PRODUCTION OF TRANSGENIC CUCUMBER PLANTLETS CONTAINING SEQUENCES FROM WATERMELON MOSAIC VIRUS-II FUSED WITH GFP GENE

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Cucumber *(Cucumis sativus* L.) is one of the most important vegetable crops that are widely cultivated throughout the world. Different diseases and pests cause significant crop losses in cucumber (Schukle *et al.,* 1995). On a worldwide basis, the three potyvirus species Zucchini yellow mosaic virus (ZYMV), Watermelon mosaic virus-II (WMV-II) and Papaya ringspot virus W (PRSV-W) are the most commonly and economically important, reported in surveys of virus infecting cucurbits in different parts of the world (Dahal *et al.,* 1997; Luis-Arteaga *et al*., 1998). WMV-II infects under natural conditions mainly cucurbits but also certain species belonging to other families, some of which are reported to be important sources of infection (Lovisolo, 1980). Conventional breeding of cucumber to improve disease resistance and other horticulture traits is limited by its narrow genetic basis and severe incompatibility barriers to related species (Kho *et al.,* 1980). Chemical control is one of the most widely used methods resulted in many problems such as environmental pollution and increased production costs. Therefore, it is important to develop a genetic transformation system for introduction of exogenous genes into cucumber in order to improve the quality of the plants and to develop new varieties. By using *Agrobacterium*-mediated method, there is no costly equipment involved and therefore it is considered a low cost method compared to other transformation methods. The widespread use of the *Agrobacterium* based strategies is also due to the efficiency with which transformation occurs and the simplicity of the plant transformation and selection protocols (Binns, 1990). Reporter genes have been used as convenient markers to visualize gene expression and protein localization *in vivo* in a wide spectrum of prokaryotes and eukaryotes (Jefferson, 1987). The transformation of cucumber was first achieved by Trulson *et al*. (1986) and then the transgenic cucumber plantlets were regenerated from cotyledons, shoot-tips and leaves that were induced by the inoculation with *Agrobacterium tumefaciens.* Hence several transgenic plants were obtained (Tabei *et al.,* 1998; Ganapathi and Perl-Treves,

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2000).

The aim of this study was to establish a n efficient *Agrobacterium*-mediated genetic transformation method for cucumber using green fluorescent protein (GFP) as a reporter gene and to produce transgenic cucumber plantlets containing virus sequence of WMV-II.

MATERIALS AND METHODS

Plant Material

Seeds and media sterilization procedures

Seeds of *Cucumis sativus* cv, Faris were provided by Indo-American Hybrid seeds, Bangalore, India. Seeds were soaked for 15 min in distilled water to render germination uniform, surface-sterilized with 5% Sodium hypochlorite (Clorox) for 20 min and rinsed three times with sterilized distilled water. The Murashige and Skoog (1962) medium (pH 5.8) with vitamins, 3% sucrose and 0.8% agar was autoclaved at (121°C) for 20 min.

Explants derived from mature seeds, cotyledons and shoot tips

Sterilized seeds were cut transversely into two unequal sections; one section (embryonic axis and one-third of the cotyledon) was eliminated and the remaining two sections (two thirds of cotyledons) were used. The cotyledon and shoot tip explants were excised from seedling grown on MS medium for 7-9 days. Cotyledons were divided into 1-cm pieces and the apices of shoot tips were cut into longitudinal halves. All explants were cultured horizontally on the MS induction medium (El-Absawy *et al*., 2012) in darkness at 25±2°C.

Cloning of construct of Catgfp-WMV into binary vector pPZPnpt

WMV-II fused with the Catgfp in the pCatgfp vector is a virus sequences located in the coat protein genes. The Catgfp-WMV controlled with double 35S promoter was first digested from pCatgfp-WMV with *HindIII*. Before cloning, the host plasmid pPZPnpt was first line a raised with the same restriction enzymes (*HindIII*) as shown in Fig. (1). Second, the complete cassettes were separately subcloned into binary vectors of pPZPnpt at the *HindIII* restriction enzyme sites and then transferred into *Agrobacterium* LBA4404 strain by heat shock method. The presence and right orientation of the cloned cassette was confirmed by digesting the recombinant plasmid either with *HindIII* or with *BamHI* and *XbaI* restriction enzymes.

Bacterial strain and plasmid

The *Agrobacterium* LBA4404 strain harboring vector pPZPnptCat-WMV carrying NPT-II gene conferring resistance to kanamycin and virus sequence of (WMV-II) fused with GFP gene driven by 35S promoter was used in transformation experiments. The recombinant DNA plasmid was prepared from *E. coli* using alkaline lysis method (Sambrook *et al*., 1989) and mobilized

into *Agrobacterium tumefaciens* prior transformation of cucumber. The plasmid concentration was determined by a spectrophotometer.

