Biological Forum – An International Journal **13(1): 293-298(2021)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Cotton Bed: An Eco friendly Tool for Eliminating *Agrobacterium tumefaciens* in Sugarcane Transformation

Niraj Nath Tiwari and Sujeet Pratap Singh
Centre for Sugarcane Biotechnology,
Sugarcane Research Institute (UP Council of Sugarcane Research), Shahjahanpur-242 001, India.

(Corresponding author: Niraj Nath Tiwari)*
(Received 08 January 2021, Accepted 27 March, 2021)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: *Agrobacterium tumefaciens* mediated genetic transformation and surface sterilization of explants with antibiotic is the key stage of genetic transformation process. Mostly it's done, biotic stresses by *Agrobacterium* and abiotic stresses by antibiotics that is causes and minimize transformation effectiveness due to necrosis and phytotoxicity of explants cells. This study was conducted to examine the effects of cotton bed over antibiotic treatment on *Agrobacterium* mediate genetic transformation in sugarcane. *Agrobacterium* strains LBA4404 were used for the infection of leaf disc explants of sugarcane somatic embryogenic calli. Bacterial removal from infected somatic embryogenic calli explants are always essential and a critical stage for survival of the plantlets. In this experiment, cefotaxime 500 mg/l was used for the washing of infected explants and removal of overgrowth of *A. tumefaciens*. At the end of the experiments, explants survival efficiency were 13%. Necrosis and phytotoxicity were not found in this cotton bed experiment. It was also observed that there was minimal secretion of phenolics compounds in media through co cultivated explants of sugarcane. This might be first report to eliminate *Agrobacterium tumefaciens* with the help of cotton bed so far.

Keywords: Sugarcane; *Agrobacterium tumefaciens;* Cotton bed, Embryogenic calli, Genetic transformation

INTRODUCTION

Sugarcane is an important farmers liking and agro-industrial cash crop that belongs to the poaceae family. It is mainly cultivated in tropical and subtropical regions of the world. Sugarcane crop is used for sugar production, paper making, chipboard, fertilizer, bio-ethanol and feed for live stocks (Chaudhry *et al.*, 2008). The major losses of the crop production are carried out by both biotic and abiotic stresses such as salinity, drought, pests and viral diseases (Nasir *et al.*, 2000, Khaliq *et al.*, 2005). It is difficult to overcome these losses by traditional methods. Traditional methods are time consuming and not well perform to overcome these losses in sugarcane. Researches of genetic engineering isolate desired gene for different traits from other living organism and transform these desired gene into crops for benefits of the human and other form of the life. Transformation of these desired genes are carried out by different mechanical methods vis. electrophoration, bombardment, and PEG methods as well as biological methods vis. *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes. Agrobacterium* mediated genetic transformation having a lot of advantages over the mechanical genetic transformation system. *Agrobacterium* prefer the single copy of gene transfer that minimize the gene silencing, enhance the precision of gene integration and help in the integration of long size of T-DNA in the host plants (Veluthambi *et al.*, 2003). Moreover, *Agrobacterium* mediated biological genetic transformation system is also critical and affected by a number of distinct factors such as cultural conditions, acetosyringone concentration, co-cultivation period, antibiotics doses and explants sources (Arencibia *et al.*, 1998; Rastogi *et al.*, 2014). Since *Agrobacterium* mediated transformation is a powerful tool for good transformation of desired gene. But this method also has some negative points. After infection and transformation, infected explants need to remove *Agrobacterium* under 48-72 hrs from their surface, otherwise it could be spoil the explants. Earlier, Karthikeyan *et al.*, (2011) reported cell necrosis and meager survival of *Agrobacterium* infected explants. Cefotaxime is belonging to beta-lactum group family and universally known as, this group causes low toxicity to plant tissue. The lower lethal efficiency of this group on plant tissue, many researchers utilize Cefotaxime for the washing of infected explants for the elimination of *Agrobacterium*. The over dose of these antibiotics in the plant regeneration medium also affects the regeneration efficiency (Ling *et al.*, 1998;

Ieamkhang and Chatchawankanphanich 2005; Rastogi *et al.*, 2014).

