International Journal of Sciences & Applied Research

www.ijsar.in

An efficient regeneration system for the genetic transformation of *Nicotiana tabacum* L. cv. Petit Havana SR1 without Auxine and optimization of Hygromycin concentration for selection

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Abstract

This is a new *in vitro* regeneration and genetic transformation protocol for Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1).Transformation and regeneration of tobacco leaf explants was done using only Benzyl amino purine (BAP) without auxin supplement as with other available protocols for tobacco. Furthermore this work evaluates the *Agrobacterium*-mediated transformation efficiency of Tobacco under the selection pressure of herbicide hygromycin. Leaves of 6 week old seedlings were used as explants. Transformation was carried out, using the *Agrobacterium tumifaciens* LBA4404 harbouring pCAMBIA1302 with hygromycin phosphor transferase gene as the selectable marker gene and the green fluorescence protein (GFP) gene as the reporter. The result showed that the optimum concentration of hygromycin for selection of transformants was 25mg L^{-1}. Multiple shoots were regenerated from each explant when Murashige and Skoog basal medium supplemented with only 3mg L^{-1} BAP had been used. Transformation efficiency of explants was 100% under the optimized conditions. The presence of the transgene was confirmed by PCR with primers corresponding to a 454bp sequence of the *gfp* gene. Integration and expression of the transgene cassette within the transformants were further confirmed by visualizing transformed tissue under fluorescence microscope with UV of specific wavelength for GFP, to visualize the expression of the *gfp* gene. Here we have standardized a suitable transformation system for tobacco with minimum hormone supplement resulting in the highest possible transformation efficiency.

Keywords: Benzyl amino purine (BAP), Green fluorescence protein (GFP), Hygromycin, pCambia, Fluorescence, transformation, tobacco

Introduction

Plant possesses highly specialized developmental plasticity. During the post-embryonic development plants can switch to diverse form of regeneration (Ikeuchi *et al*., 2016).

In certain cases initiating cells directly regenerate into new organs such as shoots and roots *via* direct organogenesis, but in other cases the initiating cell first develop a mass of dividing cells *i.e* callus, from which new embryos develops (Chowdhury *et al*.,

2014). This kind of regeneration has been widely used in agriculture as a tool for propagation of elite cultivars and for genetic engineering of crop plants (Thorpe, 2007).

Plant regeneration is an important tool in modern plant breeding and crop biotechnology. Regeneration of plants through somatic embryogenesis or organogenesis generally depends on the addition of one or more plant growth regulators to the culture medium. Manipulation of the ratio or concentration of these growth regulators is very crucial as these are the key factors leading to successful regeneration protocols (Thorpe, 2007; Srinivasan *et al*., 2007). Induction of totipotency can be obtained by exposing explants to optimum concentration of plant growth regulators (PGRs). PGRs especially auxins and cytokinins (CKs) are the most important components required for inducing organogenesis or embryogenesis (Motte *et al*., 2014; Roche *et al* .,2017). At the time of rejuvenation fully differentiated plant cells are induced to lose their final functional state (dedifferentiation), re-acquire cell proliferation potential (rejuvenation) and then re-enter a stem-cell like state (somatic embryogenesis) (Eeckhaut *et al*,. 2013). Over exposure plant tissues to higher concentration of cytokinin can exert negative effects on shoot development. The abnormalities caused by higher exposure to cytokinin are hyperhydricity (Ivanova *et al*., 2011), delayed rooting of cultured shoot (Bellini *et al* .,2014).

Transformation systems usually employ antibiotics like kanamycin for neomycin phosphotransferase II (NptII) gene or hygromycin for hygromycin phosphotransferase (*hpt*) gene for selection of transformed plants. Plant susceptibility for certain antibiotics change broadly among species, genotypes and even for specific plant tissues (Padilla *et al*., 2010). High concentration of antibiotics can kill untransformed as well as transformed tissues thus reducing the regeneration frequency of transformation. On the other hand, low concentration of antibiotic results in occurrence of escapes and hamper effective selection of transformed cells (Padilla *et al*., 2010). Therefore, it was pre requisite to select the optimum concentration of antibiotics as the selection pressure for an efficient transformation protocol.

