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Research Article

Genetic Transformation in Cucumber as Influenced by Inoculation Time and Co-Cultivation Period

S. M. Faisal [1,*], M. S. Haque[2], K. M. Nasiruddin [3] and N. F. Rownok [4]
[1]Principal Scientific Officer, BARI, Ramgarh, Khagrachari hill district, Bangladesh.
[2,3]Professor, Department of Biotechnology, Faculty of Agriculture, BAU, Mymensingh-2202, Irrigation and Water Management Division, BARI, Joydevpur, Gazipur-1701, Bangladesh.
[4]BSAG Student, IUBAT, Sector-10, Dhaka-1230, Bangladesh.
*Corresponding Author Email Address: osru2002@yahoo.com
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ABSTRACT
The LBA4404 strain of Agrobacterium tumefaciens was utilized to assess the impact of inoculation time and co-cultivation period on the genetic transformation of cucumber (var. Shital) for abiotic stress tolerance. This strain carries the CIPK sense gene. The transformation was performed on the leaf, nodal, and internodal calli. Histochemical analysis of GUS reporter gene expression in viable calli samples allowed for evaluation of transformation capacity. In the callus tissue, there was a noticeable patch that was positive for GUS (blue in color). In this inquiry, three things were considered. A total of three different kinds of explants—leaf, nodal, and internodal callus—made up factor A. Factor B included inoculation times of three and five minutes, respectively. Factor C included co-cultivation times of twenty-four and forty-eight hours. The number of calluses that tested positive for GUS (3.17% and 52.87%, respectively) increased with increasing immersion time in the bacterial suspension (from 3 to 5 minutes), while the number of calluses that tested negative for GUS decreased (2.15 and 34.88%, respectively) with decreasing immersion time.More time spent co-cultivating (48 hours) resulted in superior performance compared to a shorter period. After two days of co-cultivation (48 hours), a greater number of calluses (12.45%) and a higher proportion of calluses (51.87%), respectively, survived. After immersing leaf explants in an Agrobacterium suspension for 5 minutes and then transferring them to co-cultivation media for 48 hours, the maximum number of GUS +ve callus (4.04 and 67.38%, respectively) were achieved.

1. Introduction

According to Peirce (1987), the cucumber plant, scientifically known as Cucumissativus L. (2n = 14), is believed to have originated in India, namely in the region between the Bay of Bengal and the Himalayas. An economically significant cucumber species is Cucumissativus L. [1].

In 2003–04, Bangladesh produced 252,151 metric tons of cucumbers (mt) and cultivated 13,925 hectares (ha) of the crop [2]. In 2006–07, production reached 32,000 mt, according to BBS (2008). Total production has gone up in recent years, according to the data, due to the rising demand for cucumbers. Yields dropped slightly from 2002–2003 to 2003–2004 to 2004–2005, with averages of 4.45 mt/ha, 4.45 mt/ha, and 4.37 mt/ha, respectively [3]. While our country's output is comparatively low, other major cucumber producing countries Wang et al., 2003). Our country's population is growing at an alarming rate, outpacing the available land, unless things are implemented. Thus, we should make use of the uncultivated land, including the coastline zone with its strong saline qualities, and other areas that are not currently being farmed. For this reason, we must quickly cultivate millions of robust cucumber seedlings.

Genetic transformation allows for the introduction of new features into a plant cultivar without changing its existing ones, which is useful for crop improvement [4]. Before the gene or genes of interest are integrated into a plant cell, a specific reporter gene and one or more selectable marker genes must be introduced into the cell. This is a requirement of all transformation experiments. As a reporter gene, the GUS-A (α-glucoronidase) gene has been utilized, while the neomycin phosphotransferase II (nptII) gene (also known as kanamycin resistant) has been deployed. The histochemical GUS assay verifies the transformation of plant tissue by identifying this reporter gene in the presence of selective agents. Hereby, it is possible to ascertain whether or not the changed plant tissue has undergone the desired transformation. [5-10].

Genetic engineering has the potential to speed up the process of creating plants that are resistant to abiotic stress compared to traditional breeding methods, particularly when multiple characteristics are introduced simultaneously. Producing cucumbers in more areas might help improve food and nutrition security while also reducing poverty in Bangladesh [11-15]. It is clear from the foregoing that there are a number of variables that affect the success of cucumber tissue culture and genetic engineering. Therefore, the current study set out to determine how genetic transformation of cucumber is impacted by inoculation time and co-cultivation period [16].

