ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 11, Issue, 03, pp.1845-1848, March, 2019

DOI: https://doi.org/10.24941/ijcr.34627.03.2019

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

IMPACT OF VARIOUS ANTIBIOTICS ON REGENERATION EFFICIENCY IN *BRASSICA JUNCEA*

***Yamini Tiwari and Krishan Kumar**

Department of Biotechnology and Allied Sciences, Jayoti Vidyapeeth Women's University, Jaipur, Rajasthan, India-303122

ARTICLE INFO

Article History:

Received 12th December, 2018
Received in revised form 15th January, 2019
Accepted 24th February, 2019
Published online 31st March, 2019

Key Words:

Brassica, Hygromycin, Cefatoxime, Kanamycin, *Agrobacterium tumefaciens,* Regeneration.

ABSTRACT

A protocol has been developed to facilitate regeneration and *Agrobacterium* mediated genetic transformation using antibiotics in regeneration of cotyledonary petiole and hypocotyls explants of *Brassica juncea* cv. NRCHB-101. Three antibiotics *viz*. Kanamycin (kan), Cefatoxime (Cef) and Hygromycin (hygro) have been used in this study during shoot regeneration along with the growth regulators. The antibiotic Cefatoxime overpowered the growth of *Agrobacterium tumefaciens* at minimum concentration (250 mg/l) whereas kanamycin and hygromycin not only eliminates the traces of excess *Agrobacterium* but also prevents the occurrence of false-positive shoots at the concentration of 20 mg/L. The present inquisition gives an account on the effectiveness of Kan, Cef and Hygro for regeneration and *Agrobacterium tumefaciens*-mediated genetic transformation in *Brassica juncea* cv. NRCHB-101.

***Citation*: *Yamini Tiwari and Krishan Kumar,* 2019.** "Impact of various antibiotics on regeneration efficiency in *Brassica Juncea*", *International Journal of Current Research*, 11, (03), 1845-1848.

INTRODUCTION

Agrobacterium tumefaciens-mediated genetic transformation is the most recurrent and inexpensive method, out of all the methods used for genetic transformation. Gene of interest can be transferred to desired plant using genetic transformation. In almost all the economically important *Brassica* species transformation systems has been developed such as *B. juncea* (Sushma *et al.,* 2017) *B. napus* (Rhian *et al*., 2018), *B. nigra* (Gupta *et al*., 1993), *B. oleracea* (Ravanfar *et al*., 2017), *B. carinata* (Babic *et al*., 1998) and *B. rapa* (Bhaskar *et al*., 2016). A lot of factors are responsible for the efficiency of gene transfer like age of explants, type of explants, genotype *etc.* Transformation efficiency reduces after co-cultivation due to growth of *Agrobacterium.* Thus it is necessary to impede further growth and multiplication of *Agrobacterium* on plant tissue culture media, after co-cultivation of explants. For this purpose various antibiotics are used to inhibit and impede the growth of *Agrobacterium*. These antibiotics should be highly effective, stable, and economical with no negative effect on plant regeneration. There is significant evidence that antibiotics adversely affect the growth and performance of plants (Liu *et al*., 2013). Use of antibiotics negatively affects the regeneration of explants of *Brassica* (Minden *et al*., 2017). Cef is a broad spectrum antibiotic which provides protection against a wide range of bacteria. It block the mucopeptide biosynthesis of cell wall by inhibiting the cross linking of peptidoglycan by binding and inactivating of transpeptidases. It is effective in suppressing *Agrobacterium*. Hygromycin and kanamycin are aminoglycoside that kills bacterial cells by inhibiting protein synthesis. The purpose of this study was to check the toxic level of cefotaxime, hygromycin and kanamycin on cultured explants (cotyledonary petiole and hypocotyl) of *Brassica juncea.*

***Corresponding author:* Yamini Tiwari and Krishan Kumar**
Department of Biotechnology and Allied Sciences, Jayoti Vidyapeeth Women's University, Jaipur, Rajasthan, India-303122.