Efficiency of plant regeneration in tobacco remain challenging even though shoot organogenesis and somatic embryogenesis both had been reported decades ago in 1983 (Lemaux, 2008). There are several reports of *Agrobacterium*-mediated genetic transformation followed by in vitro regeneration in case of tobacco but very few give 100% efficiency (Panthi *et al* ., 2013). One way to improve this low efficiency is to develop protocols with optimized hormones and the use of proper selectable markers.

This study was carried out to generate an efficient regeneration and transformation protocol for Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) using minimal plant hormones in the media so that in future this system may be exploited for introduction and efficient expression of foreign gene to develop transgenic tobacco lines with desirable traits.

Materials and methods

Tobacco plant used

The seeds of tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) were used in this study.

Preparation of Explants

Seeds were surface-sterilized using 30% bleach solution and as surfactant tween-20 (0.1%) was used as previously published protocol (Basu *et al* .,2014). 5 surface-sterilized seeds were planted on the germination medium contained half strength of MS (Murashige and Skoog, 1962), 3% sucrose, 2% Phyto grade agar at pH 5.4. The jam bottles were placed at 4 °C in dark for 2

days. Then these were placed on conviron that has a temperature of 28 °C and 16/8 h (day/night) photoperiod with a light intensity of 40 l mol m^{-2} s^{-1} (cool white fluorescent tubes). Leaves of 6 week old seedlings were used as explants.

Culture media

All media were based on full-strength macro- and micronutrients as well as vitamins of Murashige and Skoog (1962) with 30 g L^{-1} sucrose and 20 g L^{-1} plant agar (Duchefa, Haarlem, The Netherlands) at pH 5.4. Sterilized seeds were grown on half strength basal medium without further supplements, while the regeneration medium contained, in addition, 3.0 mg L^{-1} 6-benzyladenine (BAP) (Duchefa Haarlem, The Netherlands). Regenerated shoots were cultured on plant growth regulator free full-strength MS-medium with 30 g L^{-1} sucrose,1.8 g L^{-1} plant agar (Duchefa Haarlem, The Netherlands), 250 mg L^{-1} cefotaxime (Duchefa Haarlem, The Netherlands) and variable concentration of Hygromycin (Duchefa Haarlem, The Netherlands) as hygromycin concentration for standardization of optimum concentration.

***Agrobacterium tumefaciens strain and vector used for transformation*:**

A.tumefaciens strain LBA4404 harboring the binary vector pCAMBIA1302 was used for transformation experiments. A single colony of *A. tumefaciens* LBA4404 was inoculated into 50 ml of liquid Luria bertini broth (LB) medium containing 50 mg L^{-1} kanamycin and 25 mg L^{-1} rifampicin, and grown overnight at 28°C on a rotary shaker at 150 rpm. An aliquot (0.5 ml) of overnight culture was inoculated into 50 ml of liquid LB medium containing the same antibiotics, and allowed to grow at 28°C with vigorous shaking until the OD600 reached about 0.6. The bacterial cells were harvested by centrifuging the culture at 3000 rpm for 4 min at 4°C. The resulted pellets were re-suspended in the infiltration medium [liquid MS medium containing 3 % sucrose pH adjusted to 5.4].

Table : Composition of different culture media used for Tobacco transformation.

Medium code	Purpose of use	Composition
GM	Seed germination	½ MS salt+ 1.8% phyto-grade agar
PIM	Pre-culture of explants	MS salt (3%sucrose)+ 1.8% phyto-grade agar +3mg L^{-1} BAP
CM	Co-cultivation media	MS medium (3% sucrose) +3mg L^{-1} BAP+1.8% phyto-grade agar
SIM	Regeneration + shoot induction + Selection media	MS media (3% sucrose)+3mg L^{-1} BAP+ 250mg L^{-1} Cefotaxime + Hygromycin (variable conc.) +1.8% phyto-grade agar
RM	Regeneration media	MS media (3% sucrose) +3mg L^{-1} BAP+ Hygromycin (Variable conc.)
RIM	Root induction media	MS media (3% sucrose) +0.1mg L^{-1} IAA+ 25mg L^{-1} Hygromycin +1.8% phyto-grade agar

Transformation Method: inoculation and co-cultivation and explants pre-culture:

Expanded leaves of Tobacco were cut in small pieces and were cultured with cut surface in contact with PIM. Each petri dish contained 8-12 excised leaf explants.on pre-incubation media, MSA supplemented with 3mg L^{-1} BPA for 48 hours. Each Petri dish contained 15 excised cotyledon explants. Pre-incubated explants were infected by the *Agrobacterium* containing pCAMBIA1302 was done following published procedure (Chowdhury *et al*., 2014). After infection was done, leaf explants were selected by hygromycin, as transformed callus have hygromycin resistance gene.

