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Combined Effect of Explant, Inoculation Time and Co-cultivation Period on *Agrobacterium* - Mediated Genetic Transformation in Cucumber (Var. Shital)

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Abstract

Combined effect of explant, inoculation time and co-cultivation periodon *Agrobacterium*-mediated genetic transformation in cucumber was observed. Transformation ability was examined by histochemical assay of GUS reporter gene in survived calli. Conspicuous GUS positive (blue colour) region were detected in callus tissue. There were 3 factors in this investigation. Factor A consisted of three types of explants viz. leaf, nodal and internodal callus, factor B consisted of two durations of inoculation time viz. 3 and 5 min and factor C consisted of two co-cultivation periods viz. 24 and 48 hours. The highest number (4.04) and percentage (67.38%) of GUS +vecalliwas obtained from leaf explants when they were immersed in *Agrobacterium* suspension for 5 min and kept into co-cultivation media for 48 hrs.The lowest number(0.95) and percentage (15.89%) of GUS +ve calluswere observed in internodal explants when they were inoculated in *Agrobacterium* for 3 min and then co-cultivated for 24 hrs.

Keywords

Cucumber, Genetic Transformation, Abiotic Stress

1. Introduction

Cucumber (*Cucumissativus* L.) (2n = 14), a member of the family Cucurbitaceae, is one of the oldest vegetable crop supposed to be originate in India, between the Bay of Bengal and the Himalayas (Peirce, 1987) [12]. *Cucumissativus* L. is a cucumber species which has commercial importance (Nonnecki, 1989) [11].

The total area and production of cucumber in Bangladesh during 2003 - 04 were 13925 ha and 25215 mt, respectively (BBS, 2005) [2]. The production has increased upto 32000 mt during the year 2006-'07 (BBS, 2008) [1]. The data indicates that total production has increased during the last few years with increased demand of cucumber. However, average yields of cucumber during 2002-'03, 2003-'04 and 2004-'05 were4.45, 4.45 and 4.37mt/ha, respectively (BBS, 2006) [3] which indicate that the yield has declined slightly. Yield of cucumber is very low in our country compared to leading cucumber producing countries like China (12.24 t/ha), former USSR (7.57 t/ha), Japan (44.23 t/ha), USA (11.06 t/ha), Turkey (16.07 t/ha), Netherlands (192.50 t/ha), Spain (30.00 t/ha) (Nonnecki, 1989) [11]. Abiotic stresses include drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and the natural states of environment (Wang *et al.*, 2003) [15]. Without these, the population of our country is increasing day by day but the land is decreasing. Therefore, we need to utilize the lands which are not under cultivation at present, such as, coastal zone which have high saline properties. That's why we need millions of healthy cucumber seedlings in a short period of time.

In crop improvement, genetic transformation offers the ability to introduce new characters into a plant cultivar without altering its existing traits (Gardner, 1993) [6]. In all transformation experiments, specific reporter gene and one or more selectable marker gene are required to be introduced into the plant cell prior to the integration of gene (s) of

interest. In this case, GUS-A (β-glucoronidase) gene and neomycin phosphotransferase IItermed as *npt* II (kanamycin resistant) gene have been used as reporter and selectable marker gene respectively. This reporter gene can be recognized in plant tissue with the help of selectable agents, confirming transformation of the plant tissue (through histochemical GUS assay). So, in this way, one can understand that the plant tissue subjected for transformation has really been transformed or not (Gardner, 1993) [6].

Ding *et al.* (1998) [24] obtained more than 100 putative transgenic plants after preculturing of the explants for three days before infection with *Agrobacterium* plasmid vector. The three days preculture period prior to inoculation of hypocotyls with *Agrobacterium* was essential for reduction of excision shock. Tsukazaki *et al.* (2002) [25] kept pre-cultured hypocotyls explants of cabbage for three days on MS medium. Kanamycin tolerant shoots were regenerated onto shoot induction medium three months after *Agrobacterium* infection. Zhang *et al.* (2000) [26] noted that in Chinese cabbage, co-cultivation for two days yielded the highest transformation frequency. The efficiency of transformation was markedly increased by co-cultivation of cell clumps with *A. tumefaciens* (Belarmino and Mii, 2000) [27] in a *Phalaenopsis* orchid. Yen *et al.* (2000) [28] mentioned that analysis of co-cultivation regimes revealed that the shorter co-cultivation periods resulted in higher number of explants survival. From the above background information it was revealed that tissue culture and genetic transformation of cucumber depend on several factors. So the present investigation was conducted to see the combined effect of explant, inoculation time and co-cultivation period on genetic transformation of cucumber.