Cotton bed is an eco friendly and economically useful for the successful *Agrobacterium* mediated genetic transformation of sugarcane. In this experiment no need of agar in media is required for surface base. Cotton bed provides a base for explants and plantlets, that's why this experiment is also economically important. No more eco friendly reports are available on successful *Agrobacterium* mediated transformation in sugarcane. In this study, we were examining the cause of cotton bed over *Agrobacterium* infection on sugarcane plant transformation. In the series of this experiment we also examine the antibiotic response on physiology of plant tissues (browning, necrosis, growth of infected tissues).

MATERIALS AND METHODS

A. Plant material and explants preparation

Six to eight months old healthy shoot tops of CoS 08272 variety were used as explants and collected from the form of the Sugarcane Research Institute, Shahjahanpur, Uttar Pradesh, India. Shoot tops were taken from the healthy plants at the upper base with some nodes.3-4 attached leaves were removed from the shoot top. 3-4 inch long apical shoot tips cut from shoot top and wipe with 70% alcohol for surface sterilization and explants preparation. Explants were washed thoroughly under running tap water for 10 minutes followed by washing with a few drops of Tween-20 for seven to eight minutes with constant shaking by hand and then again wash the explants with distilled water and then explants were transfer under aseptic condition in a laminar airflow. The pre washed shoot tips were again washed with 70% alcohol for two minute and then 2-3 times washed with sterile distilled water for 3-5 minutes. Finally, the explants was carried out with 0.1% $HgCl_2$ (Mercury chloride) for 2 minutes with gentle shaking. After thorough washing with two to three times sterile distilled water and now shoot tops are ready for leaf disc preparation. The outer 2-3 whorls of upper leaves were removed with the help of scalpel and forceps then innermost soft immature leaf rolls were cut into transverse segments in the size of 4-5mm thickness (Ali and Iqbal 2012). All the above operations were performed under aseptic conditions in laminar airflow cabinet.

B. Callus induction

For callogenesis, callus induction media have MS basal nutrients salt supplemented with 3mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) for induction of callus. Young leaf roll of CoS 08272 were cultured on 25ml callus induction semi-solid medium on petri plates. These petri plates were kept in the dark for 12-15 days. Proliferate callus were sub-cultured on to fresh MS medium containing 3mg/l 2,4-D in every 10-15 days of culture for further proliferation of somatic embryonic callus. Purple color was appearing on cultured leaf disc explants for indication of somatic embryogenesis (Paulraj *et al.*, 2014).

C. Culture preparation of Agrobacterium

Glycerol stocks of a genetic engineer, *Agrobacterium tumefaciens* having *cry1Ac* gene were inoculated to 10 ml YEP (Yeast extract peptone) media (Yeast extract powder 10 gm/l, Peptone 10 gm/l, NaCl 5 gm/l and pH 7.2) with the proper antibiotics concentration (Rifampicin 10mg/l, Kanamycin 50mg/l and Rifampicin 200 mg/l) and grown overnight in incubator shaker at 180 RPM for 28°C. This mother culture was re-grown in 50 ml LB broth with respective antibiotic concentration, till the optical density reached 0.6 to 0.8 for LBA4404 at 600nm.

D. Infection and co cultivation of embryogenic calli

In vitro grown, healthy and premature embryogenic calli as well as full of life LBA 4404 *Agrobacterium* culture was use simultaneous to incubate in a conical flask for 10 min with gentle shaking for well infection and co cultivation. After the proper infection, the co cultivated embryogenic calli were spread on autoclaved blotting sheet, under aseptic condition. Dried co cultivated embryogenic calli leaf disc were transferred on petri plates poured by MS shoot induction media (SI2 media) supplemented with MS basal as well as NAA 0.1mg/l and BAP 0.2mg/l. All plates having co cultivated embryogenic calli were wrapped with aluminum foil for dark creation and placed in BOD at 28°C for two days of co-cultivation.