Selection and regeneration:Transfer of explants to regeneration medium

Explant inoculation and co-cultivation were done as described above. Following

1 day co-cultivation, explants were grown in medium with cefotaxime 250mg L^{-1} (SIM) for 5–7 days in order to kill off any residual *Agrobacterium*. Subsequently they were selected on regeneration media (RM) supplemented with hygromycin of various concentrations (20mgL^{-1},25mg L^{-1}l, 30mg L^{-1}) for 7 days. Every passage of regeneration media were maintained for 7days followed by transfer to the next passage of hygromycin selection. Regenerating shoots were elongated on SIM Callus formation was observed from the 2nd week after the infection. Growth of calli were monitored and subsequent changes within callus were observed. From the 4th week after infection regenerated shoots were formed.

Transfer of plants on rooting media:

Regenerated shoots were cut from the callus and placed to rooting media for further development. After attaining an approximate height of at least 3 cm, and bearing 2–3 leaves, individual shoots were placed into rooting medium (RIM, Table) supplemented with IAA for rooting After plants were grown and became hardened they were removed from jar and were transfered to pots.Pots were kept in growth chamber for 2 weeks.Then pots were placed in the green house for further growth and seed production.

In order to determine the effect of different concentration of hygromycin on regeneration of transformants and shoot development, *Agrobacterium* infected explants were exposed to different concentration (20mgL^{-1},25mg L^{-1},30mg L^{-1}) of hygromycin with constant BAP concentration (3mg L^{-1}) and cultured on regeneration medium. callus growth were compared among different hygromycin concentration through compound microscopy .Shoot primordia were counted on day 14 The transformation efficiency was calculated as per the formula given below (Chowdhury *et al*., 2014).

$$\text{Regeneration frequency} = \frac{\text{Number of explants regenerating shoots}}{\text{Number of explants inoculated with } \textit{Agrobacterium}} \times 100$$

$$\text{Transformation efficiency} = \frac{\text{Number of gfp positive plants}}{\text{Number of explants inoculated with } \textit{Agrobacterium}} \times 100$$

Molecular confirmation of transgenic plants:

Genomic DNA extraction:

Genomic DNA was extracted from young leaves of transformed, *gfp*-positive regenerated shoots and also from non-transformed control plants following CTAB method with little modification (Basu *et al*., 2014). The quality and quantity of the extracted DNA were checked by 1.5 % agarose gel electrophoresis.

Polymerase chain reaction (PCR) analysis of the transformed tobacco lines:

Putative transformants were screened by PCR for presence of *gfp* sequence of the empty vector pCAMBIA1302. The coding

region of gfp with in vector pCAMBIA 1302 was amplified by using following the primer pair:

GFP(f)- 5' GGAGTTGTCCCAATTCTTGT 3'
GFP(r)- 3' ATGCCGTTCTTTTGCTTGTC 5'

Each PCR reaction was done in 25 µL of reaction mixture consisting of 2.5 µL of 10× PCR buffer, 100 ng DNA, 2.5 mM of dNTPs, 10 mM of each primer, 0.25 U of Taq polymerase .
Denaturation was done at 94 °C . The annealing temperature for the GFP primer pair was 50 °C , followed by 30 sec of extension at 72 °C with a final extension at 72 °C for 5 minutes. The PCR products were analyzed in a 1.5% (w/v) agarose gel stained with ethidium bromide. The resolved PCR products were visualized by placing the gel on the UV trans-illuminator.

Analysis of transformation by measuring fluorescence intensity

A fluorescence microplate reader (Varioskan Flash Multimode Reader, Thermo Scientific) was used to measure cell fluorescence of regeneration callus. Fluorescence intensity was measured using bottom optic readings. Excitation/ emission wavelengths were 553/574 nm for RFP and 488/507 nm for GFP. The fluorescence was quantified as relative fluorescence unit (RFU) according to published protocol (Ray *et al.*, 2015).