Transformation of chemically Agrobacterium competent cells

Agrobacterium competent cells were brought from -80°C and thawed until just becoming liquid before adding plasmid DNA. One µg of plasmid DNA mixture was added to the 0.1 mL of competent cells, mix gently, and then freeze in liquid nitrogen for 5 min, immediately making heat shock by thawing the cells in a 37°C water bath for 5 min. and put in ice for 2-5 min. About 900 µL YEP liquid media (10 g/L Bacto peptone, 10 g/L Yeast extract and 5 g/L NaCl) was added and incubated at 28°C for 2-4 h with gentle shaking, then all cells were transferred and spread on YEP plate containing 200 mg/L spectinomycin and streptomycin and incubated 2 to 3 days at 28°C.

Agrobacterium-mediated transformation and selection procedures of cucumber explants

The *Agrobacterium* suspension and explants were then mixed and gently shaken to ensure all the explants were fully submerged (De Bondt *et al.,* 1994). After immersion for (15, 30, 45, 60, 75 and 90 min), the explants were blotted dry on sterile filter paper and transferred to the co-cultivation medium. The cultures were incubated at 25±2°C under 16 h light/8 h dark photoperiod. In this research, the effects of the different parameters to influence the transformation efficiency were assessed: Callus, cotyledon, leaf and shoot-tip explants were prepared and pre-cultured for 0, 1, 2, 3, 4 and 5 days on MS basal medium prior to co-cultivation for 0, 1, 2, 3, 4 and 5 days with *Agrobacterium*. Subsequently, *Agrobacterium* concentration was adjusted to 0.2, 0.4, 0.6, 0.8 and 1.0 at OD 600 nm. All the parameters were optimized by screening for transient GFP expression using a fluorescence stereomicroscope. All experiments were carried out with 50 explants and repeated three times. After co-cultivation with *Agrobacterium* for three days, the explants were transferred and placed onto selection and regeneration medium (MS + 1 mg/L N6-Benzyladenine (BA) + 200 mg/L kanamycin + 300 mg/L cefotaxime) for 4-week. After elimination of *Agrobacterium* by cefotaxime, the transformed explants were transferred on the same fresh regeneration medium without cefotaxime and incubated under light for shoot initiation.

PCR analysis

The presence of transformed and control (non-transformed) plantlets were analyzed by the Polymerase Chain Reaction (PCR). Genomic DNA was extracted using CTAB method as described by Roger and Bendich (1985). The PCR was used here to ensure the presence of virus resistance gene into cucumber plantlets. The PCR amplification was carried out in 25 µL reactions as follows: about 20 ng (1 µL) of DNA template (plasmid) which

contained WMV-2 sequences in separated reaction tubes; 12 pmol (0.25 µL) of two specific primers (the nucleotide sequence of primer was F, 5´-ATG GAT CCA GGT TAC TTC CAA AAC ACC-´3 and R, 5´-ATT CTA GAC GTC CCT TGC AGT GTG CCT-´3.); 1µL of a mixture of four deoxyribonucleoside triphosphates (dNTPs); 0.25 µL of *Taq* (Eppendorf) DNA polymerase, which adds a single deoxyadenosine (A) to the 3 ends of the PCR product to allow it to be efficiently ligated to a linearized vector contains 3 deoxythymindine (T) overhangs; 2.5 µL of 10 X Taq buffer and 20 µL of distilled water. The mixture was transferred to a 0.2 mL PCR tube. The PCR mixtures were denatured at 94°C for 5 min followed by 30 cycles for (30 sec. at 94°C, 30 sec at 52°C for annealing, 30 sec at 72°C for extension).

RESULTS AND DISCUSSION

Optimization of parameters influencing Agrobacterium transformation using GFP as a reporter gene.

The efficiency of transformation is greatly influenced by the compatibility between plant and *Agrobacterium* and was influenced by several factors such as *Agrobacterium* concentration, pre-culture period, co-cultivation period and immersion time. The results obtained were based on the percentage of GFP-positive transformants. Cotyledon, shoot-tip, leaf and callus explants were cultured on MS medium supplemented with 200 mg/L kanamycin in order to be completely blocking the non transformed cells.