E. Bacterial removal from targeted explants

Effect of cotton bed techniques were examined over infected sugarcane tissue for the removal of *Agrobacterium* after co-cultivation. 500mg/l cefotaxime were used for the washing of co cultivated embryonic calli. 50 ml of ½ MS basal liquid media having 500 mg/l cefotaxime were used and put it in conical flask in the shaker incubator for 30 minutes at 50 RPM at 35-37°C. Wash the tissues 3-4 times with autoclaved distilled water and dry them on blotting paper then transferred on sterile petri plates having cotton bed with liquid SI2 media (MS basal, NAA 0.1mg/l and BAP 0.2mg/l) for stopping the growth of bacteria on washed co cultivated explants. Every 18-20 days interval, explants were sub cultured on fresh liquid SI2 media medium having cotton bed and lacking cefotaxime. After first subculture (18-20 days), if required, again wash once more time with 500mg/l cefotaxime for proper killing of *Agrobacterium.*

RESULTS AND DISCUSSION

A. Formation of somatic embryogenic calli

Young leaf disc explants (6-8 months old) were suitable for good embriogenic calli formation. Callus induction media along with MS basal nutrients salt was implemented for callogenesis in dark period. Callus tissue were initiated and visible at the scratch end of leaf roll disc explants, after a period of 10-12 days on MS basal medium containing 3mg/l 2,4-D Whereas, in the absence of 2,4-D in a medium, not a single or in a small quantity of callus were appear on leaf roll disc explants. (Fig. 1). Mayerni *et al.,* (2020) also reported 2,4-D alone or together with BAP given better result for the formation of callus induction. During the embryogenic calli formation there was various types of calluses seen that was differ on the basis of color and texture. (I) Dense callus- In 3-4 weeks, milky, solid and compact like structure was appeared, that was further produce somatic embryo like structure. On a later stage, it produces purple color and do confirm the right way for the development of embryogenic calli (Fig. 2).

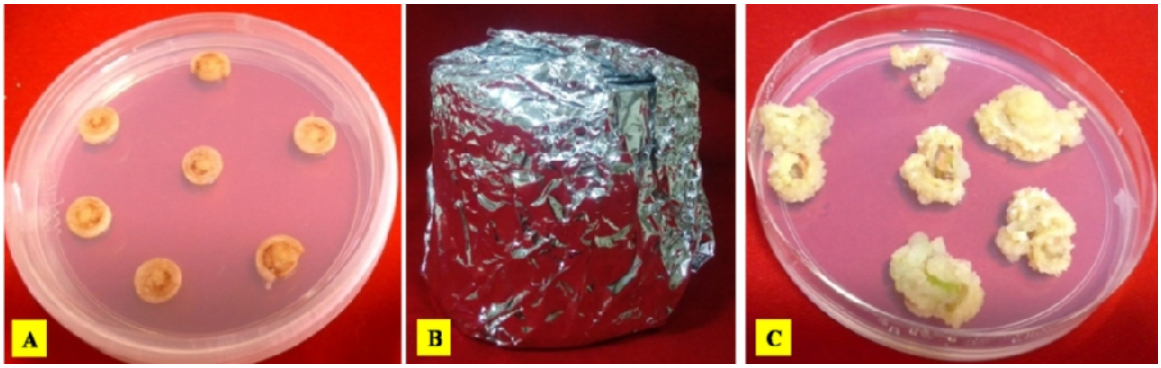

Fig. 1. Callus induction process **(A).** Inoculated leaf disc on MS+30mg/l sucrose+3mg/l 2,4D **(B).** Wrapped leaf disc in aluminum foil for proper darkness **(C).** Appeared calluses in pretreated leaf disc explants.