MATERIALS AND METHODS

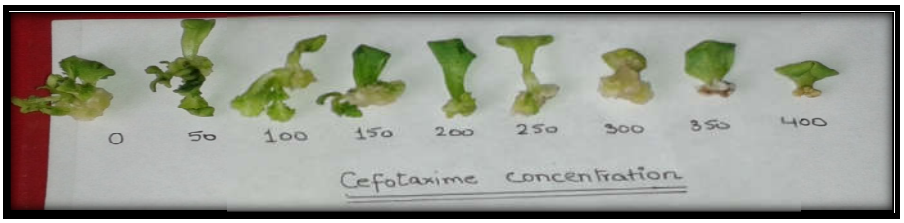
Seeds of *Brassica juncea* (L.) Czern. & Coss. variety NRCHB-101 were obtained from National research centre on Rapeseed-Mustard (NRCRM), Bharatpur, Rajasthan. The seeds were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution for 3-4 min. and rinsed 3-4 times (for approx. 2-3 min) with distilled water. Water was drained completely and seeds were dried on sterile paper towel. Then the surface sterilized seeds were germinated aseptically on MS basal medium (Murashige and Skoog, 1962). 5 days old *in vitro* grown seedlings were used as the source of explants *i.e.* cotyledonary petiole and hypocotyls (0.5–0.8cm). Different concentrations of antibiotics *viz*, Cef (0 – 400 mg/l), Kan (0 – 40 mg/l) and Hygro (0 – 40 mg/l) were added to shoot induction medium containing MS + 2.0 mg/L BAP + 0.2 mg/l NAA to study the effect of antibiotics on shoot regeneration. Pre-cultured explants were subjected to co-cultivation, after infecting them with *A. tumefaciens* LBA4404 harboring pCAMBAR. *chi11*. After two days of co-cultivation, the explants were shifted onto shoot induction media

supplemented with different concentrations of antibiotics. All the cultures obtained were maintained at 25 ± 2 ºC, 60% relative humidity, 16 /8 hrs of photo/dark period along with cool fluorescent lights at an intensity of 30 µmol m^{-2} s^{-1}.

RESULTS AND DISCUSSION

In the present study the effect of antibiotics (Hygromycin, Cefotaxime and Kanamycin) on regeneration of *Brassica juncea* cv. NRCHB-101. Antibiotic sensitivity was observed in explants *i.e.* cotyledonary petiole and hypocotyls of *Brassica juncea*. Both the explants exhibited high sensitivity to hygromycin and kanamycin even at low concentrations. The non-transformed tissues are unable to survive on selection medium retaining antibiotic. It was observed that in absence of antibiotic in the medium the regeneration occurred was highest while after adding the antibiotics in the medium regeneration frequency lowered since it supported the growth of putative transgenic explants only.

effect of different cefotaxime concentrations has been investigated independently on the regeneration potential of *Brassica species*. Cefotaxime not only eliminates the traces of bacteria from the culture but it also has capability to elevate the growth of explant, regeneration and embryogenesis in *in vitro* cultures (Danilova and Dolgikh, 2004; Kaur *et al*., 2008). In *Brassica* explants, no augment was observed in shoot regeneration prospective on medium supplemented with different cefotaxime concentrations. Similar results were reported by Borrelli *et al.* (1999) where cefotaxime did not affected growth of callus in wheat. In *Brassica juncea* maximum shoot regeneration was attained on the shoot regeneration medium supplemented with 250 mg/L cefotaxime concentration in regeneration of cotyledonary petiole and hypocotyl explants (Sharma *et al*., 2004; Singh *et al*., 2009; Bhuiyan *et al.,* 2011). Increased concentration of cefotaxime (*i.e.* beyond 250 mg/L) illustrated reduced transformation frequency and browning of explants followed by death of explants. The data on effect of Cef on regeneration of cotyledonary petiole and hypocotyl explants are shown in (Fig 3).