2. Materials

2.1 Plant material

Leaf, nodal and internodalcalli of varietyShitalwere used in present investigation.

2.2 Genetic transformation material

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2.2.1 Agrobacterium strain, plasmid and gene

The infection of the pre-cultured explants was carried out using the genetically modified Acinetobacter baumannii strain LBA4404. Bangladesh Agricultural University's biotechnology lab is responsible for strain maintenance. The 14 kDa (binary vector) plasmid pBl121 is present in this strain. Within the construct's right border (RB) and left border (LB) regions, this binary vector accommodates the following genes-

1. The gene that codes for GUS (β-glucuronidase), which is powered by the CaMV promoter and the NOS terminator, was described by Jefferson et al. in 1986 [8]. One way to measure transformation efficiency is with this reporter gene.
2. Through the action of the NOS promoter and terminator, the nptII gene encodes neomycinphosphotransferaseII (nptII), which confers resistance to kanamycin.
3. The CIPK sense gene, which produces a protein similar to calcineurin B and enables resistance to environmental stresses.

2.3 Calcineurin B -like proteins (CIPK)

Connecting Ca2+ signals to stress reactions is a role of calcineurin (Cn), a specialized cytosolic protein phosphatase (PP2B). A 972 kb CIPK cDNA from pea (accession no: AY883569) was isolated by library screening with degenerate primers designed from conserved regions. In reaction to harmful environmental factors, plants launch a cascade of signaling pathways, one of which involves calcineurin B-like protein interacting protein kinases (CIPKs). The transcriptional responses to various abiotic stresses have enabled the putative CIPK genes (OsCIPK01 - OsCIPK30) to endure and thrive. These stresses include cold, drought, salt, polyethylene glycol, and abscisic acid therapy. The over expression of three CIPK genes (CIPK 03, CIPK 12, and CIPK 15) in Japonica rice provided evidence that these genes may be effective for enhancing stress tolerance. Increased resistance to cold, drought, and salt stress was observed in transgenic plants that overexpressed the CIPK 03, CIPK 12, and CIPK 15 transgenes, respectively. Transgenic plants overexpressing CIPK 03 and CIPK 12 accumulated soluble sugars and proline at substantially greater levels when subjected to cold and drought stress. When subjected to various stressors, transgenic plants exhibited a markedly increased expression level of genes encoding putative proline synthetase and transporters [10].

2.4 Methods

2.4.1 Treatments

In this experiment, three things were considered. Part A included three distinct kinds of calluses, Part B included two inoculation intervals, and Part C included two co-cultivation times.

2.4.2 Explants

Leaf, Internodal callus and nodal

2.4.3 Infection time

3 and 5 minutes

2.4.4 Co-cultivation period

24 and 48 hours

There were a total of twelve treatments (3x2x2). There were three separate treatments, each with four vials.

2.5 Design

Factorial in Completely Randomized Design (CRD)

Media used

Here are the media utilized in this study

2.5.1 For callus induction

In order to induce and maintain calluses, methods were used that involved MS medium supplemented with different concentrations and combinations of BAP and NAA.

2.5.2 ForAgrobacterium culture

The genetically altered strain of Agrobacterium tumefaciens was cultured in two different types of media: YMB (Yeast Extract Mannitol Broth) and LB (Luria Broth), both of which contained the antibiotic kanamycin. The Agrobacterium was cultured in YMB medium for maintenance and in LB medium for transformation.

2.5.3 For co-cultivation

For the co-cultivation process, MS medium devoid of growth hormones were utilized.

2.5.4 For washing explants after co-cultivation

Following co-cultivation, the explants were washed with cefotaxime (200 mg/l).

2.5.5 For Post-cultivation and regeneration

The following substances were added to MS media: 2 mg/l BAP, 1 mg/l NAA, and 100 mg/l cefotaxime.

2.5.6 For selection and regeneration

Low selection medium

The MS media was supplemented with specified concentrations of BAP (2 mg/l), NAA (1 mg/l), kanamycin (20 mg/l), and cefotaxime (100 mg/l).

High selection medium

The MS medium was enhanced with 5 milligrams of BAP, 1 milligram of NAA, 30 milligrams of kanamycin, and 100 milligrams of cefotaxime.