Study of GFP expression in transformed plants: visualization of green fluorescent protein:

The GFP expressing plant calli were visualized under UV and photographs were taken.

Results and discussion

In earlier reports it has been shown that when *N. tabacum* explants were cultured on medium supplemented with 2 mg L^{-1} naphthalene acetic acid (NAA), 2 mg L^{-1} 6 benzyladenine (BAP) maximum regeneration can be achieved (Shiro-Fukuta *et al.,* 2012). In tobacco, cytokinin mediated induction of shoots via organogenesis is well known although there are several reports about *Agrobacterium*-mediated Tobacco transformation but no single study had been investigated to transform Tobacco using only BAP in the regeneration medium with 100% regeneration frequency. Hence, the focus shifted towards transformation strategies utilizing BAP solely in the regeneration medium, in this work we used the concentration of BAP 3mg L^{-1} in subsequent growth medium for transformation and regeneration.
Prior to infection with *Agrobacterium*, leaf explants were cultured on pre incubation media as earlier reports confirmed that pre incubation increase transformation efficiency (Kumar *et al.,* 1999). After 48 hours of pre incubation explants were infected with *Agrobacterium* and co-cultivated for 24 hours in dark according published protocol on sesame transformation (Chowdhury *et al.,* 2014). Time and condition for co-cultivation are very crucial parameters for the efficiency of transformation. After co cultivation explants were incubated on media containing cephotaxim 250mg L^{-1}
Leaf explants were placed on MS medium containing 3mg L^{-1} BAP and different concentration of hygromycin. The explants began to enlarge significantly after 1 weeks of incubation (Fig. 1).
On the second week of culture, initiation of callus formation was observed from the cut edges of the explants. Three weeks after infection, shoot regeneration was observed on regeneration medium supplemented with hygromycin (different concentrations).