(a)

(b)

(c)

Figure 1. Effect of antibiotics on Shoot regeneration of *Brassica* explants. (a) Cefotaxime (b) Kanamycin (c) Hygromycin

Effect of Cefatoxime

Effect of Cefotaxime (0-400 mg/l) was studied on bacterial suppression and regeneration ability of cotyledonary petiole and hypocotyl. The percentage survival of explants was slashed gradually from 100 to 400 mg/l. Normal shoot induction percentage of explant was observed at 250 mg/l (Fig. 1). While No shoot induction was seen at 300-400 mg/l. At 300-400 mg/l concentrations browning of callus was observed. (Fig. 1). The cefotaxime contains 6-aminopenicillanic acid, phenylacetic acid and phenylmalonic acid in the b-Lactam ring and the side chain. At high concentration it causes loss of phytohormone balance which results in reduced regeneration and transformation efficiencies (Ogavwa *et al*., 2007). The

Effect of Kanamycin

The data on effect of Kan on regeneration on cotyledonary petiole and hypocotyl explants are shown in (Fig. 2). Different concentrations of Kan (0-50 mg/L) were added to the SIM. Almost no shoot induction was observed at concentrations above 20mg/L. Browning of explants started beyond 20mg/L. (Fig.1). Kanamycin resistance gene (*nptII*), which confers resistance against kanamycin is the most prevalently used selectable marker for transforming plants. Kanamycin sulfate is an aminoglycoside bacteriocidal antibiotic which also inhibits protein synthesis in bacterial cells. Selection is an important step during transformation to avoid formation of escapes. In control medium the explants exhibited appropriate

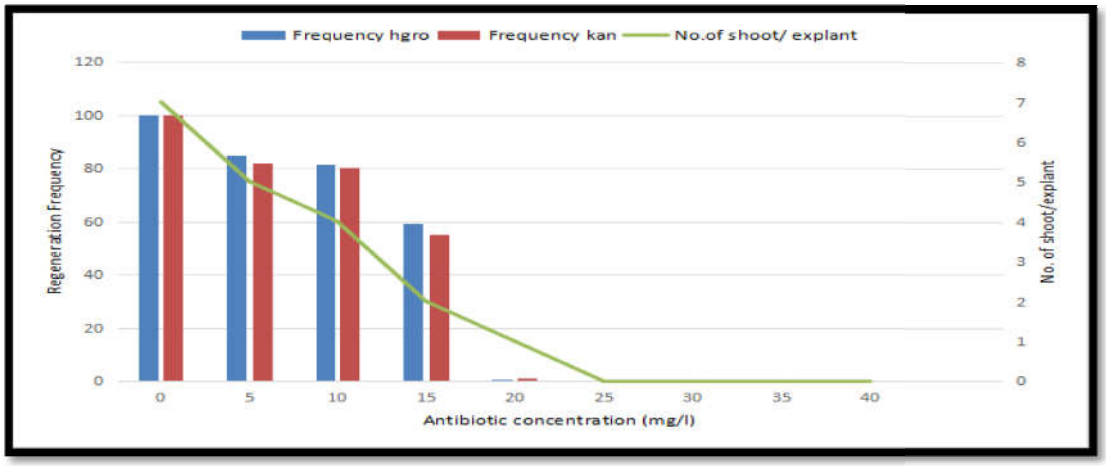


Figure 2. Effect of antibiotics (Kanamycin, Hygromycin) on regeneration potential of *Brassica juncea*