2. Materials

2.1. Plant Material

Leaf, nodal and internodal calli of variety Shital were used in present investigation.

2.2. Genetic Transformation Material

2.2.1. *Agrobacterium* Strain, Plasmid and Gene

Genetically engineered *A. tumefaciens*strain LBA4404 was used for infection in the pre-cultured explants. The strain is being maintained at the Biotechnology lab. Under Bangladesh Agricultural University. This strain contains plasmid pBl121 of 14 kDa (binary vector). This binary vector contains following genes within the right border (RB) and left border (LB) region of the construct-

i. The *uidA*gene (Jefferson, 1986) [7] encoding *GUS*(*β-glucuronidase),* driven by CaMV promoter and *NOS* terminator. This reporter gene can be used to assess the efficiency of transformation.
ii. The *npt*II gene encoding *neomycinphosphotransferase* II *(npt*II*)*conferring kanamycin resistance, driven by *NOS* promoter and *NOS* terminator.
iii. The CIPK sense gene encoding calcineurin B-like protein conferring abiotic stress tolerance.

2.2.2. Calcineurin B-like Proteins (CIPK)

Calcineurin (Cn) is a unique Ca^{2+} dependent serine/threonine protein phosphatase (PP2B) of cytosol, which plays an important role in the coupling of Ca^{2+} signals to stress responses. Using degenerate primers from the conserved domains and by library screening a full-length cDNA (CIPK, 972 bp) was isolated from pea (accession no: AY883569). Plants respond to adverse environments by initiation a series of signaling processes that often involves diverse protein kinases, including calcineurin B-like protein interacting protein kinases (CIPKs). Putative CIPK genes (O_sCIPK01 - O_sCIPK30) survived for their transcriptional responses to various abiotic stresses, like drought, salinity, cold, polyethylene glycol and abscisic acid treatment. To prove that some of these stress-responsive CIPK genes are potentially useful for stress-tolerance improvement, three CIPK genes (CIPK 03, CIPK 12, CIPK 15) were over expressed in Japonica rice. Transgenic plants over expressing the transgenes CIPK 03, CIPK 12, CIPK 15 showed significantly improved tolerance to cold, drought and salt stress, respectively. Under cold and drought stresses, CIPK 03, CIPK 12, over expressing transgenic plants accumulated significantly higher content of proline and soluble sugars. and putative proline synthetase and transporter genes had significantly higher expression level in the transgenic plants, against different stresses (Mahajan and Tuteja, 2005) [8].

3. Methods

3.1. Treatments

There were 3 factors in this experiment. Factor A consisted of three types of callus, factor B consisted of two inoculation times and factor C consisted of two co-cultivation periods.

A. Explant: Leaf, nodal and Internodal calli

B. Infection time: 3 and 5 minutes

C. Co-cultivation period: 24 and 48 hours

Total no. of treatments were 12 (3x2x2). Each treatment consisted of 4 vials and replicated three times.

Design: Factorial in Completely Randomized Design (CRD)

3.2. Media Used

Media used in the present study were as follows.

A. For callus induction

For induction and maintenance of callus, MS(Murashige and Skoog, 1962) [9] medium supplemented with different concentrations and combinations of BAP (benzyl amino purine) and NAA (α–napthalene acetic acid) were used.

B. For *Agrobacterium* culture

Two types of culture media, namely, YMB (Yeast Extract Mannitol Broth) medium and LB (Luria Broth) medium were used with kanamycin as antibiotic to grow the strain of genetically engineered *Agrobacterium tumefaciens.* YMB

medium was used for *Agrobacterium* maintenance and LB medium was used as *Agrobacterium* working culture medium for transformation work.

C. For co-cultivation

MS media without growth hormones were used for co-cultivation.