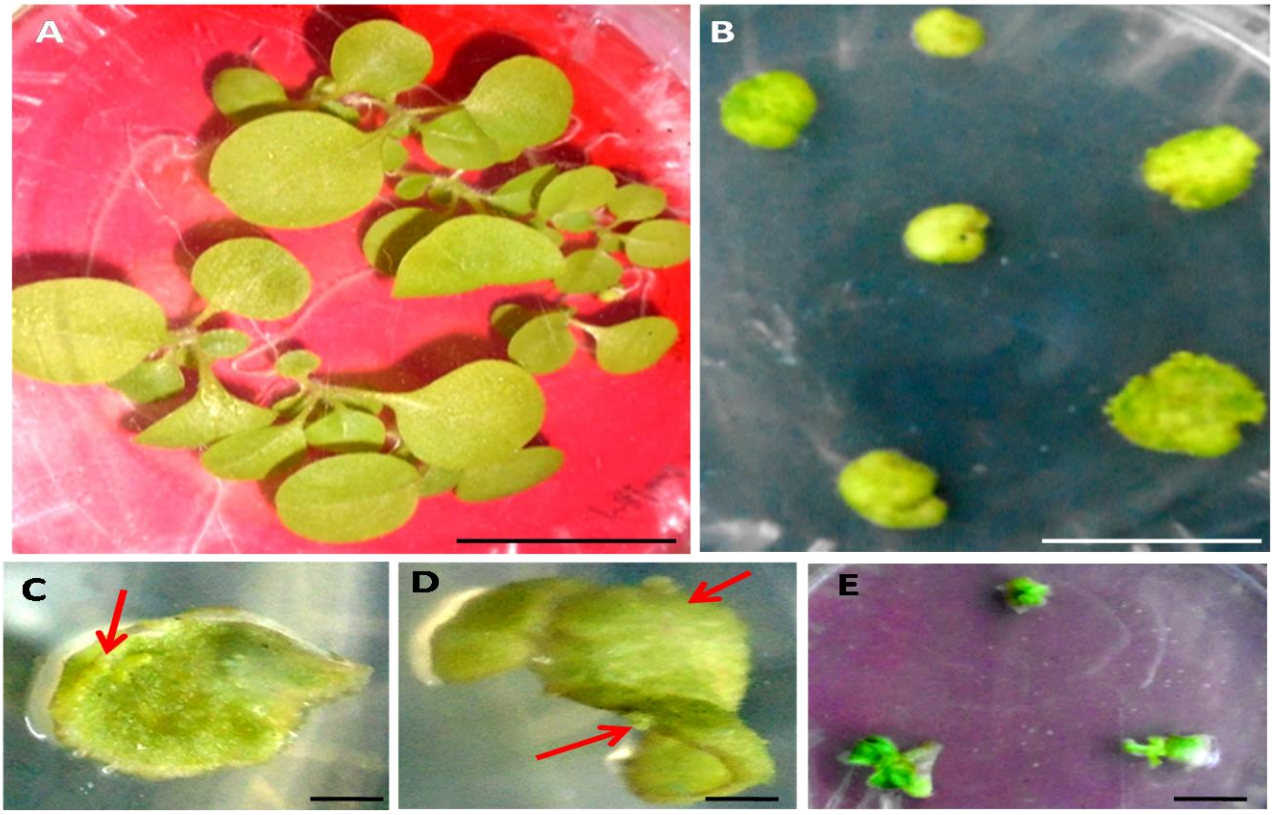

Figure 1: Stages of transfromation of tobacco (A) Tobacco plants grown in aseptic culture, as sources of leaf explants. (B) Leaf explants set for preincubation treatment on MS solid medium supplemented with 3mg L-1 BAP. (C, D) initiation of callus formation form cut surface of leaf explant shown by red arrows.(E) Regeneration of transformed plantlets on selection mediaum. (Bar= 0.5 cm).

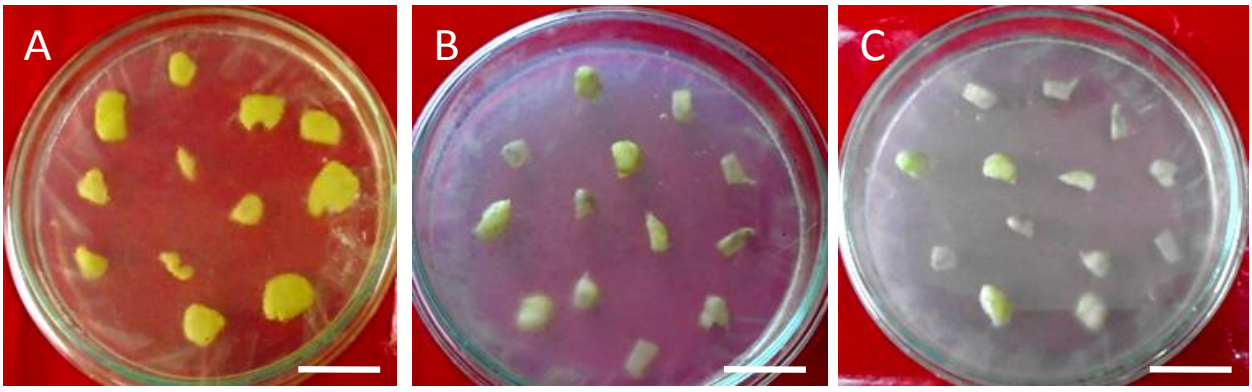

Figure 2: Phenotypes of control (untransformed) explants treated with 20, 25 and 30 mg/L Hygromycin. (A) control explants gown on medium supplemented with 3mg/L BAP and 20mg/L Hygromycin showing pale coloration. (B) Explants grown on optimal subculture medium supplemented with 3 mg/L BAP and 25mg /L Hygromycin showing almost white coloration.(C) explants become completely white being grown on optimal subculture medium supplemented with 3 mg/L BAP and 30mg/L Hygromycin. No regeneration took place in any of the three media. White bar indicates 0.5 cm.

