Shoot Tip Transformation in Papaya (*Carica papaya* L.)

R. Chandra[1], M. Mishra[1], R. Pati[1], S. Agarwal[2] and R.K. Jain[2]
[1]Biotechnology Laboratory, Central Institute for Subtropical Horticulture, Lucknow- 227 107, India
[2]Indian Agricultural Research Institute, New Delhi, India

Keywords: *Carica papaya*, transformation, shoot tip, acetosyringone, spermidine

Abstract

In vitro regeneration and transformation protocol has been developed in papaya across the globe using somatic embryo as explant. Transgenic papaya has been developed from hypocotyle or immature zygote. The transformation efficiency remains very low through embryo mediated transformation due to lack of synchronized maturity and subsequently poor germination. We report here for the first time transformation of young, in vitro grown shoot tips of papaya with dual gene (*cp*+*rep*) construct through *Agrobacterium*.

INTRODUCTION

India is one of the leading papaya producing countries of the world contributing 25% of world's total production. Papaya cultivation is threatened world over by a deadly viral disease, *Papaya ringspot virus* (PRSV). This disease is transmitted by aphid with characteristics ring spot lesions on fruit and leaves which affects productivity and marketability. *Papaya leaf curl virus* (PaLCuV) is another viral disease that affects papaya production but it is mostly confined to North India. The details of this geminivirus were studied by Saxena et al. (1998). This disease was discovered in the papaya orchards of southern Taiwan in 2002. Infected papaya developed symptoms such as downward curling of leaves, twisted petioles, veinenation, and stunting. Diseased plants produced small and distorted fruits that tend to fall prematurely.

Efforts have been made to combat these diseases. Cultural practices cross protection and resistant breeding has not yielded promising results for PRSV. r-DNA technology has successfully produced papaya conferring resistance against PRSV in Hawaii (Fitch et al., 1993; Cai et al., 1999; Gonsalves, 1998). However, similar efforts have not been made in India. In order to deliver gene of interest in the plant genome, somatic embryos developed from immature zygotic embryo or hypocotyle segment (Fitch et al., 1993; Cai et al., 1999) has been the preferred explant world over. However, high recovery of transformants through embryogenesis mediated pathway remains low due to lack of synchronized maturity and poor germination of somatic embryos and finally poor rooting in embryos derived plantlets. We report here a method of *Agrobacterium* mediated shoot tip transformation of papaya cv. Pusa Delicious using dual gene (*cp*+*rep* genes) in pBINAR binary vector.

MATERIALS AND METHODS

Papaya seedlings were germinated in vitro from immature seeds of cultivar Pusa Delicious grown in the germplasm block of CISH, Lucknow on half strength Murashige and Skoog medium.

Dual genes construct (*cp*+*rep*) in pBINAR binary vector mobilized in *Agrobacterium tumifaciens* strain LBA 4404 developed at Indian Agricultural Research Institute, New Delhi, was utilized for transformation. It has npt II selection marker and CMV 35S promoter. For activation of bacterial culture, single colony of *Agrobacterium* containing dual gene (*cp* and *rep*) was inoculated in 50 ml of LB liquid medium and left overnight at 28°C (OD 0.8 at 600 nm) in incubator shaker at 100 rpm. Overnight grown *Agrobacterium* culture was centrifuged (10,000 rpm) and pellets were dissolved in liquid MS medium supplemented with different concentration of spermidine (1, 2 and 3 µM), acetosyringone (50, 100 and 150 µM) and kept for 3 h prior to infection. 0.5 cm long

shoot tips were taken from 1 week old seedlings, meristems were excised and wounded with different methods such as vortexing the shoot tip with carborandum, bombardment of tungsten from gene gun (Gene Pro-HE 2000) and wounding the meristems tip with fine needle. The wounded tissues were inoculated on MS liquid medium fortified with acetosyringone for 30 min under agitated condition for infection. After infection, the explants were blotted dry on sterile filter paper and further inoculated on agarified MS medium (without hormone) containing acetosyringone (50, 100 and 150 µM) for co-cultivation under dark for different periods (24, 48 and 72 h).