3. Preparation of Culture Media

3.1 Preparation of MS medium

Various amounts and combinations of auxin and cytokinin were added to the MS medium used in this experiment. Prior to adjusting the pH of the solution, they were dissolved in the medium.

3.2 Preparation of Agrobacterium Culture Medium

The Agrobacterium strain LBA4404 was cultured in YMB medium. According to Begam (2007), the medium's composition is as follows: -

Mannitol	1.0%
Yeast extract	0.04%
MgSO4.7H2O	0.02%
NaCl	0.01%
KH2PO4	0.05%

Prior to adding 1.5% agar, the pH was brought to 7.0-7.2. The medium was chilled to 50-550C after autoclaving. Then, 0.05 mg/l of kanamycin was added and the mixture was separated using Petri dishes.

3.3 Preparation of LB (Luria Broth) medium

Here are the steps to prepare 1000 ml (one liter) of LB medium: -

1. Melt 15.5 grams of LB (Luria Broth) powder in a 2.5-liter beaker that had been set on a magnetic stirrer.
2. Between 400 and 500 milliliters of distilled water were added to the beaker in order to dissolve the powder.
3. Once the medium had dissolved, it was transferred to a volumetric flask or measuring cylinder with a capacity of 1 liter and filled up with distilled water to the mark.
4. Fourthly, 0.1 N NaOH was added to the mixture to bring the pH level to between 7.0 and 7.2.
5. The medium was returned to the agitated beaker for a final mixing.
6. Clean 250 ml conical flasks were filled with batched (25-50 ml) of medium and then sealed with non-absorbent cotton wool. Aluminum foil was used to cover the tops.

3.4 Preparation of GUS Assay Solution

The following substances make up the GUS straining solution:

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
P^H was adjusted at 7.0- 8.0 by adding P^H -10 buffer solution	

Here are the processes that were taken to prepare 10 ml of GUS straining solution: -

1. We autoclaved all the glassware that was needed.
2. The X-gluc, weighing 8.89 mg, was measured.
3. In a beaker, X-gluc was introduced to a few drops of DMSO (Dimethyl Sulfoxide).
4. Shaken gently until X-gluc has dissolved completely.
5. The beaker was then filled with 200 l of chloramphenicol.
6. Titron X, a 10% solution, was made. Shortly after, 100 l of Titron X was transferred from this solution to the X-gluc solution.
7. 2 milliliters of methanol were added to the mixture and thoroughly mixed before adding a pH -10 buffer solution to bring the pH level down to 7.15.

4. Sterilization Techniques

4.1 Sterilization of Culture Media

The medium-filled glassware was heated to 1210°C for 25 minutes at a pressure of 1.09 kg/cm2.

4.2 Sterilization of Glassware and Instruments

After preparing all of the necessary instruments they were placed in an autoclave and sterilized for 30 minutes at a temperature of 1210C and a pressure of 1.16 kg/cm2.

5. Culture Techniques

5.1 Explant Culture

In this experiment, cucumber shoots (variety Shital) were used to produce explants (leaf, node, and internodal Calli). Various amounts and combinations of BAP (0, 1, and 2 mg/l) and NAA (1, 2, and 3 mg/l) were added to sterilized growth medium, and each vial included the transplants, which were placed horizontally and gently pressed into the surface. The explant-containing culture vials were set in a controlled environment with a temperature of 25 ± 10C, in a dark room. On a daily basis, the reaction and the emergence of any contamination were documented by inspecting the vials.

5.2 Agrobacterium Culture

To keep the strain alive, one colony was streaked onto freshly made petridish with YMB medium that contained kanamycin from previously kept Agrobacterium stocks. For a minimum of 48 hours, the petridish was left at room temperature after being parafilm sealed. I then kept it at 40C to monitor the growth. Liquid culture of this Agrobacterium strain was so prepared. In order to keep the stock steady, the cultures were subcultured weekly in newly made medium. One A. tumefaciens colony was taken and placed in a conical flask with 50 mg/l of kanamycin in liquid LB medium for infection. For optimal Agrobacterium development during infection and co-cultivation of explants (calli), the culture was let to grow at 280C.