Effect of Agrobacterium concentration

Different concentrations of *Agrobacterium* had different effects on transformation efficiency and percentage of GFP positive transformants as shown in Fig. (2). OD 600 nm 0.8 of *Agrobacterium* concentration gave the highest average percentage of GFP positive transformants (51.5%) in all type of explants, while OD 600 nm (0.2) gave the lowest mean percentage of GFP positive transformants (9.5%) in all type of explants. Increasing *Agrobacterium* concentration above OD 600 nm (0.8) resulted in decreasing the GFP positive transformants percentage. Overall, the interaction between *Agrobacterium* concentration and explant types showed that the highest GFP positive transformants percentage (78%) were given when the cotyledon explants were inoculated with OD 600 nm (0.8) of *Agrobacterium* (Fig. 2A). On other hand, the lowest number of GFP positive transformants percentage (6%) was observed when shoot tip explants were inoculated with OD 600 nm (0.2) of *Agrobacterium* (Fig. 2C). High *Agrobacterium* concentration may causes bacterial overgrowth which becomes problematic for the elimination of the *Agrobacterium* from the *in vitro* culture post-infection. Bacterial overgrowth can lead to the damage of the infected tissues or can result in tissue necrosis and interference with tissue regeneration. These data are in agreement with Yin *et al.* (2005), they showed that the frequency of *Agrobacte-*

rium-mediated transformation ranged from 0.8 to 10% and was influenced by bacteria cell density. Chakravarty and Wang-Pruski (2010) reported that from various parameters investigated to increase transformation efficiency was concentration of bacterial cultures which used for transformation. Different concentration of bacterial suspension (OD600 0.2-0.6) were used.

Effect of pre-culture period

According to data in Fig. (3), the highest average percentage of GFP positive transformants (30.5%) was recorded when all explants were pre-culture with *Agrobacterium* for 4 days. While the lowest percentage of GFP positive transformants (3.5%) was resulted from all explants which were pre-culture with *Agrobacterium* for zero days. Increasing the pre-culture period above 4 days led to decreasing the GFP positive transformants percentage. The interaction between pre-culture period and explant types showed that the highest GFP positive transformants percentage (46%) were given when the cotyledon explants were pre-cultured with *Agrobacterium* for 4 days (Fig. 3A). On other hand, the lowest value of GFP positive transformants percentage (0.0%) was observed when shoot-tip explants were pre-culture with *Agrobacterium* for zero days (Fig. 3C). Pre-culture allowed proliferation of the plant cells to provide a large population of competent cells as potential targets for transformation, and high cell division occurred when these explants was sub-cultured on the new medium. This cell division may reaches to maximum at 3 and 4 days resulted in high number of dividing cells. These results are in agreement with Yong *et al.* (2006), they mentioned that four days of pre-culture was optimum for *M. malabathricum* transformation, while 3 days of pre-culture for *T. semidecandra*. Khan *et al.* (2009) mentioned that the transformation experiment was performed by optimizing pre-culture time. Infection was most effective when explants were pre-cultured for 72 hours (80% *GUS* positive).

Effect of co-cultivation period

Concerning to results presented in Fig. (4)**,** the highest mean percentage of GFP positive transformants (60%) was recorded when all explants were co-cultivated with *Agrobacterium* for 3 days. While the lowest mean percentage of GFP positive transformants (6.5%) was resulted from all explants which were co-cultivated with *Agrobacterium* for zero day. Increasing the co-cultivated period above 3 days led to decrease GFP positive transformants percentage. Overall, the interaction between co-cultivation period and explant types showed that the highest GFP positive transformants percentage (82%) were given when the cotyledon explants were co-cultivated with *Agrobacterium* for 3 days (Fig. 4A). On other hand, the lowest number of GFP positive transformants percentage was observed when shoot-tip explants were co-cultivated with *Agrobacterium* for zero days (Fig. 4C). Co-cultivation period led

to the induction of virulence and gene transfer. These data are in agreement with Men *et al*. (2003) and Weber *et al*. (2003), they showed that normally 2-3 days of co-cultivation are standard for most transformation protocols, Where longer period than that may cause necrosis and cell death. Vasudevan *et al.* (2007) reported that the infected explants were co-cultivated for 2 days with *Agrobacterium* for best transformation. González *et al.* (2008) showed that optimal transformation conditions were obtained for sweet potato cultivars (Jewel and CEMSA 78354) by co-cultivating leaf explants with *Agrobacterium tumefaciens* in liquid MS medium for 24 hours at 28°C in stationary cultures in the dark. Suma *et al.* (2008) reported that co-cultivation was carried out for 15 min, 1, 2, 3 and 4 days in darkness**.**

Effects of immersion time in Agrobacterium suspension

Data presented in Fig. (5) revealed that the highest mean percentage of GFP positive transformants (38%) was recorded when all explants were immersed in *Agrobacterium* suspension for 60 min. While the lowest mean percentage of GFP positive transformants (5.5%) was resulted from all explants which were immersed in *Agrobacterium* suspension for 15 min. Increasing the immersion time above 60 minutes led to decrease the GFP positive transformants percentage. The highest GFP positive transformants percentage (52%) was given when the cotyledon explants were immersed in *Agrobacterium* suspension for 60 minutes (Fig. 5A). On other hand, the lowest number of GFP positive transformants percentage was observed when shoot tip explants were immersed in *Agrobacterium* suspension for 15 min. (Fig. 5C). This may be due to immersion time also varied between plant species and tissue types and immersion of explants in *Agrobacterium* suspension enhanced the attachment of *Agrobacterium* to the explants. These results are in agreement with Kaneyoshi *et al.* (1994); they used 15 min. of immersion time to transform epicotyl segments of trifoliate orange by using *Agrobacterium tumefaciens* LBA4404, whereas Aida *et al.* (1999) used 5 min. immersion time to transform etiolated petioles of *Cyclamen persicum* with the same *Agrobacterium* strain. Young *et al.* (2006) reported that 60 min of immersion time gave the highest percentage of positive transformants for both *M. malabathricum* and *T. semidecandra*.