After co-cultivation the shoot tips were washed with different antibiotics (cefotaxime 500 mg/L, carbenicillin 500 mg/L and augmantin 500 mg/L) and transferred to regeneration medium (1/2 MS + BAP 0.2 mg/L + NAA 0.1 mg/L + glutammin 400 mg/L) containing cefotaxime 500 mg/L, carbenicillin 500 mg/L and augmantin 500 mg/L for controlling growth of *Agrobacterium*. After one week shoot tips were shifted to selection medium having antibiotics (1/2 MS + 1.5 mg/L BAP, 0.2 mg/L NAA and 75 mg/L kanamycin). The shoots were selected up to 12 weeks in kanamycin. Initially they were exposed to 75 mg/L kanamycin for 4 weeks than survived shoots were further transferred to 100 mg/L for 4 weeks and finally the surviving shoot tips were shifted to 150 mg/L Kanamycin another for 4 weeks. Kanamycin selected shoot tips were enlongated in regeneration medium for another 4 weeks. Finally elongated shoot tips (1-2 cm) were further inoculated on kanamycin free MS medium supplemented with different concentrations of IBA (1, 2 and 3 mg/L) + activated charcoal (500 mg/L) for roots induction.

Molecular Analysis

Total genomic DNA kanamycin resistant plantlets were isolated (Qiagen, Plant genomic DNA Isolation kit). Approximately 250 ng of genomic DNA was used as template for PCR. In order to show the integration of T_L-DNA and T_R-DNA of Ti plasmid in the transformed plantlets, one region (npt II) was amplified with a pair of gene specific primers. npt II specific gene was detected by PCR with primers specific to npt II (~800 kb), such as forward primer (5'-TCTCACCTTGCTCCTGCC-3') and reverse primer (5'-AGGCGATAGAAGGCGATG-3'). The amplification conditions for npt II, initial duration was at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 54°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR products were analyzed by electrophoretic separation on 1% agarose gel (w/v) in 1X TBE buffer and staining with ethidium bromide.

RESULTS AND DISCUSSION

One week old wounded seedlings explant is prerequisite for *Agrobacterium* mediate transformation. The data (Fig. 1) clearly indicated that shoot tips vortexed with carborandum (for 1 min) gave higher transformation efficiency (11.20%) compared to other wounding methods. Zhu et al. (2006) described an *Agrobacterium*-mediated plant transformation by carborandum wounding in papaya embryogenic calli. While in another experiment in *C. papaya*, *Agrobacterium tumefaciens* has been used in conjunction with carborundum in a liquid phase which improved transformation efficiency (Yeh et al., 1998). This method gave the higher transformation efficiency which on average produced at least one positive plant during PCR analysis and southern blot analysis from 10-20% of callus cluster co-cultivated with *Agrobacterium tumefaciens*. The density of bacterium used for infection is adjusted either by monitoring the time of overnight cultures during incubation or by diluting the overnight bacterial cultures (Yeh et al., 1998). The bacterial density and inoculation time is directly correlated to each other. The optimum bacterial culture densities varied in different fruit crops. The explant after infection (30 min) with *Agrobacterium* is blotted dry on sterile filter papers and then transferred to agarified regeneration medium for co-cultivation at different hours (24, 48 and 72 h). The data (Fig. 2) clearly revealed that tissues co-cultivated for 72 h in dark with *Agrobacterium*, gave higher putative transformants up to 8.80%. In citrus, explants after agro infection were