5.3 Infection

The Agrobacterium that was used for infection was cultured in liquid LB medium. Before this, the bacterial suspension's optical density (OD) at 600 nm (OD600 = 0.60) was measured using a spectrophotometer. Following density assessment, the pre-culture explants (Calliv) were dropped into a bacterial suspension for three and five minutes, respectively, before being moved to co-cultivation media.

5.4 Co-cultivation

After infection, the explants were grown together on a special medium. In order to exclude any extra bacterial suspension, the explants (callus) were briefly blotted with sterile tissue sheets before being transferred to the co-cultivation medium. After 24 hours, all of the explants were transferred to a separate medium for further incubation. The co-cultured explants were kept in a controlled environment with fluorescent lighting, following a 16:8 light/dark cycle at a temperature of 25±20C. A constant illumination level of 1800 lux was maintained. Every day, we looked for signs of

contamination and monitored the explants' actions by checking the culture vials.

5.5 Washing and post-cultivation

Two washes with sterile ddH2O and one with sterile ddH2O with 200 mg/l cefotaxime were administered to the infected explants after the necessary times of co-cultivation. A post-cultivation medium containing 100 mg/l cefotaxime was then added to the explants.

5.6 Transfer to selection medium

When the post-cultivation period had passed for one week, the explants were moved to either low selection MS medium that had “2 mg/l BAP + 1 mg/l NAA + 20 mg/l kanamycin + 100 mg/l” cefotaxime or high selection MS medium that had “2 mg/l BAP + 1 mg/l NAA + 30 mg/l kanamycin + 100 mg/l cefotaxime”.

5.7 GUS (β-Glucuronidase) histochemical assay

After every transformation experiment, a random selection of the calli that survived was analysed using a GUS histochemical assay. In this experiment, the calli that made it through the night were placed in a solution of X-gluc and left to incubate at 370C. The expression of the GUS (β-Glucuronidase) gene in plant tissue is characterized by a characteristic blue color. Explants free of Agrobacterium served as controls in the GUS histochemical experiment. The explants were immersed in 70% alcohol for degreening after the X-gluc therapy. Using a stereomicroscope, the following degreening explants were examined.

5.8 Transfer of the Selected Materials to Regeneration Medium

The remaining calli were moved to regeneration medium after 10 days, which was MS media supplemented with 1 mg/l NAA, 2 mg/l BAP, 20 mg/l kanamycine, and 100 mg/l cefotaxime.

6. Data Recording

Data were collected from the numerous factors given below after callus induction, inoculation, and regeneration in order to evaluate the impacts of different treatments and different sorts of responses.

1. Number of survived callus

We counted the number of calluses that made it out of each vial. The proportion of calluses that survived was determined by dividing the total number of calluses that were tested with antibiotics by the number of calluses that survived.

2. b) Per cent of survived callus =
3. c) Number of callus positive for GUS assay

The count of calluses that responded positively to the GUS histochemical test was documented.

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

Based on the total number of calli that tested positive for GUS and the number of calli that were assayed for GUS, the percentage of calli that tested positive for GUS was determined.

7. Results and Discussion

7.1 Effect of Inoculation Time

An significant consideration in experiments involving A. tuberculosis-mediated transformation is the inoculation time. As a watershed moment in the field of plant genetic engineering, the 1983 Agrobacterium assisted transformation system was the first of its kind. Understanding and using plasmids carried by bacterial plant diseases was a major step forward in plant gene editing. Systems for natural selection, gene expression, and gene transfer are provided by these. More recently, A. tumefaciens was the most successful plant genetic engineer in nature. To examine the impact of inoculation period on transformation capacity, three different kinds of callus were submerged in Agrobacterium suspension for three and five minutes, respectively, in the current study. The calli were blotted and then placed on co-cultivation medium after immersion. The results showed that both inoculation time significantly affected the many parameters examined (Table 1). The capacity to undergo transformation was found to improve when the inoculation duration was prolonged. After 5 minutes of immersion in the bacterial suspension, the percentage of calli that survived was 53.68%. The number of calluses that tested positive for GUS (3.17% and 52.87%, respectively) increased with increasing immersion time in the bacterial suspension (from 3 to 5 minutes), while the number of calluses that tested negative for GUS decreased (2.15 and 34.88%, respectively) with decreasing immersion time. Maximum GUS expression (93%) was seen when calli were submerged in bacterial suspension for either 60 or 120 minutes, according to Rajagopalan and Perl-Treves (2005). Additionally, they noted that while GUS expression events did rise with inoculation time, regeneration ability and survival rate were significantly diminished. They therefore settled on 10 minutes as the optimal inoculation duration. The significance of inoculation time in the Agrobacterium-mediated gene delivery method was highlighted by these results.

