showing positive GUS expression conspicuous blue color in whole explant. F- Same as fig. E, showing localized blue color within the internal tissue (arrow) of CADE explant

4. Conclusion

An efficient protocol for *Agrobacterium* mediated genetic transformation in lentil was developed which showed transfer of marker genes (GUS and *npt*II). DE explants showed highest transformation frequency having an optical density (O.D.) of 1.0 with 45 minutes of incubation and 3 days of co-cultivation. Next to DE, CADE showed positive response towards *Agrobacterium* mediated genetic transformation but this explants showed highest frequency of regeneration and survival rate. Further molecular analysis like PCR, southern and northern hybridization, RT PCR etc. to be needed to confirm transformation of putative transformants. Thus in further investigation agronomically and economically important traits could be transfer to the locally grown lentil varieties using this protocol.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no conflict of interests. Subroto K. Das conducted the experiments, including statistical analyses and mainly wrote the manuscript. R. H. Sarker and M. I. Hoque supervised the experiments and gave suggestion in preparing manuscript.

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