co-cultivated for 3 days (Cervera et al., 1998; Perez et al., 1998; Han et al., 1999), *C. sinensis* and *C. reticulate* (3 days) and sweet orange (2 days). In grapes, co-cultivation period of 1-5 days have been reported. In *V. vinifera*, co-cultivation periods of 1 day (Baribault et al., 1990), 2 days (Harst et al., 2000), 3 days (Nakano et al., 1994) and 5 days (Hoshino et al., 2000) have been reported. The data (Fig. 3) clearly shows that cefotaxime 500 mg/L is very effective in control of *Agrobacterium* from tissue surface and gave higher putative transformants (8.80%) compared to other antibiotics (carbanicillin and augamantin). Lin et al. (1995) used 10 mg/L carbenicillin or 5 mg/L cefotaxime in suspension cultures for complete inhibition of *Agrobacterium*. However, higher concentrations of carbenicillin or cefotaxime, 250-500 mg/L, have been widely used in plant tissue culture, e.g., *Arabidopsis thaliana* (Akama et al., 1992) and *C. papaya* (Fitch et al., 1993; Cabrera-Ponce et al., 1996; Cheng et al., 1996; Yang et al., 1996). For *Agrobacterium* mediated gene transfer in papaya, carbenicillin (Fitch et al., 1990, 1993; Yang et al., 1996; Cheng et al., 1996; Tsong-Ann et al., 2001) and cefotaxime (Fitch et al., 1993; Cabrera-Ponce et al., 1996) are often added to the medium during plant regeneration to control the growth of *Agrobacterium*. The usage of phenolics (acetosyringone) may raise the expression of the *Vir* region and the transformation rate of the explants (Bolton et al., 1986). Acetosyringone has been routinely used in transformation experiments. It is evident from our results that (Fig. 4) acetosyringone (100 µM) added during co-cultivation period enhanced putative transformants (11.20%). Fitch et al. (1993) also suggested that overnight grown bacterial cultures induced for 4 h with 50 µM acetosyringone prior to co-cultivation improved the transformation efficiency. In tamarillo, addition of acetosyringone to bacterial culture and co-cultivation medium increased transformation efficiency (35%) significantly (Atkinson and Gardner, 1993). Our results showed (Fig. 5) that polyamine such as spermidine at 1.0 mM concentration increased the transformation efficiency when used during co-cultivation process. The spermidine (1 mM) enhances the *vir* gene induction when *Agrobacterium* cells were treated prior to acetosyringone addition (Kumar, 2003; Kumar and Rajam, 2006). It has been suggested that the optimum cellular polyamine level in the host plant through the modulation of polyamine metabolism either by using exogenous polyamine may be helpful in enhancing transformation frequency. The supplementation of spermidine in the selection medium has led to the enhancement of transformation frequency in wheat by *Agrobacterium* (Khanna and Daggard, 2003). Transformed shoots (1-2 cm long) subjected to IBA (2.0 mg/L) in the absence of kanamycin produced more roots (3.0 roots/culture). Such plants were easily acclimatized on sterilized coconut husk supplemented with MS salt mixture.

ACKNOWLEDGEMENTS

The authors are grateful to ICAR Networking Project on Transgenics in Crops for financial assistance.

Literature Cited

Akama, K., Shiraishi, H., Ohta, S., Nakamura, K., Okada, K. and Shimura, Y. 1992. Efficient transformation of *Arabidopsis thaliana*: comparison of the efficiencies with various organs, plant ecotypes and *Agrobacterium* strains. Plant Cell Rep. 12:7-11.

Atkinson, R.G. and Gardner, R.C. 1993. Regeneration of transgenic tamarillo plants. Plant Cell Rep. 12:347-351.

Baribault, T.J., Skene, K.G.M., Cain, P.A. and Scott, N.S. 1990. Transgenic grapev: regeneration of shoots expressing beta-glucoronidase. J. Exp. Bot. 14:1045-1059.

Bolton, G.W., Nester, E.W. and Gordon, M.P. 1986. Plant phenolic compounds induce expression of *A. tumecaciens* loci needed for virulence. Science 232:983-985.

Cabrera-Ponce, J.S., Vegas-Garcia, A. and Herrera-Estrella, L. 1996. Regeneration of transgenic papaya plants via somatic embryogenesis induced by *Agrobacterium rhizogenes*. In Vitro Cell. Dev. Biol. Plant 32:86-90.