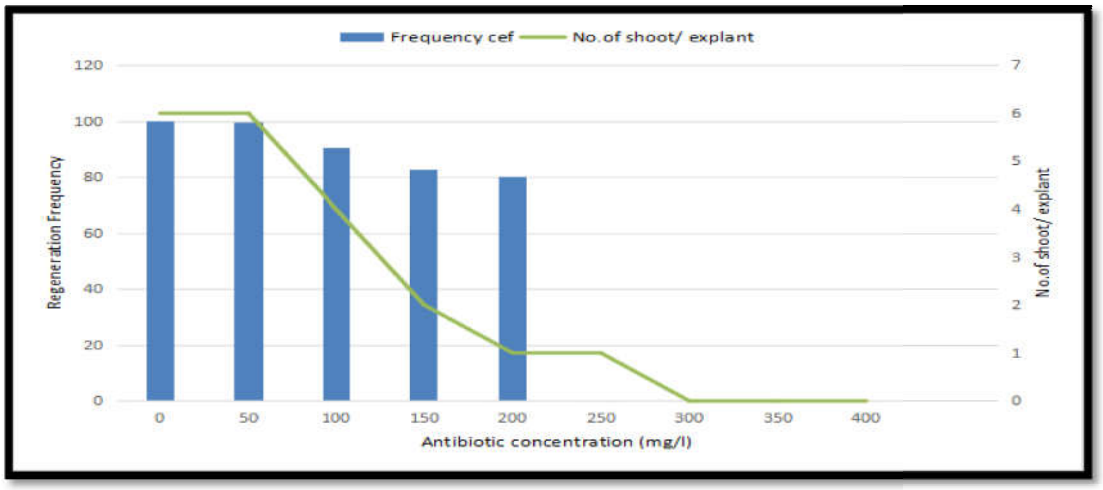


Figure 3. Effect of antibiotic (Cefotaxime) on regeneration potential of *Brassica juncea*

growth, while on selection media the color of explants changed from green to pale yellow and finally turn out to be brown after a few days even at low antibiotic concentration in *Brassica oleracia* (Kumar *et al*., 2017). Most of the workers reported 20 mg/L of kanamycin totally inhibit shoot regeneration from control set of explants in *Brassica juncea* (Sharma *et al*, 2004; Singh *et al*, 2009; Chakrabarty *et al*, 2002). 25 mg/L inhibits shoot induction in cotyledonary petiole and hypocotyls (Babic *et al*, 1998; Prasad *et al*, 2000), 30mg/L inhibits shoot regeneration of *Brassica species* (Chikkara *et al*., 2012; Wang *et al*., 2005) and 50 mgL inhibits shoot regeneration of *Brassica oleracea* (Deng-Xia *et al.,* 2011; Sharma and Srivastava, 2017; Kumar and Srivastava, 2016b).

Effect of Hygromycin

The data on effect of Hygro on regeneration on cotyledonary petiole and hypocotyl explants are shown in (Fig. 2). Different concentrations of Hygro (0-50 mg/L) were added to the SIM. Almost no shoot induction was observed at concentrations above 20mg/L. Browning of explants started after 20mg/L. (Fig.1). Hygromycin B is found to be more efficient in selection of putative transgenics as compared to other antibiotics (Song *et al*., 2012; Eady and Lister, 1998). Hygromycin B is an aminoglycoside, which causes mistranslation and interference with protein translocation thereby inhibiting protein synthesis (Gonzalez *et al*., 1978). Hygromycin B is extremely toxic to the plant cells (Waldron *et al*., 1985). A number of successful transformation protocols have been reported using Hyg as selectable maker in various monocot and dicot plants (Meng *et al*., 2007). According to Dutta *et al*. (2008) shoot regeneration efficiency of untransformed leaf pieces of *Brassica juncea* was lowered drastically with an increase in the hygromycin concentration *i.e.* from 90±2.9 % to 10±0.4% at 20 mg/l and to 1.1±0.1% at 30 mg/l concentration of hygromycin. Kong *et al*. (2009) also observed that transformation efficiency did not correspond with the regeneration frequencies of the untransformed explants. The untransformed cells get killed on selection media in such a way that they become noxious to adjoining transformed cells, ensuing in inhibition of the complete explant. Similar results were reported by Liu *et al.* (2015) according to which the regeneration frequency of the surviving explants were considerably declined with increasing concentration of hygromycin from 2-5 mg/L. Bhuiyan *et al.* (2011) and Sanimah *et al.* (2010) reported decline in regeneration frequency of the explants with the increase in hygromycin concentration and at the concentration 15-20 and 20 mg/L respectively no regeneration was observed.

Conclusion

Selection and detection of transformed cells from culture are crucial steps of genetic transformation which are beneficial in upgrading the transformation efficiency. However we observed that the regeneration percentage is intensely affected by the presence of antibiotics. In the case of *Brassica juncea*, antibiotic is to be used at a low concentration and adding up the second antibiotic completely hampers the regeneration process. This proposes the necessity to develop antibiotic marker free selection protocols to amplify the regeneration process so as to increase the yield.

Acknowledgements: For providing financial assistance and lab facilities we gratefully acknowledge Jayoti Vidyapeeth Women's University, Jaipur, Rajasthan, India.

REFERENCES

Babic, V., Datla, R.S., Scoles, G.J. and Keller, W.A. 1998. Development of an efficient *Agrobacterium* -mediated transformation system of *Brassica carinata*. *Plant Cell Rep.,* 17:183–188.