Regeneration of plantlets after transformation by Agrobacterium.

After co-cultivation with *Agrobacterium* for three days, the explants were transferred and placed onto selection and regeneration medium (MS + 1 mg/L BA + 200 mg/L kanamycin + 300 mg/L cefotaxime) for four week. After elimination of *Agrobacterium* by cefotaxime the transformed explants were transferred on the same fresh regeneration medium without cefotaxime and incubated under light for shoot initiation. After four weeks shoots formed initially at the longitudinal-

ly cut of the leaf, cotyledon and shoot-tip and then transferred onto the same fresh medium without cefotaxime for shoot elongation for another four weeks. Calli started to initiate shoot buds after 50-60 days of incubation. A few of the transformed calli and leaves continued to grow and differentiated into shoots. Multiple shoots were induced from cotyledons and shoot-tips as shown in Fig. (7). This study obtained finally twenty putative transgenic plantlets from leaf, cotyledon, callus and shoot-tip explants, and it could be confirmed by screening of putative transgenic plantlet by PCR analysis.

Screening of putative transgenic plantlet by PCR analysis

PCR analysis was further used to confirm the presence and the integration of virus sequence (WMV-2) in cucumber genome, as well as the copy number integrated. The PCR was used here to amplify 150 bps of the virus nucleotide sequence WMV-2. From twenty putative transgenic plants, four of them were selected for PCR analysis. DNA was extracted from 4 putative transgenic plantlets which was carrying plasmid pPZPnptCat-WMV, a non-transgenic plantlet as negative control, and plasmid pPZPnptCat-WMV as positive control were used as templates for PCR amplification as shown in Fig. (8). Finally, this work developed tools to establish virus resistance in plant. Using the optimized *Agrobacterium*-mediated transformation procedure together with the introducing of virus-targeted sequence into a potential host plant genome confer resistance of plants against this virus according to Wilson (1993); Gonsalves *et al*. (1994) and Fuchs *et al*. (1997), they found that the ability to confer resistance against an otherwise devastating virus by introducing a single pathogen-derived or virus sequence into the DNA of host plant.

SUMMARY

Cucumber (*Cucumis sativus* L. cv. Faris) explants were transformed by LBA4404 strain of *Agrobacterium tumefaciens* harboring the binary vector pPZPnptCat-WMV**.** The T-DNA region contains Neomycin Phosphotransferase II (NPT II) as a selectable marker gene and sequences of Watermelon Mosaic Virus-II (WMV-II) fused with Green Fluorcent Protein (GFP) gene under control of 35S promoter. *Agrobacterium*-mediated transformation was optimized using GFP as a reporter gene. The optimized parameters were *Agrobacterium* concentration, preculture period, co-cultivation period and immersion time. Results were recorded based on the percentage of green fluorescent protein (GFP) expression. *Agrobacterium* concentration at (OD 600 nm 0.8), four days of pre-culture, three days of co-cultivation and sixty minutes of immersion time gave the highest percentage of GFP areas (78%, 46%, 82% and 52%, respectively). Cotyledon was the best explants to give the highest percentage of GFP areas in all tested parameters. Following co-cultivation, leaf, cotyledon, callus and shoot-tip explants were cultured on selective and regeneration

Murashige and Skoog (MS) medium containing 1mg/L 6-Benzylaminopurine (BA), 200 mg/L kanamycin and 300 mg/L cefotaxime. Kanamycin resistant shoots were induced from these explants after four weeks. Putative transgenic plantlets were produced from leaf, cotyledon and shoot-tip explants at 8 weeks and at 12 weeks from callus. Integration of the transgenes in the cucumber genome was confirmed by PCR analysis. This study showed that the *Agrobacterium*-mediated gene transfer system and regeneration via organogenesis is an effective method for producing transgenic cucumber plantlets.

CONCLUSION

Cloning construct of Catgfp-WMV-2 into binary vector pPZPnpt was done successfully. *Agrobacterium* concentration at 0.8, four days of pre-culture, three days of co-cultivation and sixty minutes of immersion time gave the highest number of GFP positive percentage (78%, 46%, 82% and 52%, respectively). Cotyledon was the best explants to give the highest number of GFP positive percentage in all tested parameters. Putative transgenic plantlets were obtained from (leaf, cotyledon and shoot-tip explants after 8 weeks) and after 12 weeks from callus.