Cai, W.Q., Gonsalves, C., Tenent, P., Fermin, G., Jr. Souza, M., Sarindu, N., Jam, F.J.,

Zhu, H.Y. and Gonsalves, D. 1999. A protocol for efficient transformation and regeneration of *Carica papaya* L. In Vitro Cell Dev. Biol. Plant 35:61-69.
Cervera, M., Pina, J.A., Juarez, J., Navarro, L. and Pena, L. 1998. *Agrobacterium*-mediated transformation of citrange: factors affecting transformation and regeneration. Plant Cell Rep. 18:271-278.
Cheng, Y.H., Yang, J.S. and Yeh, S.D. 1996. Efficient transformation of papaya by coat protein gene of papaya ring spot virus mediated by *Agrobacterium* following liquid-phase wounding of embryogenic tissue with caborundum. Plant Cell Rep. 16:127-132.
Fitch, M.M.M., Manshardt, R.M., Gonsalves, D. and Slightom, J.L. 1993. Transgenic papaya plants from *Agrobacterium*-mediated transformation of somatic embryos. Plant Cell Rep. 12:245-249.
Fitch, M.M.M., Manshardt, R.M., Gonsalves, D., Slightom, J.L. and Sanford, J.S. 1990. Stable transformation of papaya via microprojectile bombardment. Plant Cell Rep. 9:189-194.
Gonsalves, D. 1998. Control of *Papaya ring spot virus* in papaya: A case study. Annual Rev. Phytopatho. 36:415-437.
Han, M., Lu, R., Zhu, J., Qiu, X., Huang, H., Han, M.L., Lu, R.S., Zhu, J.Y., Qui, X.J. and Huang, H.Y. 1999. Studies on factors effecting gene-transformation by *Agrobacterium*-mediated in citrus. Acta Botynica Yunnanica 21:491-496.
Harst, M., Barnhoff, B.A., Zyprian, E., Topfer, R., Jach, G., Bouquet, A. and Bourtsiquot, J.M. 2000. Regeneration and transformation of different explants of *Vitis vinifera* L. Transgenic Res. 10:105-112.
Hoshino, Y., Zhu, Y.M., Mii, M., Takahashi, E., Nakano, N., Bouquet, A.E. and Boursiquot, J.M. 2000. Transgenic grapevine plants (*Vitis vinifera* L.) produced by selecting secondary embryos after co-cultivation of embryogenic callus with *Agrobacterium tumifaciens*. Acta Hort. 528:361-366.
Khanna, H.K. and Daggard, G.E. 2003. *Agrobacterium tumifaciens* mediated transformation of wheat using a super binary vector and polyamine supplemented medium. Plant Cell Rep. 21:429-436.
Kumar, S.V. 2003. Transgenic manipulation polyamine biosynthesis in *Solanum melongena* and *Chlamydomonas reinhardtii*. Ph.D. Thesis, University of Delhi.
Kumar, V.S. and Rajam, M.V. 2006. Polyamines enhance *Agrobacterium tumefaciens vir* gene induction and T-DNA transfer. Plant Sci. 162:769-775.
Lin, J.J., Assad-Garcia, N. and Kou, J. 1995. Plant hormone effect of antibiotics on the transformation efficiency of plant tissue by *Agrobacterium tumefaciens* cell. Plant Sci. 109:171-177.
Nakano, M., Hoshino, Y. and Mii, M. 1994. Regeneration of transgenic plants of grapevine (*Vitis vinifera* L.) via *Agrobacterium rhizogenes*-mediated transformation of embryogenic calli. J. Exp. Bot. 45:649-656.
Pang, S.Z. and Sanford, J.C. 1988. *Agrobacterium* mediated gene transfer in papaya. J. Am. Soc. Hort. Sci. 113:287-291.
Perez Molphe Balch, E. and Ochoa Alejo, N. 1998. Regeneration of transgenic plants of Mexican line from *Agrobacterium rhizogenes*-transformed tissues. Plant Cell Rep. 17:591-596.
Saxena, S., Hallan, V., Singh, B.P. and Sane, P.V. 1998. Leaf curl disease of *Carica papaya* from India may be caused by a bipartite geminivirus. Plant Dis. 82(1):126.
Tsong-Ann, Y., Shyi-Dong, Y. and Jiu-Sherng, Y. 2001. Effects of carbenicillin and cefotaxime on callus growth and somatic embryogenesis from adventitious roots of papaya. Bot. Bull. Acad. Sin. 42:281-286.
Yang, J., Yu, T., Cheng, Y., Yeh, S., Yang, J.S., Yu, T.A., Cheng, Y.H. and Yeh, S.D. 1996. Transgenic papaya plants from *Agrobacterium* mediated transformation of petioles of in vitro propagated multishoots. Plant Cell Rep. 15:459-464.
Yeh, S.D., Bau, H.D., Cheng, Y.H., Yu, T.A., Yang, J.S., Yeh, S.D., Bau, H.J., Cheng, Y.H., Yu, T.A., Yang, J.S. and Drew, R.A. 1998. Green-house and field evaluation of coat-protein transgenic papaya resistant to papaya ring spot virus. Acta Hort. 461:321-