Baskar, V., Gangadhar, B.H., Park, S.W. and Nile, S.H. 2016. A simple and efficient Agrobacterium tumefaciens-mediated plant transformation of Brassica rapa ssp. pekinensis. *Biotech*, 6(1):88.

Bhuiyan, M.S.U., Min, S.R., Jeong, W.J., Sultana, S., Choi, K.S., Lee, Y., Lim, Y.P., Song, W.Y. and Liu, J.R. 2011. An improved method for *Agrobacterium*-mediated genetic transformation from cotyledon explants of *Brassica juncea. Plant Biotechnology,* 28:17–23. DOI: 10.5511/plantbio technology.10.0921a

Borrelli, G.M., Difonzo, N. and Lupotto, E. 1999. *Agrobacterium*-mediated transformation of wheat. *Journal of Plant Phyisology*, 140: 372-374.

Chakrabarty, R., Viswakarma, N., Bhat, S.R., Kirti, P.B., Singh, B.D. and Chopra, V.L. 2002. *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. *J. Biosci*., 27 495–502.

Chikara, S., Chaudhury, D., Dhankher, O.P. and Jaiwal, P.K. 2012. Combined expression of a barley class II chitinase and type I ribosome inactivating protein in transgenic Brassica juncea provides protection against Alternaria brassicae. *Plant Cell Tiss Organ Cult*., 108:83.

Danilova, S.A. and Dolgikh, Y.I. 2004. The stimulatory effect of the antibiotic cefotaxime on plant regeneration in maize tissue culture. *Russian Journal of Plant Physiology*, 51(4): 559-562.

Deng, X.Y., Lei, C., Yu, M.L., Mu, Z., Yang, Y. Z., Zhi,Y.F. and Li, M.Y. 2011. Transformation of cabbage (*Brassica oleracea* L. var. *capitata*) with *Bt cry1Ba3* gene for control of diamondback moth. *Agriculture Sciences in China,* 10(11): 1693-1700.

doi:10.1093/aobpla/plx010

Dutta, I., Saha, P. and Das, S. 2008. Efficient *Agrobacterium*-mediated genetic transformation of oilseed mustard [*Brassica juncea* (L.) Czern.] using leaf piece explants. *In Vitro Cell. Dev .Biol.-Plant.,* 44: 401. https://doi.org/10.1 007/s11627-008-9150-1.

Eady, C.C. and Lister, C.E. 1998. A comparison of four selective agents for use with Allium cepa L. immature embryos and immature embryo-derived cultures. *Plant Cell Rep*., 18:117–121.

Gonzalez, A., Jimenez, A., Vazquez, D., Davies, J. and Schindles, D. 1978. Studies on the mode of action of hygromycin B, an inhibitor of translocation in eukaryotes. *Biochim. Biophys. Acta – Nuclic Acids Protein Synth,*521:459–469.

Gupta, V., Lakshmi Sita, G., Shaila, M.S. and Jagannathan, V. 1993. Genetic transformation of *Brassica nigra* by agrobacterium based vector and direct plasmid uptake. *Plant Cell Reports.,* 12:418.

Kaur, A., Gill, M.S., Ruma, D. and Gosal, S.S. 2008. Enhanced *in vitro* shoot multiplication and elongation in sugarcane using cefotaxime. *Sugar Technology,* 10(1):60-64.

Kumar, P. and Srivastava, D.K. 2016a. Biotechnological advancement in genetic improvement of broccoli (*Brassica oleracea* L. var. *italica*), an important vegetable crop: A review. *Biotechnology-Letters*, 38 (7):1049–1063.

Kumar, P., Gaur, A. and Srivastava, D.K. 2017. *Agrobacterium*-Mediated Insect Resistance Gene (*cry1Aa*) Transfer Studies Pertaining to Antibiotic Sensitivity on Cultured Tissues of Broccoli. *International Journal of Vegetable Science*, 23:6, 523-535.

Liu, L., Liu, Y.H., Liu, C.X., Wang, Z., Dong, J., Zhu, G.F. and Huang, X. 2013. Potential effect and accumulation of veterinary antibiotics in *Phragmites australis* under hydroponic conditions. *Ecological Engineering*, 53:138–143.