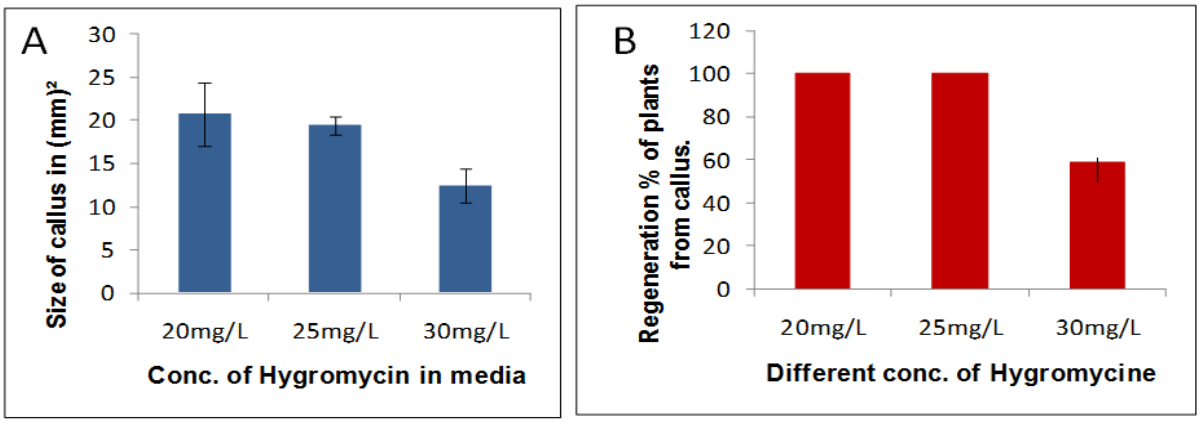

Figure 3: Effect of different hygromycin concentration of callusing and transformation efficiency. A. Graph showing comparison of sizes of Hygromycin resistant calli grown on different concentration of Hygromycin. B. Graphical representation for the frequency of regenerating transformed plants from each callus grown in different concentration of Hygromycin.