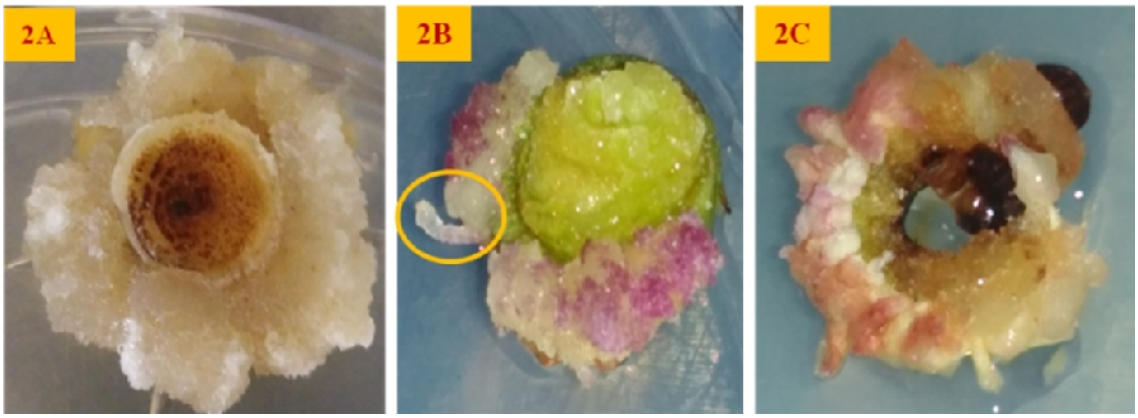

Fig. 2. Dense callus; **(A).** white compact callus; **(B&C).** Purple in later stages and was shown to be embryogenic in nature.

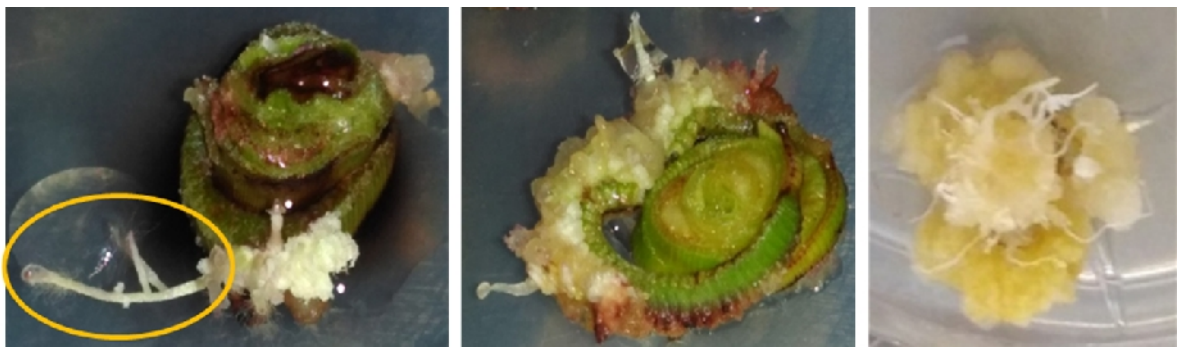

Fig. 3. Spongy callus; friable, whitish, semi-translucent callus tissue and occasionally developed roots (in circle).

Earlier Paulraj *et al.,* (2014) also reported the related result in *Arabidopsis thaliana*. (II) Spongy callus- Among these calluses some of the calluses were differ from dense callus and showing a whitish and loosely arranged like manner while some of the calluses having hairy root (Fig. 3). Most of the calluses were originated on the lower surface of leaf roll disc explants which was attached with medium whereas a small quantity of calluses were also appear on the upper surface of explants. A good and healthy embryogenic calli were obtained in a period of 50-60 days. Leaf disc were taken from upper part of the sugarcane shoot tip, develop a little amount of solid and milky callus (Fig. 3).

B. Bacterial removal from co-cultivated explants

Cefotaxime effectively kill the *Agrobacterium*, but not completely removed the Bacteria from the co cultivated plant tissue under given concentration and time period. Cefotaxime concentration and washing time was increased, as a result of this, sugarcane explants were shown immediate depigmentation and secretion of secondary metabolites, which was responsible for tissue death. Rastogi *et al.,* (2014) reported cefotaxime @ 200 mg/l effective to eliminate *A. tumefaciens* from sugarcane leaf disc explants and promote the growth of healthy regenerated sugarcane plantlet. High concentration or long time use of cefotaxime in a regeneration medium widely exhibited browning of explants and affected regeneration of plantlets as well as survival of the regenerated plantlets (Fig. 4A). This is also agreement with the finding of Mangena (2018). Cotton beds not only kill or eliminate the *Agrobacteria* from co-cultivated explants but also help to do separate the bacterium from infected explants. As a result healthy shoot induction efficiency was increase in co-cultivated explants (Fig. 4B). Without cefotaxime wash, co-cultivated explants were transferred on cefotaxime free MS-SI2 media, which show high rate of necrosis due to the over growth of *Agrobacterium* on somatic embryogenic calli. As a result not a single shoot was obtained through this method (Fig. 4C).