328.
Zhu, Y.J., Fitch, M.M.M. and Moore, P.H. 2006. Papaya (*Carica papaya* L.). Methods Mol. Biol. 344:209-18.

Figures

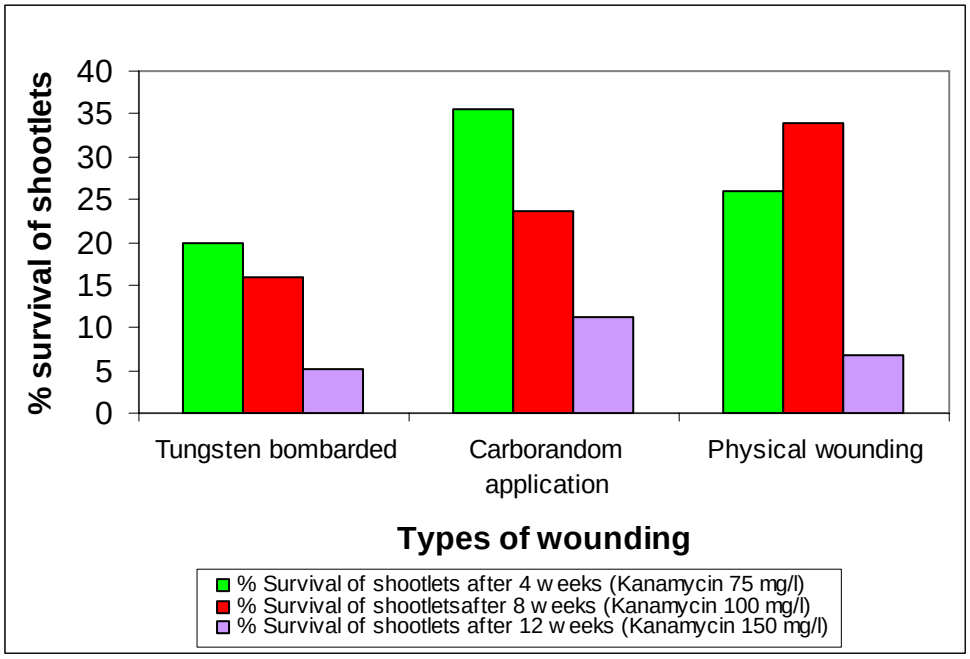

Fig. 1. Effect of wounding method on transformation efficiency.