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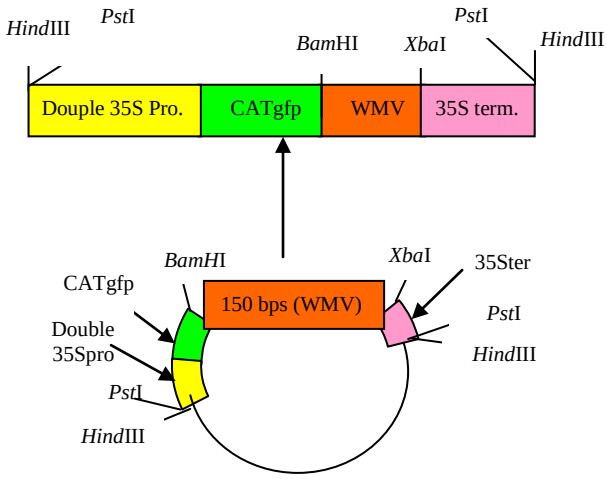
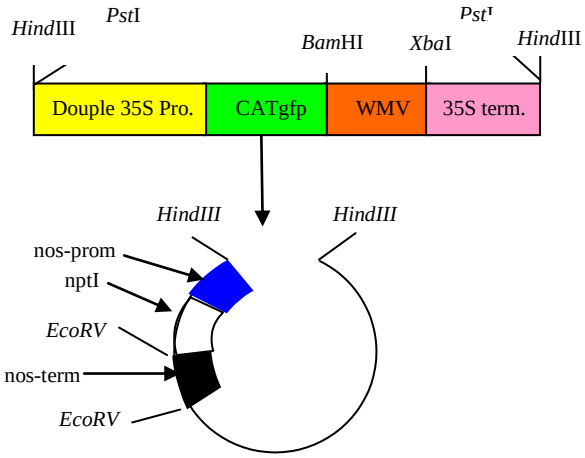


Step 1): Digestion of Catgfp-WMV from pCatgfp-WMV with *HindIII*.



Step 2): Ligation of Catgfp-WMV into pPZPnpt plasmid binary vector

Fig. (1): Schematic representation of cloning strategy of WMV into binary plasmid vector pPZPnpt.

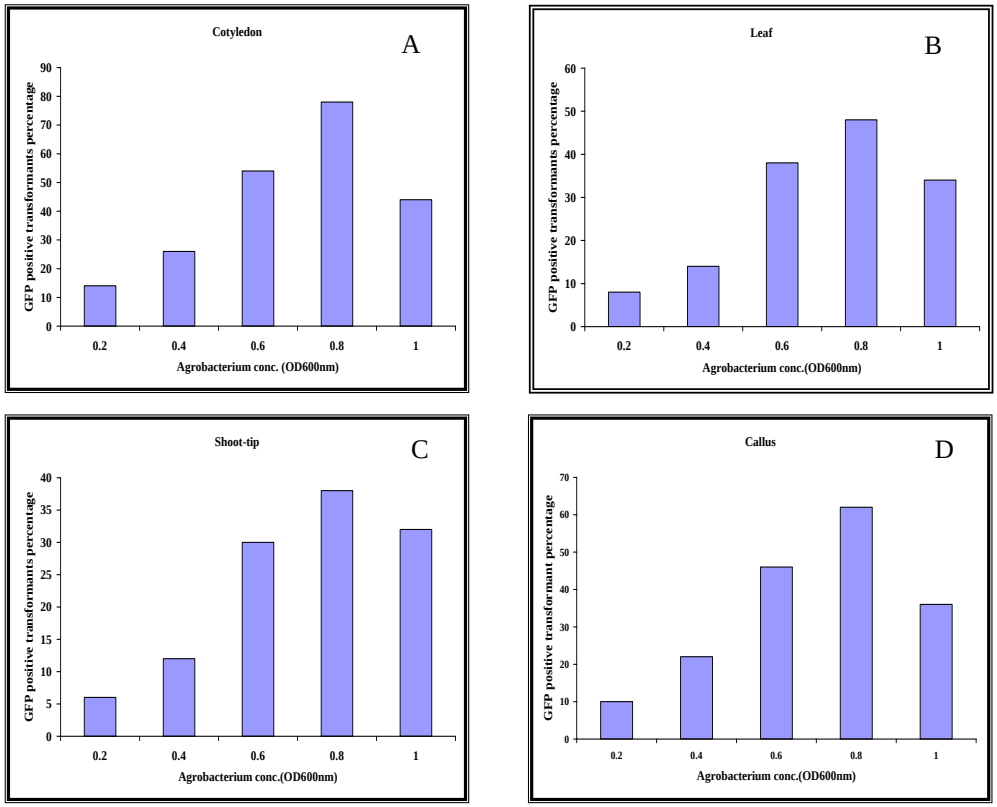


Fig. (2): Effect of *Agrobacterium* concentration on GFP positive transformants percentage of different cucumber explants, (A: cotyledon, B: leaf, C: shoot tip, D: callus).