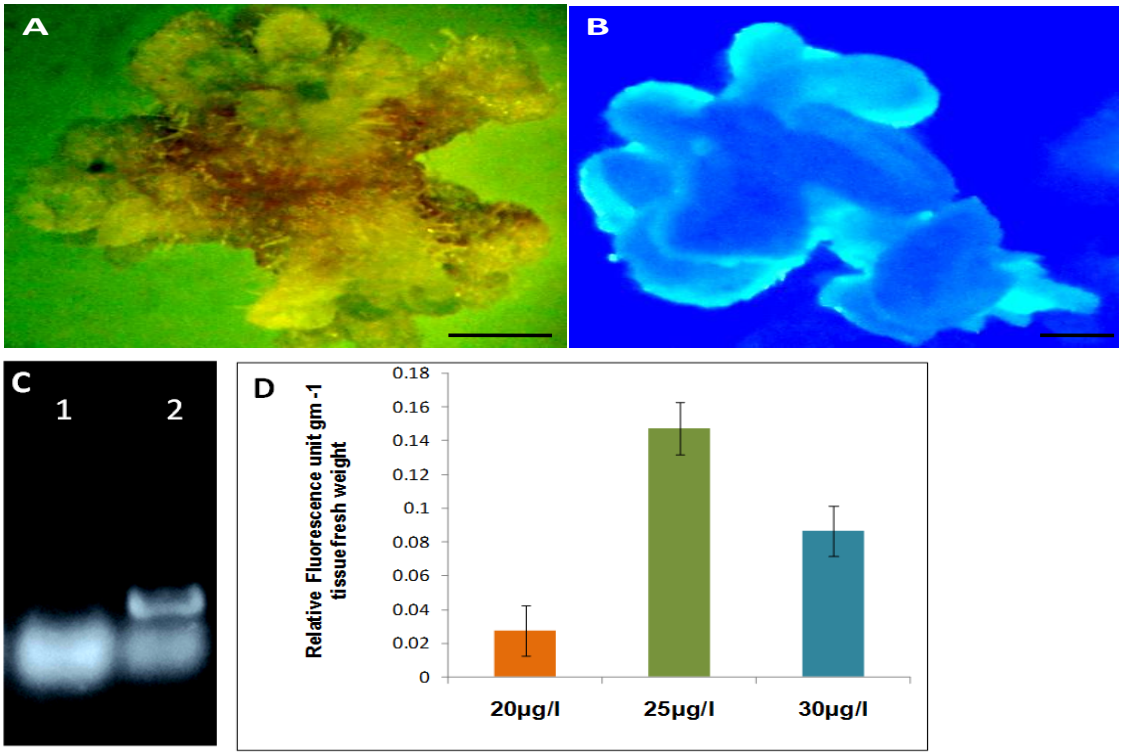

Figure 4: Assay of transformed tobacco calli for integration and expression of *gfp* gene (A) A transformed callus under visible light and (B) A transformed calli as seen under UV light showing GFP fluorescence. (C) PCR confirmation of the presence of *gfp* sequence in transformed tobacco calli; Lane 1: Control tobacco showing only primer dimer and lane 2: PCR of transformed tobacco showing band corresponding to 454 bp of *gfp* sequence. (D) Graphical representation of the comparative effect of different hygromycin concentration on transformation efficiency in terms of relative fluorescence unit of transgenic calli . Data represented mean of three repeated experiments, with 10 calli for each set.(Bar =5mm)

The T-DNA of the transforming vector pCambia1302, including the *hptI* marker gene, was integrated into the chromosome of plant cells, so that only transformed cells survived in the medium supplemented with hygromycin (Fig 1). To set up an effective regeneration system suitable for *Agrobacterium*-mediated transformation, it was necessary to determine the minimal concentrations of hygromycin in the selection medium. From the 2nd week significant differences were observed between the explants incubated on different percentage of hygromycin (Fig 2 A,B,C). The control explants failed to regerated on these concentrations specially on 25 mg/l and 30 mg/l hygromycin. Therefore both of these conenctrations were able to suppress all of the untransformed control tissue from regeneraing.

Transformation of tobacco using the GFP construct

The calli sizes were affected by the hygromycin concentration in the media. Increasing concentration resulted in decrease in callui sizes (Fig 3 A). Transformation frequency was in Hygromycin concentration of 20 and 25 mg L^{-1}. The regeneration as well as transformation frequency was 100% under these optimized conditions (Fig. 3B). With higher Hygromycin concentration, the efficiency was reduced. Hence the highest Hygromycin concentration where regeneration frequency was still 100% was 25 mg L^{-1}.

Assay of integration of the gfp gene through PCR and expression of the GFP protein through fluorescence

The expression of the GFP protein in the transformed calli was visualized under UV which showed high fluorescence (Fig. 4 A,B). Integration of the *gfp* sequence was further confirmed by PCR using *gfp* sequence specific primers (Fig 4 C). This showed a distinct 454 bp band corresponding to the amplicon size expected for the PCR.

The expression levels of GFP of calli from different Hygromycin concentration were further analyzed and compared by measuring the relative fluorescence unit of GFP protein inserts through microplate reader. Data from microplate reader (Fig 4 D) shows that the highest RFU was observed in transformants selected on 25mg L^{-1} hygromycin showed 1200 relative fluorescence unit (RFU) followed by transformants grown on 30mg L^{-1} having 820 RFU and 20mg L^{-1} showed the least, 250 RFUmg L^{-1} . Non-transformed plant tissues were used as controls.

The shoot apical meristem (SAM) consisting a group of pluripotent stem cells are important for postembryonic formation of aerial organs and tissues. It has been found that cytokinin plays a crucial role in maintaining balance between cell proliferation and differentiation in SAM (Werner *et al*., 2009).

Contrary to the promotive activity of cytokinin on SAM, it has an inhibitory effect on Root Apical Meristem (RAM) (Warner *et al*.2009). A high cytokinin to auxin ratio triggers shoot formation instead of root. So for the root formation in the regenerating plants external auxin source is required so that 0.1 mg L^{-1} of IAA had been applied to the basal medium (Root inducing medium).After 4-5 day root formation can be seen. From the result we had got it was very clear that the concentration IAA used here support root formation. It can be concluded that by following this method of transformation it was possible to get the highest percentage possible, of transformed plants from leaf explants.

Conclusion

In conclusion it can be said that this is a new and simple method of transformation of tobacco. This method avoids the use of

several hormones and different media compositions. This method requires only one hormone namely BAP for regeneration of transformed plantlets. The antibiotic concentration for selection of transformants was also optimized. We confirmed the integration of the transgene that is *gfp* gene by PCR. The expression GFP was confirmed by fluorescence microscopy. The transformation efficiency is the highest possible percentage i.e., 100%. Therefore this is a simple method of transformation of tobacco with GFP as reporter that can be applied to introduce genes of desirable traits.