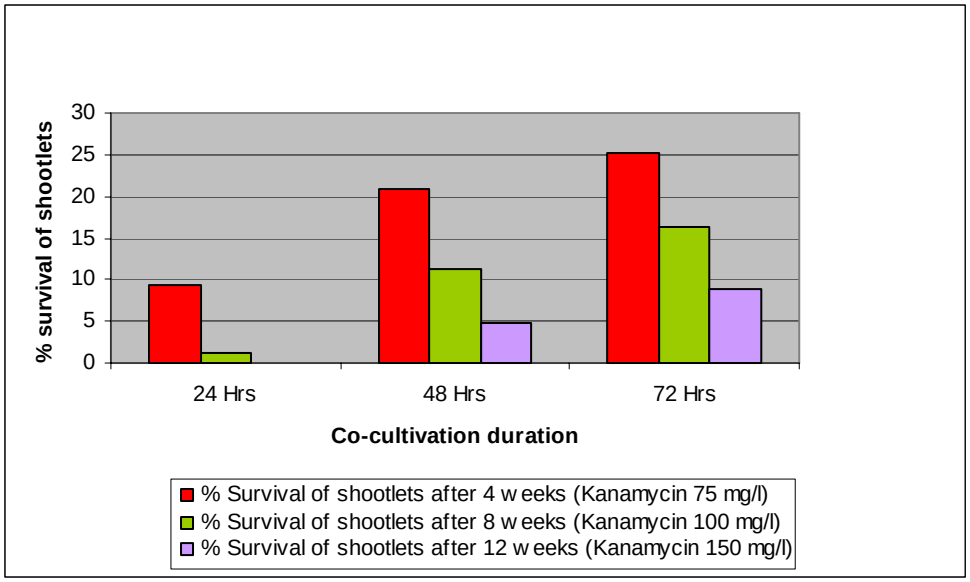

Fig. 2. Effect of co-cultivation periods on transformation efficiency.

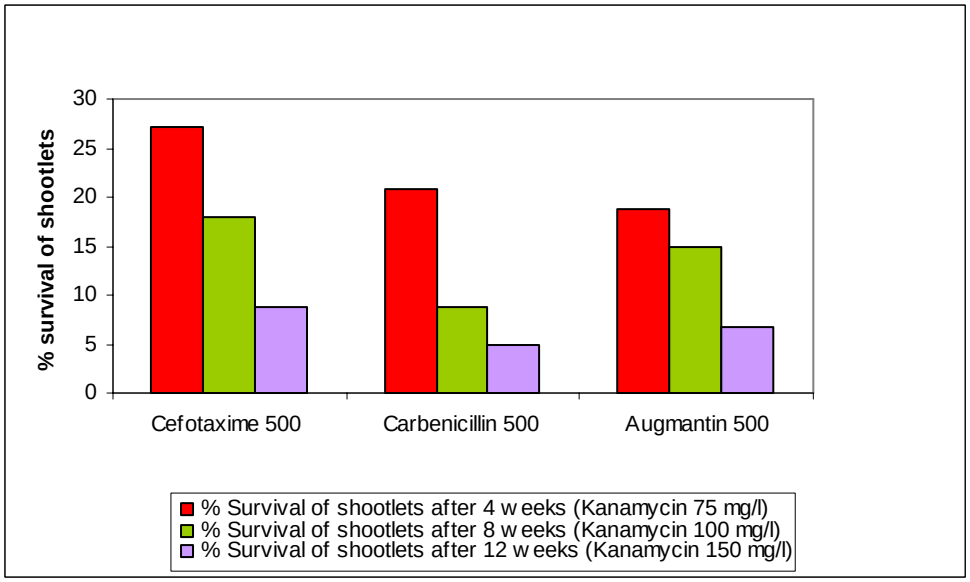

Fig. 3. Effect of antibiotics on control of growth of *Agrobacterium*.