D. For washing explants after co-cultivation

Cefotaxime (200 mg/l) was used for washing the explants after co-cultivation.

E. For Post-cultivation and regeneration

MS media supplemented with 2 mg/l BAP, 1 mg/l NAA and 100 mg/l cefotaxime were used for this purpose.

F. For selection and regeneration

Low selection medium: MS media supplemented with 2 mg/l BAP, 1 mg/l NAA, 20 mg/l kanamycin and 100 mg/l cefotaxime were used.

High selection medium: MS media supplemented with 2 mg/l BAP, 1 mg/l NAA, 30 mg/l kanamycin and 100 mg/l cefotaxime were used.

3.3. Preparation of Culture Media

3.3.1. Preparation of MS Medium

The MS media used in this investigation were fortified with different concentrations and combinations of required auxin and cytokinin. They were added in the medium before the adjustment of P^H of the solution.

3.3.2. Preparation of *Agrobacterium* Culture Medium

YMB medium was used for the maintenance of *Agrobacterium* strain LBA4404. The composition (Begam, 2007) [4] of the medium given below-

Mannitol	1.0%
Yeast extract	0.04%
$MgSO_4.7H_2O$	0.02%
NaCl	0.01%
KH_2PO_4	0.05%

The pH was adjusted to 7.0-7.2 before adding agar at 1.5%. After autoclaving the medium was cooled to 50-550C and kanamycin was added at a rate of 0.05 mg/l and separated in petridishes.

3.3.3. Preparation of LB (Luria Broth) Medium

To prepare one liter (1000 ml) of LB medium, the following steps were followed-

i. 15.5 g of LB (Luria Broth) powder was taken into a 2-liter beaker on a magnetic stirrer.
ii. 400-500 ml of distilled water was poured in the beaker to dissolve the powder
iii. After dissolution the medium was transferred to a 1 liter measuring cylinder or volumetric flask and volume was made up to the mark with distilled water.
iv. Then the pHof the medium was adjusted to 7.0-7.2 with 0.1 N NaOH
v. The medium was transferred back to stirred beaker to allow full mixing.
vi. Batched (25-50 ml) of medium was transferred to clean 250 ml conical flasks and plugged with non-absorbent cotton wool. The tops were covered with aluminum foil.

3.3.4. Preparation of GUS Assay Solution

The GUS straining solution is composed of the following chemicals.

Components	Amount/10ml
X-gluc (solvent: DMSO)	8.89 mg
Chloramphenicol	200 µl.
NaH_2PO_4	119.8 mg
Triton X (10%)	100 µl
Methanol	2 ml

pH was adjusted at 7.0- 8.0 by adding pH -10 buffer solution

For the preparation of 10 ml GUS straining solution, the following steps were followed-

- All necessary glasswares were autoclaved.
- The 8.89 mg X-gluc was weighted.
- Few drops of DMSO (Dimethyl Sulphoxide) were taken in a beaker and X-gluc was added.
- Gently shaken until all the X-gluc was dissolve.
- 200 µl of chloramphenicol was added into the beaker.
- 10% titron X was prepared. Then 100 µl Titron X from this solution was added to the X-gluc solution.
- 2 ml methanol was added to the solution and gently mixed and pH was adjusted to 7.15 by adding pH -10 buffer solution.

3.4. Sterilization Techniques

3.4.1. Sterilization of Culture Media

The glasswares with medium were sterilized under 1.09 kg/cm^2 pressure at 121°C for 25 min.

3.4.2. Sterilization of Glasswares and Instruments

Beakers, test tubes, conical flasks, pipettes, metallic instruments like forceps, scalpels, and inoculation loop, micropipette tips, eppendorf tubes, needles, spatulas were wrapped with aluminium foil, vials were capped with plastic cap and then sterilized in an autoclave at a temperature of 121°C for 30 minutes at 1.16 kg/cm^2 pressure.