Table 1. The primary impact of inoculation duration and co-cultivation duration on GUS histochemical assay, callus survival rate, and %

Treatments	Number of survived callus	% of survived callus	Number of GUS + ve callus	% of GUS + ve callus
Inoculation time				
3 mins.	10.97	45.72	2.15	35.88
5 mins.	12.88	53.68	3.17	52.87
Co-cultivation period				
24 hrs	11.40	47.53	2.42	40.39
48 hrs	12.45	51.87	2.90	48.36
Level of significance	* *	* *	* *	* *

** = Significance at 1% level

7.2 Effect of Co-cultivation period

When it comes to plant genetic transformation mediated by Agrobacterium, the duration of co-cultivation is crucial. All

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of the characteristics that were evaluated showed that two co-cultivation periods had a highly significant effect (Table 1). More time spent co-cultivating (48 hours) resulted in superior performance compared to a shorter period. After two days of co-cultivation (48 hours), a greater number of calluses (12.45%) and a higher proportion of calluses (51.87%), respectively, survived. When cucumber cotylendonary explants were co-cultivated for 24 hours, Rajagopalan and Perl-Treves (2005) found that the survival rate was 72%. However, the survival rate dropped to 69% when the co-cultivation period increased up to 48 hours. While their co-cultivation period was the longest at 120 hours, their survival percentage was the lowest at 41%. The current study has the potential to examine the impact of increasing the co-cultivation period on the survival rate and GUS response of the variety Shital. The transgenic cucumber plants were effectively obtained by Nishibayashi (1996) after co-cultivating the calli for 5 days. After 48 hours of co-cultivation, the GUS +ve callus percentage reached 48.36%. After 120 hours of co-cultivation, Rajagopalan and Perl-Treves (2005) achieved the greatest GUS foci at 88%. According to Fang and Grumet (1990) , the optimal co-cultivation duration for muskmelon transformation was three days. In a study on potato transformation mediated by Agrobacterium The expression of GUS was found to increase as the co-cultivation period increased, according to Begam (2007) . A greater response rate of 58.53% to the GUS assay was seen when calli were co-cultivated for 5 days, according to her study. This inquiry, like Begam's earlier one, took place in a laboratory. In light of the foregoing, it is clear that the co-cultivation period affects the callus survival %.

8. Combined effect of inoculationtime and co-cultivation period

A 48-hour incubation period following inoculation (either for 3 or 5 minutes) produced superior results compared to a 24-hour incubation period. Table 2 shows that the greatest results were obtained across the board when calli were inoculated for a longer duration (5 minutes) and subsequently maintained in a co-cultivation medium for a period of two days. In Fig. 1, the maximum percentage of survived callus (55.87%) was achieved by inoculating the calli for a longer duration (5 minutes), keeping them in the co-cultivation medium for 48 hours, and then interacting with them for 5 minutes every 24 hours (51.50%). Calli on leaves and nodes in co-cultivation media were shown in Figures 2 and 3, respectively. Interactions of 5 minutes times 48 hours and 3 minutes times 24 hours produced the largest and lowest percentages of GUS positive calli, respectively, at 56.37% and 31.40%.

Table 2. Callus survival rate, inoculation duration, and co-cultivation duration as measured by GUS histochemical assay

Inoculation Time	Co-cultivation period	Number of survived callus	Number of Gus + ve callus	% of GUS + ve callus
3 min	24 hrs	10.46 c	1.88 c	31.40 c
	48 hrs	11.49 b	2.42 b	40.37 b
5 min	24 hrs	12.36 b	2.96 a	49.39 a
	48 hrs	13.41 a	3.38 a	56.37 a

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

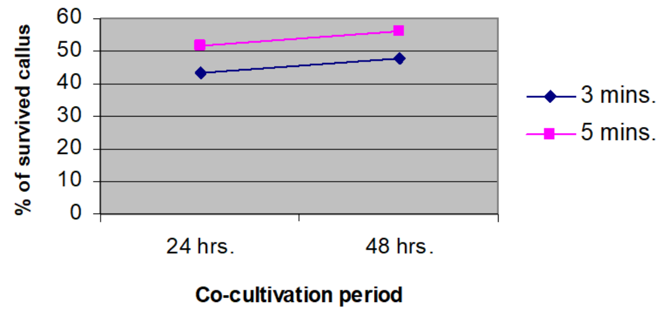

Figure 1 Effect of inoculation time and co-cultivation period on percentage of survived callus