C. Effect of cotton bed for shoot regeneration and bacterial elimination

Agrobacterium infected explants showing difficulties for shoot regeneration and multiplication from somatic embryogenic calli. Although, sugarcane variety CoS 08272 used in this study is a highly shoot inducing variety (Singh *et al.,* 2014), but more than 50% of explants exhibiting shoot growth inhibition due to oxidative browning and induced compact callus (Fig. 4). Since, phenolic compounds secreted in high amount by co-cultivated explants due to the presence of *Agrobacterium* or presence of antibiotics in liquid SI2-MS medium. Probably combined inhibitory effects of bacterial infection and tissue browning caused by oxidation and accumulation of phenolics compounds and affected shoot initiation. This is negative effects on the rate of shoots and, thus the regeneration of transformed micro shoots was dramatically decreased in all cultures tested for *Agrobacterium* mediated genetic transformation. Several approaches have been reported for the shoot regeneration such as; the addition of dithiothreitol, polyvinylpyrrolidone (PVP), activated charcoal and other antioxidant mixtures like ascorbic acid (Huh *et al.,* 2017), but economically these are cost effective than cotton. Liquid SI2 MS media with cotton bed was given significantly result to overcome the secretion of phenolic compounds in media. Cotton bed provides a porous base for infected tissue and these porous base help to bacterium to settle down in bottom to consume the food from the media. As a result infected somatic embryogenic calli started shoot induction and suffer very less stresses as well as secreted very less amount of phenolic compounds.

Fig. 4. Shoot induction, bacterial elimination and overgrowth of *Agrobacteria* at different mode of treated co-cultivated explants. **(A).** Solid MS-SI2 media containing 500mg/l cefotaxime, *Agrobacteria* absent and shoot induction very slow; **(B).** Cotton bed with liquid MS-SI2 media without cefotaxime, *Agrobacteria* not appears and shoot induction very fast **(C).** Solid MS-SI2 media without cefotaxime, *Agrobacteria* appear and killed the co-cultivated somatic embryogenic calli.

In present study, explants survival efficiency were observed 13 per cent and least necrosis and phytotoxicity were found in this cotton bed experiment. It was also observed the minimal secretion of phenolics compounds in media through co-cultivated explants of sugarcane. Depigmentation was not observed in media and tissues as well as induced shoots were brownish green. Persistence and regrowth of *Agrobacterium* were observed in liquid regeneration media, but their negative impact was not observed on regenerated putative transgenic shoots (Fig. 5). Cotton bed was provided a base for support for plantlets and pores for capillarity of medium. Plant take their food from medium via pores of cotton and *Agrobacteium* get settle down through this pores in medium to consume their food. The final result of this cotton bad during *Agrobacterium* mediated genetic transformation, both bacteria and plantlets get free from each other and plant survival efficiency get increased. This finding could be an efficient protocol for suppressing *Agrobacterium tumefactions* without using any antibiotics in further genetic transformation or another works of sugarcane. This report might be a first report to eliminate *Agrobacterium tumefaciens* with the help of cotton bed so far.

Fig. 5. (A&B). Co-cultivated healthy embryogenic calli on regeneration media with cotton bed **(C).** 1st day Clean and transparent half MS medium and root were absent; **(D).** Loosing transparency of half MS media and roots present due to the detachment of *Agrobacterium* from plants in cotton bed and healthy roots were appeared.

Conflict of Interest statements: There is no any conflict of Interest.

ACKNOWLEDGEMENTS

The authors gratefully thanks to Additional Chief Secretary, Cane Development and Sugar Industry, U.P. Government, India for their financial support.

REFERENCE

Alsheikh, M.K., Suso, H.P., Robson, M., Battey, N.H. & Wetten, A. (2002). Appropriate choice of antibiotic and Agrobacterium strain improves transformation of antibiotic-sensitive *Fragaria vesca* and F.v. semperflorens. *Plant Cell Rep.*, **20**: 1173–1180.