3.5. Culture Techniques

i. Explant culture

Explants (Leaf, node and internodal calli) were produced in present experiment from the shoots of cucumber seeds (variety Shital). Explants were separately placed horizontally on each vial and gently pressed into the surface of the sterilized culture medium supplemented with various concentrations and combinations of BAP (0, 1 and 2 mg/l) and NAA (1, 2 and 3 mg/l). The culture vials containing explants were placed under dark in growth room with controlled temperature (25 ± 1°C). The vials were checked daily to note the response and the development of contamination if any.

ii. Agrobacterium culture

For maintenance the strain, one single colony from previously maintained *Agrobacterium* stocks was streaked onto freshly prepared petridish containing YMB medium having kanamycin. The petridish was sealed with parafilmand kept at room temperature for at least 48 hours. This was then kept at 4°C to check over growth. Such culture of *Agrobacterium* strain was thus ready to use for liquid culture. The cultures were subcultured regularly at each week in freshly prepared media to maintain the stock.For infection single colony of *A. tumefaciens* was picked and inoculated in a conical flask containing liquid LB medium with 50 mg/l kanamycin. The culture was allowed to grow at 28°C to get optimum growth of *Agrobacterium* for infection and co-cultivation of explants (calli).

iii. Infection

The *Agrobacterium* grown in liquid LB medium was used for infection. Prior to this, optical density (OD) of the bacterial suspension was determined at 600 nm (OD_{600} = 0.60) with the help of a spectrophotometer. Following the determination of density, to pre-culture explants (calli) were dipped into bacterial suspension for 3 and 5 min, respectively, before transferring them to co-cultivation medium.

iv. Co-cultivation

Following infection, the explants were co-cultured on co-cultivation medium. Prior to transfer of all explants (callus) to co-cultivation medium they were blotted with sterile tissue papers for a short period to remove excess bacterial suspension. All the explants were maintained in co-cultivation medium for 24 and 48 hours, respectively. Co-cultured explants were placed under fluorescent illumination with 16/8 hours light/dark cycle at (25±2°C). The intensity of light was maintained at 1800 lux (approximately). The culture vials were checked daily to observe any contamination and the behaviors of the explants.

v. Washing and post-cultivation

After co-cultivation for required periods, the infected explants were washed twice with sterile ddH_2O (double distilled water) and once with sterile ddH_2O containing 200 mg/l cefotaxime. Then the explants were transferred onto post-cultivation medium containing 100 mg/l cefotaxime.

vi. Transfer to selection medium

Following one week of post-cultivation, the explants were transferred onto low selection MS medium supplemented with 2 mg/l BAP + 1 mg/l NAA + 20 mg/l kanamycin + 100 mg/l cefotaxime and also onto high selection MS medium fortified with 2 mg/l BAP + 1 mg/l NAA + 30 mg/l kanamycin + 100 mg/l cefotaxime.

vii. GUS (β-Glucuronidase) histochemical assay

From each batch of calli following each transformation experiment, randomly selected survived calli were examined for GUS histochemical assay. For this test survived calli were immersed in X-gluc (5-bromo-4-chloro-3-indoly-1-glucuronide) solution and were incubated at 37°C for overnight. A characteristic blue color would be the expression of GUS (*β-Glucuronidase*) gene in the plant tissue. Proper control for GUS histochemical assay was done with the explants having no *Agrobacterium* infection. After X-gluc treatment explants were transferred to 70% alcohol for degreening. Following degreening explants were observed under stereomicroscope (Begam, 2007) [4].

viii. Transfer of the selected materials to regeneration medium

After ten days, the survived calli were transferred to regeneration medium consisting of MS medium supplemented with 1 mg/l NAA + 2 mg/l BAP + 20 mg/l kanamycine + 100 mg/l cefotaxime for regeneration.

3.6. Data Recording

To investigate the effects of different treatments and responses of different varieties to callus induction subsequent inoculation and regeneration, data were collected from the different parameters as given below.

a) Number of survived callus

The number of callus that is survived in each vial was recorded. The percentage of survived callus was calculated on the basis of the number of callus survived and the total number of callus tested with antibiotics.

b) Per cent of survived callus = $\frac{No.\ of\ survived\ \ callus}{Total\ no.\ of\ cultured\ callus} \times 100$

c) Number of callus positive for GUS assay

The number of callus giving positive response to GUS histochemical assay was recorded.

d) Percentage of callus positive for GUS (Percent GUS expression) assay

The percentage of GUS positivecalli were calculated on the basis of the number of calli assayed for GUS and the total number of calli positive for GUS.