Acknowledgement

This work is partially funded by DBT, Government of India and UGC for providing junior and senior research fellowship under RGNF scheme to M.M, and also UGC-CAS Department of Botany, University of Calcutta.

Conflict of interest

There is no conflict of interest.

References

Basu, A., Ray, S.,Sarkar,S., Ray Chaudhuri, T., Kundu, S., 2014. *Agrobacterium* mediated genetic transformation of popular Indica Rice Ratna (IET 1411).African Journal of Biotechnology.13:3187-3197.

Bellini, C., Pacurar, D.I., Perrone,I., 2014. Adventitious Roots and Lateral Roots: Similarities and Differences. Annual Review of Plant Biology.65:639-666.

Chowdhury,C., Basu ,A., S. Kundu., 2014.A new high-frequency *Agrobacterium*-mediated transformation technique for *Sesamum indicum* L. using de-embryonated cotyledon as explants. Protoplasma.251:1175-1190.

Eeckhaut, T., Lakshmanan, P.S., Deryckere, D., van Bockstaele, E., Van Huylenbroeck, J., 2013. Progress in plant protoplast research. Planta .238: 991-1003.

Ikeuchi, M., Ogawa,Y., Iwase,A., Sugimoto, K., 2016.Plant regeneration: cellular origins and molecular mechanisms. Development.143:1442-1451

Ivanova, M., & van Staden, J., 2011. Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*. Plant Cell, Tissue and Organ Culture.104:13-21.

Kumar, G., Vijayachandra, K., Veluthambi, K., 1999. Preincubation of cut tobacco leaf explants promotes *Agrobacterium*-mediated transformation by increasing vir gene induction. Plant Science.

Lemaux, P. 2008. Genetically Engineered Plants and Foods: A Scientist's Analysis of the Issues (Part I). Annual Review of Plant Biology.59: 771–812.

Motte, H., Vereecke, D., Geelen, D., Werbrouck,S., 2014. The molecular path to in vitro shoot regeneration. Biotechnology Advances. 32:107–121.

Murashige, F., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473–492.

Padilla, I.M.G., Burgos, L., 2010. Aminoglycoside antibiotics: structure, functions and effects on in vitro plant culture and genetic transformation protocols. Plant Cell Reports.29:1203.

Pathi, K.M., Tula, S., Tuteja, N., 2013. High frequency regeneration via direct somatic embryogenesis and efficient *Agrobacterium*- mediated genetic transformation of tobacco. Plant Signaling & Behavior 8:6.

Ray, S., Lahiri, S., Halder, M., Mondal, M., Ray Choudhuri, T., Kundu,S., 2015. An Efficient Method of Isolation and Transformation of Protoplasts from Tomato Leaf Mesophyll Tissue using the Binary vector pCambia 1302. International Advanced Research Journal

in Science, Engineering and Technology. 2:6.

Roche,J., Turnbull,M.H., Guo,Q., Novák,O., Späth,J., Gieseg, S.P., Jameson, P.E., Love,J., 2017.Coordinated nitrogen and carbon remobilization for nitrate assimilation in leaf, sheath and root and associated cytokinin signals during early regrowth of *Lolium perenne*. Annals of Botany 119 :1353-1364.

Shiro Fukuta,S.,, Kei-ichi, K. , Yuko, M. , Yukie, Y., Jun-ichi, U., Michio, K., F2012. Transgenic tobacco plants expressing antimicrobial peptide bovine lactoferricin show enhanced resistance to phytopathogens. Plant Biotechnology. 29:383–389.

Srinivasan, C., Liu, Z., Heidmann, I., Supena, E., Fukuoka, H., Joosen, R., Lambalk, J., Angenent, G., Scorza, R., Custers, J.B., Boutilier, K. 2007. Heterologous expression of the ap2/erf transcription factor baby boom alters the development and enhances the regeneration capacity of transgenic tobacco (*Nicotiana tabacum* L.). Planta. 225:341-351.

Thorpe, T.A., 2007. History of Plant tissue culture. Molecular Biotechnology 37: 169-180.

Werne,T., Schmülling,T., 2009. Cytokinin action in plant development. Current Opinion in Plant Biology 12:527–538.