Arencibia, A.D., Carmona, E.R., Tellez, P., Chan, M.T., Yu, S.M., Trujillo, L.E. & Oramas, P. (1998). An efficient protocol for sugarcane (*Saccharum* spp. L.) transformation mediated by *Agrobacterium tumefaciens*. *Transgenic Res*, **7**: 213-222.

Chaudhry, S.M. & Naseer, Z. (2008). Safety of ensiling poultry litter with sugar cane tops. *Pak J Agri Sci*, **45**(2): 322-366.

Huh, Y.S., Lee, J.K. & Nam, S.Y. (2017). Effect of plant growth regulators and antioxidants on in vitro plant regeneration and callus induction from leaf explants of purple passion fruit (*Passiflora edulis* Sims). *J Plant Biotechnol*, **44**: 335-342.

Ieamkhang, S. & Chatchawankanphanich, O. (2005). Augmentin as an alternative antibiotic for growth suppression of *Agrobacterium* for tomato (*Lycopersicon esculentum*)

transformation. *Plant Cell Tiss Organ Cult,* **82**: 213-220.

Karthikeyan, A., Pandian, S.K. & Ramesh, P.M. (2011). *Agrobacterium*-mediated ransformation of leaf base derived callus tissues of popular indica rice (*Oryza sativa* L. sub sp. indica cv. ADT 43). *Plant Sci*, **181**: 258–268.

Khaliq, A., Ashfaq, M., Akram, W., Choi, J.K. & Lee, J. (2005). Effect of plant factors, sugar contents and control methods on the Top Borer (*Scirpophaga nivella* F.) Infestation in selected varieties of sugarcane. *Entomol Res,* **35**: 153-160.

Ling, H.Q., Kriseleit, D. & Ganal, M.W. (1998). Effect of ticarcillin/ potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* Mill.). *Plant Cell Rep*, **17**: 843-847.

Mangena, P. (2018). The role of plant genotype, culture medium and *Agrobacterium* on soybean plantlets regeneration during genetic transformation, *Book chapter-Licensee Intech Open*, p. 2-25.

Mayerni, R., Satria, B., Wardhani, D.K. and Chan, S.R.O.S. (2020). Effect of auxin (2,4-D) and cytokinin (BAP) in callus induction of local patchouli plants (*Pogostemon cablin* Benth). *IOP Conf. Series: Earth and Environmental Science,* doi:10.1088/1755-1315/583/1/012003.

Nasir, N.M., Qureshim R.H. & Aslam, M. (2000). Effect of salinity on emergence of sugarcane lines. *Pak Sugar J*, **15**: 12-14.

Paulraj, S., Lopez-Villalobos, A. & Yeung, E.C. (2014). Abscisic acid promotes shoot regeneration in *Arabidopsis* zygotic embryo explants. *In Vitro Cell.Dev.Biol.-Plant*, **50**: 627–637.

Rastogi, J., Tiwari, N.N., Gupta, S., Bubber, P. & Singh, R.K. (2014). Eco-friendly dose of antibiotics for suppression of *Agrobacterium tumefaciens*, **16**: 85-87.

Singh, R.K., Singh, S.P., Pal, K., Karma, N.K. & Ram, B. (2014). CoS 08272 - An early maturing high yielding rich sugar variety for Uttar Pradesh. *Procc. 73rd Ann. Conv. STAI, Bangalore*, p. 39-45.

Tang, H., Ren, Z. & Krezal, G. (2000). An evaluation of antibiotics for the elimination of *Agrobacterium tumefaciens* from walnut somatic embryos and for the effects on the proliferation of somatic embryos and regeneration of transgenic plants. *Plant Cell Reports*, **19**: 881-887.

Veluthambi, K., Aditya, K.G. & Sharma, A. (2003). The current status of plant transformation technologies. *Curr Sci*, **84** (3): 368–37.

How to cite this article: Tiwari, N.N. and Singh, S.P. (2021). Cotton Bed: An Eco friendly Tool for Eliminating *Agrobacterium tumefaciens* in Sugarcane Transformation. *Biological Forum – An International Journal*, **13**(1): 293-298.