4. Results and Discussion

Combined effect of explant, inoculation time and co-cultivation period

All the explants performed better when they were infected in *Agrobacterium* suspension for 5 min then co-cultivated for 48 hrs (Table 1). The highest number (14.22) survived calli were obtained from leaf explants when they were immersed for 5 min in *Agrobacteirum* suspension and then transferred into co-cultivation medium for 48 hrs and the lowest number (9.65) survived calli were obtained from internodal explants when they were inoculated for 3 min and co-cultivated for 24 hrs. The highest and the lowest percentage (59.26% and 40.22%, respectively) of survived calli were found from the combined effect of leaf explant x 5 min inoculation time x 48 hrs co-cultivation period and internodal explants x 3 min inoculation time x 24 hrs co-cultivation period, respectively (Fig. 1).

Mohiuddin *et al.*(2000) [16], Rajagopalan and Perl-Treves (2005) [13], Vengadesan *et al.* (2004) [17], Nishibayashi *et al* . (1996) [10], Raharjo *et al.* (1996) [18], Chee (1990) [19], Chee and Slightom (1991) [20] and Trulson*et al.* (1986) [21] had successfully accomplished the *Agrobacterium* mediated genetic transformation work on cucumber. So, the results obtained from present investigation were partially agreed

with and partially differed from the different findings of cucumber reported by different researchers. This study was carried out to develop a genetic transformation protocol as well as to emphasized the importance of tissue culture conditions for transformation efficiency and demonstrated that the success in genetic transformation studies depend on existing tissue culture conditions of Bangladesh. The *Agrobacterium* mediated transformation system may be used to develop different biotic and abiotic stress tolerance/resistance transgenic cucumber with economically important genes either to increase production or to improve nutritional qualities.

From the last one and half decades the bacterial gene uidA encoding B-Glucuroridase (GUS) has become the most frequently used reporter gene for the analysis of plant gene expression. It's wide acceptance has mainly resulted from the availability of a highly sensitive nonradioactive assay using fluorogenic substrate 4-MU gluc and of a histochemical assay using X-gluc, that allows a quantitative analysis of cell and tissue specific expression. The major advantage of this reporter gene is that it does not require DNA extraction, electrophoresis or autoradiography (Chawla, 2002) [5]. The highest number and percentage (4.04 and 67.38%, respectively) GUS +vecalli obtained from leaf explants when they were immersed in *Agrobacterium* suspension for 5 min and following immersion transferred into co-cultivation media for 48 hrs. Figure 2 showedtransformed and non transformed leaf calli in eppendorf tubes. Figures 3 and 4 showed blue patches that indicated GUS activity and confirming GUS expression in transgenic leaf and nodal callus, respectively. The lowest number and percentage (0.95 and 15.89%, respectively) of GUS + ve calluswere observed in internodal explants when they were inoculated in *Agrobacterium* for 3 min and then co-cultivated for 24 hrs. In an investigation of *Agrobacterium* mediated transformation of lentil Sarker *et al.* (2003) [22] observed the highest number and percentage (72 and 84.7%, respectively) GUS +vecalli from epicotyl explantsfollowed by decapitated embryo (70 and 77.7%, respectively). Previously Rachmawati and Anzai (2006) [23] reported that genetic transformation as well GUS histochemcial assay varied in plant to plant, genotype to genotype, species to species and depend on many other factors.

Table 1. *Interaction effect of explant, inoculation time and co-cultivation period on number and percentage of survived callus and GUS histochemical assay.*

Explant	Inoculation time	Co-cultivation period	Number of survived callus	Number of GUS +ve callus	% of GUS +ve callus
Leaf callus	3 min	24 hrs	11.18 d-f	2.57 c	42.83 c
		48 hrs	12.41 b-d	2.92 bc	48.72 bc
	5 min	24 hrs	13.17 a-c	3.67 ab	61.22 ab
		48 hrs	14.22 a	4.04 a	67.38 a
Nodal callus	3 min	24 hrs	10.54 ef	2.13 cd	35.50 cd
		48 hrs	11.64 c-e	2.76 bc	46.05 bc
	5 min	24 hrs	12.63 a-d	3.01 bc	50.27 bc
		48 hrs	13.58 ab	3.64 ab	60.66 ab
Internodal callus	3 min	24 hrs	9.65 f	0.95 e	15.89 e
		48 hrs	10.42 ef	1.58 de	26.33 de
	5 min	24 hrs	11.28 d-f	2.20 cd	36.66 cd
		48 hrs	12.43 b-d	2.46 cd	41.05 cd