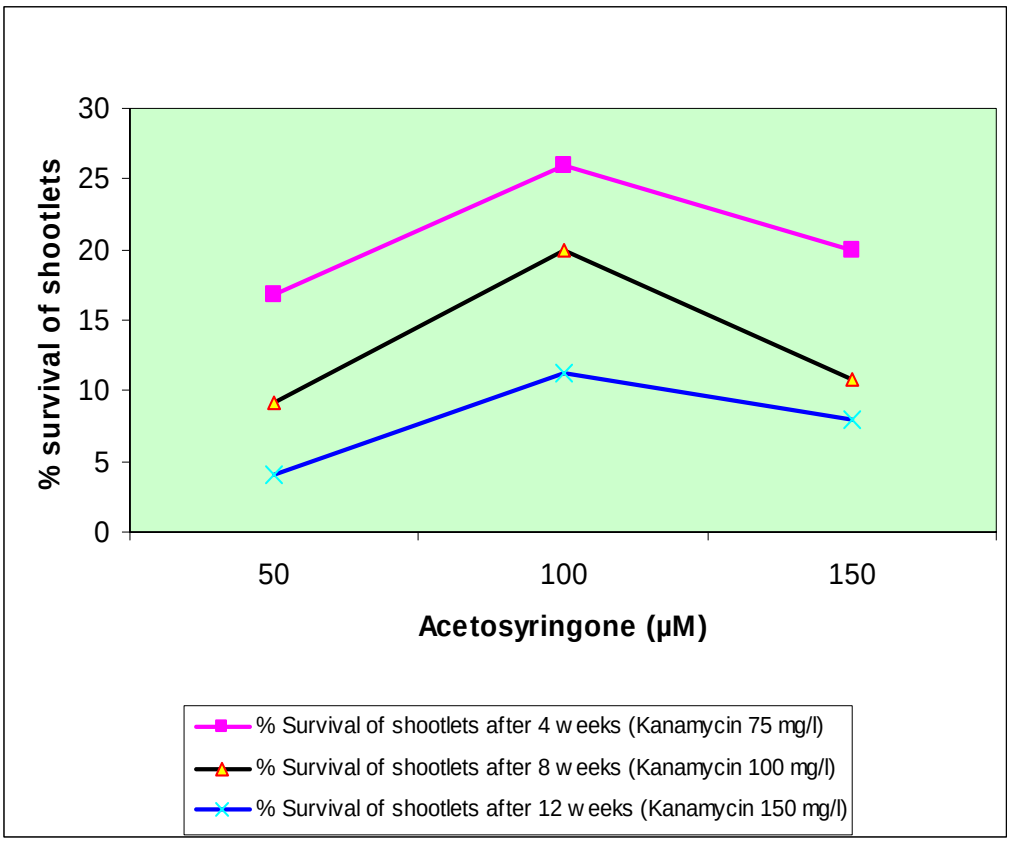

Fig. 4. Effect of acetosyringone on transformation efficiency.

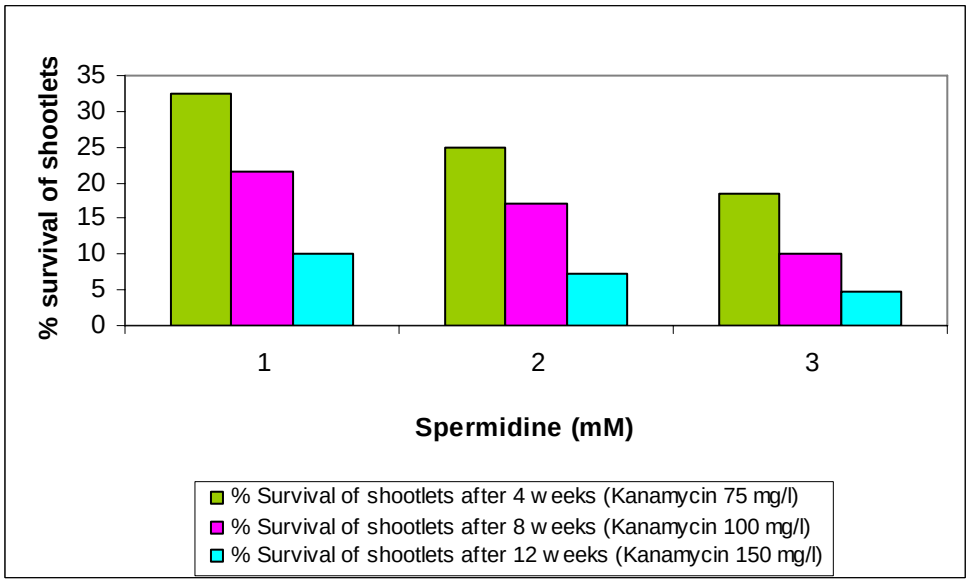

Fig. 5. Effect of spermidine on transformation efficiency.