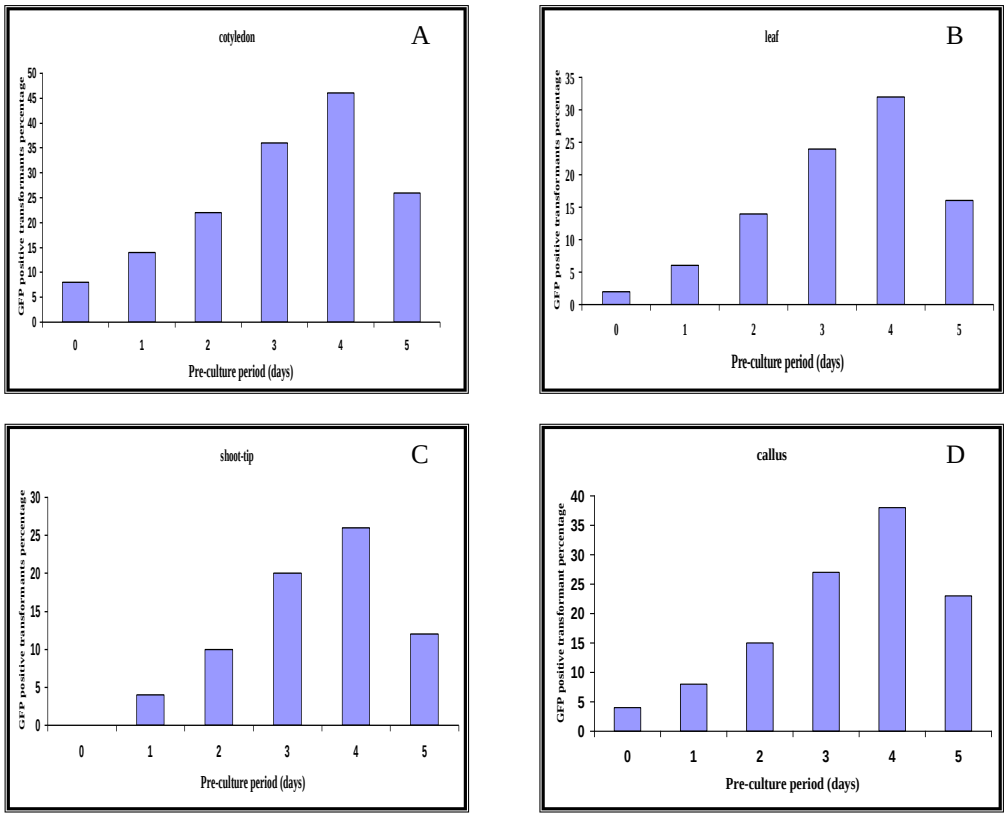


Fig. (3): Effect of pre-culture period on GFP positive transformants percentage of different cucumber explants, (A: cotyledon, B: leaf, C: shoot tip, D: callus).