Means in a column followed by uncommon letter (s) varied significantly at 5% level of significance

From last two decades, *A. tumefacines* mediated plant genetic transformation has become well established in numerous laboratories and at present the most preferred method for cucumber transformation. The advantages of this method includes high transformation ability, minimal rearrangement of the transgene and a relatively high percentage of the transgenic plants that harbour a single copy of the transgene (Roy *et al.* 2000) [14]. The future aim of cucumber breeding may be use this transformation technique to develop value-added transgenic cucumber varieties by transforming single or many transgenes into commercially important cucumber varieties of Bangladesh or any part of the world.

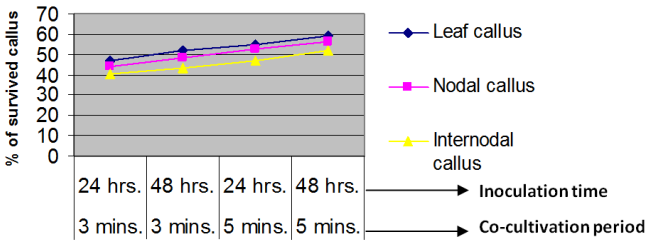

Fig. 1. *Interaction effect of explant, inoculation time and co-cultivation period on percentage of survived callus.*

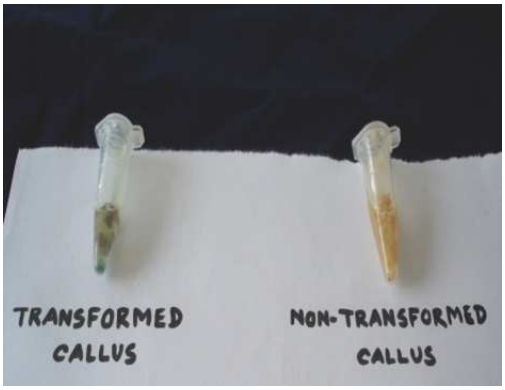

Fig. 2. *Successfully transformed (GUS +ve) (left)and non-transformed leaf calli (GUS –ve) (right).*

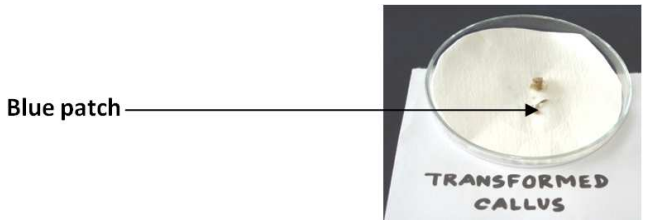

Fig. 3. *Transformed leaf callus showing GUS +ve response.*

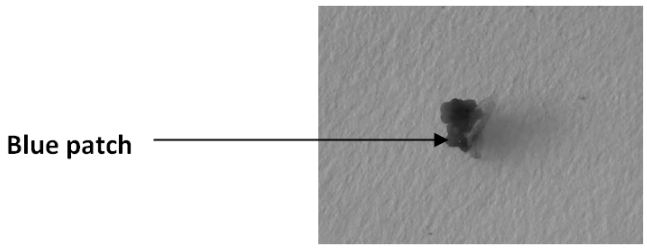

Fig. 4. *Transformedleaf callus showing GUS +ve response.*

5. Conclusion

An efficient protocol for genetic transformation in cucumber was developed which showed transfer of CIPK sense gene in variety Shital and integration of two marker genes (GUS and npt II). To obtain the highest number and percentage (4.04 and 67.38%, respectively) of GUS +vecalli leaf explants will be immersed in *Agrobacterium* suspension for 5 min and then keep into co-cultivation media for 48 hours.From the study it was observed that higher inoculation time and co-cultivation period were suitable than lower one. Further transgenecity confirmation test like PCR, southern blotting, sequencing 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 cucumber varieties using this protocolin Bangladesh.

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