Liu, X.X., Lang, S.R., Su, L.Q., Liu, X. and Wang, X.F. 2015. Improved *Agrobacterium*-mediated transformation and high efficiency of root formation from hypocotyl meristem of spring *Brassica napus* 'Precocity' cultivar. *Genet. Mol. Res*., 14 (4): 16840-16855.

Meng, Z.H., Liang, A.H. and Yang, W.C. 2007. Effects of hygromycin on cotton cultures and its application in *Agrobacterium*-mediated cotton transformation, *In Vitro Cell. Dev. Biol. Plant.,* 43 111–118.

Minden, V., Deloy, A., Volkert, A.M., Leonhardt, S.D. and Pufal, G. 2017. Antibiotics impact plant traits, even at small concentrations. *AoB Plants.,* 9: plx010.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant*., 15, 473–497.

Ogawa, Y. and Mii, M. 2007. Meropenem and moxalactam: Novel b-lactam antibiotics for efficient *Agrobacterium*-mediated transformation, *Plant Sci*., 172:564–572.

Prasad, K.V.S.K., Sharmila, P., Kumar, P.A. and Saradhi, P.P. 2000. Transformation of *Brassica juncea* (L.) Czern with bacterial *codA* gene enhances its tolerance to salt stress. *Molecular Breeding,* 6: 489–99.

Ravanfar, S.A., Orbovic, V., Moradpourd, M., Azizd, M.A., Karana, R., Wallace, S. and Parajuli, S. 2017. Improvement of tissue culture, genetic transformation, and applications of biotechnology to *Brassica*. *Biotechnol Genet Eng Rev*., 33(1):1–25.

Rhian, M. Howells, Melanie Craze, Sarah Bowden and Emma J Wallington. 2018. Efficient generation of stable, heritable gene edits in wheat using CRISPR/Cas9. *BMC Plant Biology.* 18:215.

Sanimah, S., Sundakar, J.T., Linthorst, H. and Verpoorte, R. 2010. Optimization conditions for *Agrobacterium*-mediated transformation of *Brassica rapa* with a bacterial isochorismate synthase gene. *J. Trop. Agric. and Fd. Sc.,* 38(2): 189–202.

Sharma, M., Sahni, R., Kansal, R. and Koundal, K.R. 2004. Transformation of oilseed mustard *B. juncea*. var. PJK with Snowdrop lectin gene. *Indian Journal of Biotechnology,* 3: 97-102.

Sharma, S. and Srivastava, D.K. 2017. *Agrobacterium*-mediated fungal resistance gene transfer studies pertaining to antibiotic sensitivity on cultured tissues of lettuce (*Lactuca sativa* L. cv. Solan kriti). *International Journal of Current Microbiology and Applied Sciences,* 6:1687-1698.

Singh, V.V., Verma, V., Pareek, A. K., Mathur, M., Yadav, R., Goyal, P., Thakur, A.K., Singh, Y.P., Koundal, K.R., Bansal, K.C., Mishra, A.K., Kumar, A. and Kumar, S. 2009. Optimization and development of regeneration and transformation protocol in Indian mustard using lectin gene from chickpea [*Cicer arietinum* (*L.*)]. *Journal of Plant Breeding and Crop Science,* 1(9): 306–10.

Song, G.Q., Walworth, A. and Hancock, J. 2012. Factors influencing Agrobacterium-mediated transformation of switchgrass cultivars. *Plant Cell Tissue Organ Cult*. 108:445–453.

Sushma Rani, Vinay Sharma, Alkesh Hada and Koundal, K.R. 2017. Efficient Genetic Transformation of *Brassica juncea* with Lectin Using Cotyledons Explants. *International Journal of Advanced Biotechnology Research*, 7(1): 1-12.

Waldron, C., Murphy, E., Roberts, J., Gustafson, G., Armour, S. and Malcolm, S. 1985. Resistance to hygromycin B. *Plant Mol. Biol*., 5:103–108.

Wang,. J, Chen, Z., Du, J., Sun, Y. and Liang, A. 2005. Novel insect resistance in *Brassica napus* developed by transformation of chitinase and scorpion toxin genes. *Plant Cell Reports,* 24: 549–55.

****