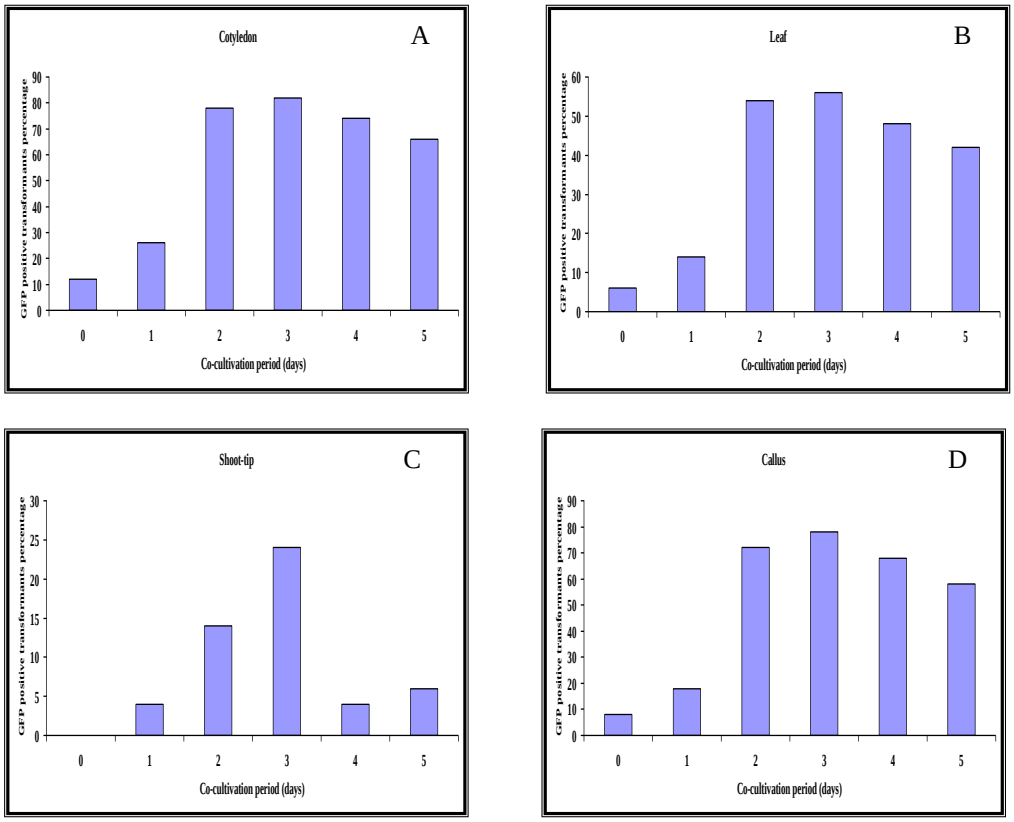


Fig. (4): Effects of co-cultivation period on GFP positive transformants percentage of different cucumber explants, (A: cotyledon, B: leaf, C: shoot tip, D: callus).

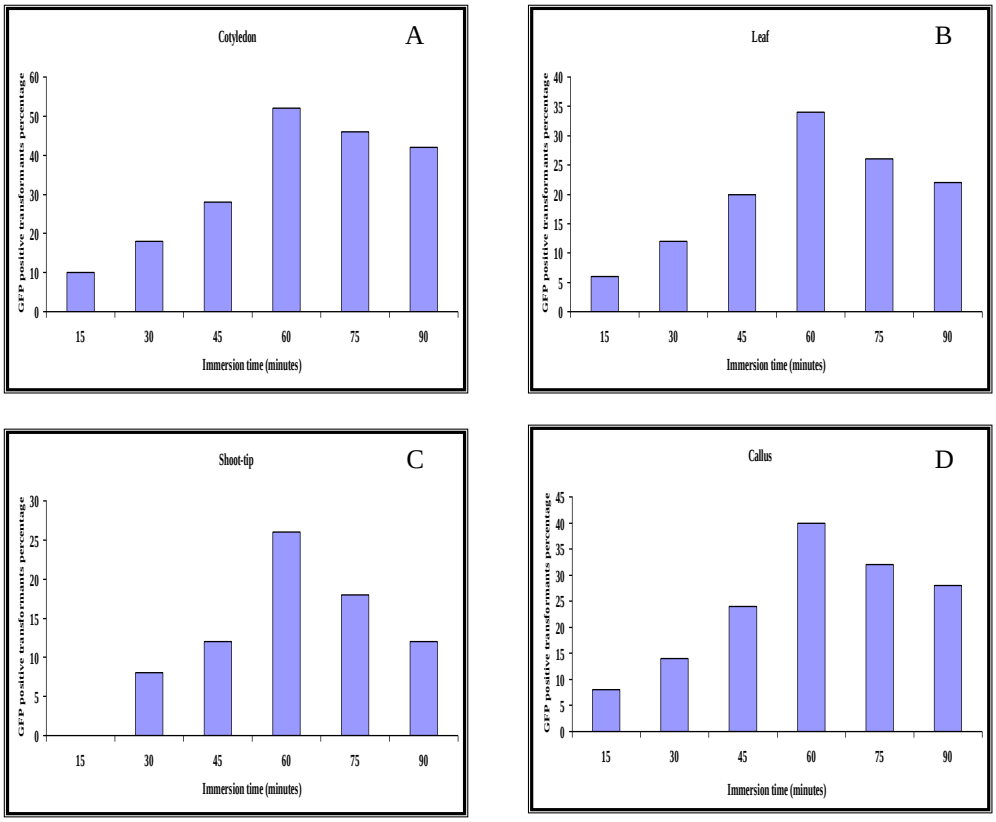


Fig. (5): Effects of immersion time in *Agrobacterium* suspension on GFP positive transformants percentage of different cucumber explants, (A: cotyledon, B: leaf, C: shoot tip, D: callus).