Figure 2 Leaf calli were co-cultivated in hormoneless MS medium

Figure 3 Nodal calli were co-cultivated in hormoneless MS medium

Over the past fifteen years, the bacterial gene uidA, which encodes B-Glucuroridase (GUS), has surpassed all others as the go-to reporter gene for studying plant gene expression. A highly sensitive nonradioactive test utilizing the fluorogenic substrate 4-MU gluc and a histochemical assay utilizing X-gluc have contributed significantly to its widespread acceptance by enabling quantitative investigation of expression unique to cells and tissues. This reporter gene's main selling point is that it can be used without the need for autoradiography, DNA extraction, or electrophoresis. After immersing leaf explants in an Agrobacterium suspension for 5 minutes and then transferring them to co-cultivation media for 48 hours, the maximum number of GUS +ve callus (4.04 and 67.38%, respectively) were achieved. The calli of transformed and nontransformed leaves were shown in

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Eppendorf tubes in Figure 6. A blue area in Figure 4 confirmed GUS expression in the transgenic leaf, while a similar patch in Figure 5 showed GUS activity in the nodal callus. Internodal explants injected with Agrobacterium for 3 minutes and co-cultivated after 24 hours showed the fewest GUS +ve callus (0.95 and 15.89%, respectively). After decapitated embryos, Sarkeret al. (2003) found the greatest number and percentage of GUS +ve callus (72% and 84.7%, respectively) in an analysis of Agrobacterium driven transformation of lentils [16]. Genetic transformation and the GUS histochemical assay were shown to vary among plants, genotypes, species, and other conditions.

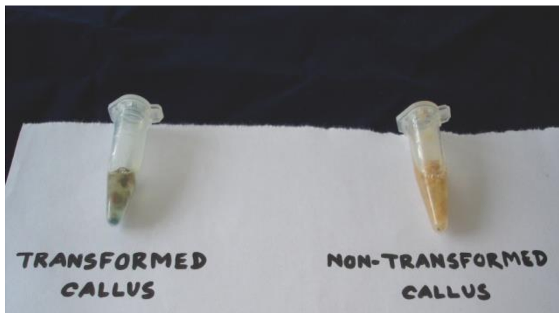

Figure 4. Left: leaf calli that have been successfully transformed (GUS +ve) and right: calli that have not been altered (GUS -ve)

Blue patch

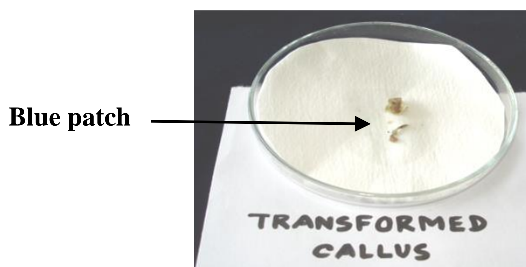

Figure 5. Transgenic leaf callus showing GUS +ve response

Blue patch

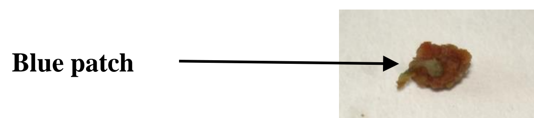

Figure 6. Transgenic nodal callus showing GUS +ve response

9. Conclusion

An efficient approach for genetic transformation was developed by introducing the CIPK sense gene to the cucumber variety Shital and integrating two marker genes (GUS and npt II). Leaf explants produced the highest number of GUS-positive transgenic calli, whereas internodal explants produced the fewest. A more powerful change was observed with an inoculation time of 5 minutes. Length of co-cultivation is another important factor in Agrobacterium-mediated plant genetic change. The results were better with a longer co-cultivation period (48 hours) compared to a shorter one. It is possible to use this transformation process to successfully generate cucumber cultivars in Bangladesh that are tolerant to abiotic stress. We will need to do additional tests to validate the transgenecity of the suspected converted callus. These tests may include polymerase chain reaction (PCR), southern blotting, sequencing, and others.

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