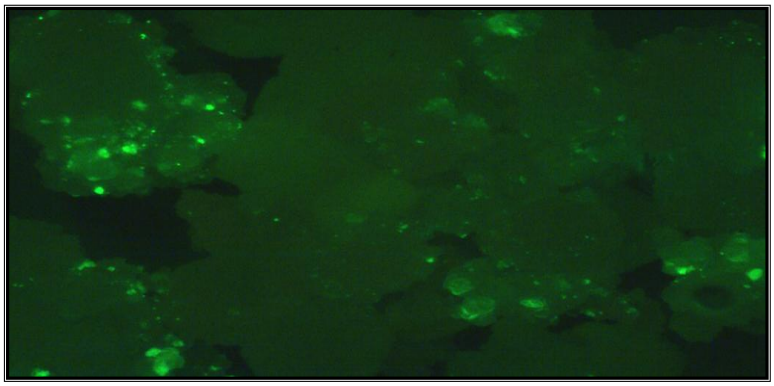


Fig. (6): Embryogenic calli GFP positive transformant, three days after co-cultivation.

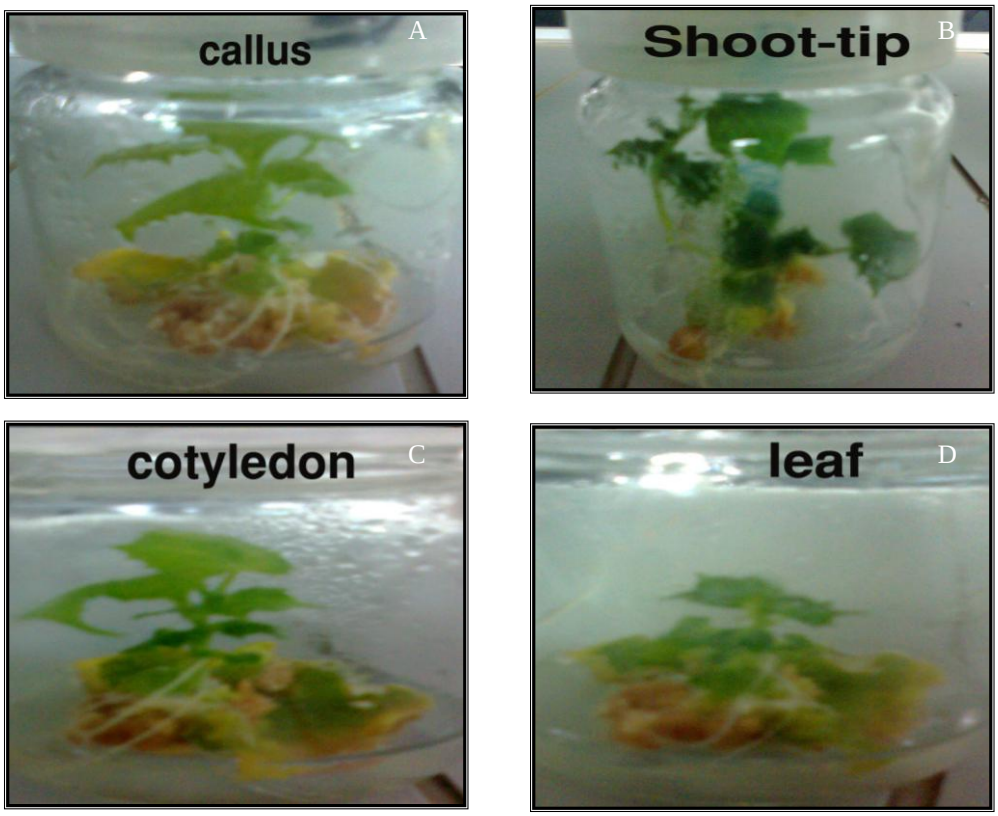


Fig. (7): Regeneration from transgenic cucumber explants (transgenic plantlets) after 12 weeks for callus (A) and after 8 weeks for shoot-tips (B), cotyledons (C), leaf (D).

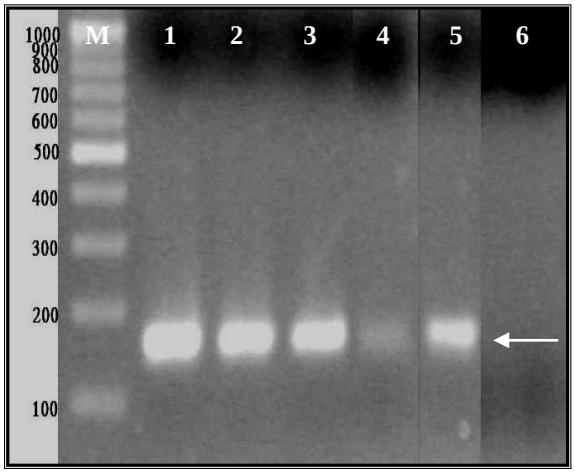


Fig. (8): PCR products (150bp) amplified from the total extracted nucleic acids prepared from transformed plants. (M, 100bp DNA ladder marker; 1-4, putative transgenic plantlets; 5, Positive control and 6, negative control).