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Effect of Various Factors on the Efficiency of Agrobacterium-Mediated Transformation of Grape (*Vitis vinifera* L.)

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Abstract

The present investigation was conducted to study the effect of inoculation time, co-cultivation period and various concentrations of hygromycin and kanamycin on genetic transformation of grape (*Vitis vinifera* L.) cv. King's Ruby through *Agrobacterium tumefaciens.* The Chitinase gene (for fungal resistance) and GUS gene (for phenotypic expression of transgenic) were introduced in the embryogenic calli developed from leaf discs, through *Agrobacterium* strain LBA4404 harboring plasmid pBI121 *nptII* as selectable marker for GUS gene and *hptII* for Chitinase gene. Regarding transformation efficiency rate, 10 minutes inoculation period with bacterial suspension showed the highest transformation efficiency rate i.e. 2.83% with Chitinase gene and 2.5% with GUS gene. Infected calli with Chitinase gene and GUS gene co-cultivated for 2 days showed the maximum transformation efficiency of 2.75% and 3.25%, respectively. For elimination of excess bacteria, cefotaxime treatment (300 mg L^{-l}) showed the highest survival rate of 3.16% for Chitinase gene and 2.5% for GUS gene. The maximum i.e.2.83% transformation efficiency rate was achieved at 10 mg L^{-l} of hygromycin for selection of transformed calli. The highest 2.25% transformation efficiency was yielded when kanamycin was used at the rate of 100 mg L^{-l}. Five out of 8 calli showed positive expression with 62.5%transformation efficiency through histochemical GUS assay. Present results may be helpful in improving genetic transformation efficiency of grape through *Agrobacterium tumefaciens* against fungal diseases and reduce the use of fungicides.

Keywords: Genetic transformation, Chitinase gene, GUS gene, Grape, Transformation efficiency

Introduction

The grape (*Vitis vinifera* L.*)* belongs to the family Vitaceae, which comprises of 12 genera and 60 species (Gray 1995, Sajid *et al.* 2006). It is one of the most widely grown fruit crop in the world. Plant pathogens are a real threat to worldwide agriculture. Significant yield losses due to fungal attacks occur in most of the agricultural and horticultural crops. More than 70% of all major crop diseases are caused by fungi (Agrios 2005). A number of fungal diseases limit worldwide production and storage of grape cultivars (Pearson and Goheen 1988). Fungal diseases, which cause extensive losses in yield and quality, are the most severe problems for grape cultivation (Pearson and Goheen 1988). The most frightening among these diseases are powdery mildew, anthracnose, downy mildew and gray mold rot. Methods of genetic transformation could be used to enhance defence mechanism of plants against diseases.

The use of genetic engineering for plant improvement permits introduction of useful agronomic traits without altering the features of the cultivar, necessitating the development of *in vitro* systems for genetic transformation and plant regeneration. Shoot regeneration from excised shoot apices has been profitably applied to several grape species and hybrids (Barlassand Skene, 1978). Different growth stages also occurred when cultures were initiated from the explants taken from field grown muscadine grapes (Gray and Benton, 1991). Transformation is the introgression of exogenous DNA into plant cell, tissue or organs employing direct orindirect means (Alves *et al.* 1999). Indirect gene transfer involves the introduction of exogeneous DNA by biological vector such as *Agrobacterium*, whereas direct gene transfer involves physical and

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chemical processes such as polyethylene glycol mediated DNA uptake, microinjection, silicon carbide fibres and microprojectile bombardment (Taylor and Fauquet 2002).

One of the main aims of plant transformation has been to solve agricultural problems without environmental damages (Alves *et al.,* 1999). However, the efficiency of the transformation protocol depends on several factors, including the genotype (Harst *et al.* 2000), choice of strategies (Perl *et al.* 1996, Yamamoto *et al.* 2000, Fan *et al.* 2008), *Agrobacterium* strain (Torregrosa *et al.* 2002), culture method (Harst *et al.* 2000) and use of antioxidants (Perl *et al.* 1996). The bacterial concentration is also important issue affecting transformation efficiency, even though only in few cases comparisons of bacterial density were made (Aguero *et al.* 2006). So, the purpose of present research was to optimize inoculation time, co-cultivation period and antibiotics concentration for bacterial elimination and selection of transformed calli of grape cv. King's Ruby for both Chitinase gene with selectable marker *hptII* which confers resistance to hygromycin and GUS gene with selectable marker *nptII* which confers resistanceto kanamycin.

Material and Methods

Initiation and maintenance of embryonic cultures

For callus induction, leaf discs were excised from *in vitro* cultured shoots of grapevine (*Vitis vinifera* L.) cv. King's Ruby. These were cut into pieces of 0.5 cm square and placed on MS (Murashige and Skoog 1962) medium containing macro-, micro-elements and vitamins) supplemented with 30 g L^{-1} sucrose, 2.0 mg L^{-1} 2, 4-D, 0.3 mg L^{-1} BAP and 0.2 mg L^{-1} NAA, and solidified with 7 g L^{-1} agar. The pH of medium was adjusted to 5.8 ± 0.1 prior to autoclaving. The cultures were maintained in dark at 26 ± 2 °C. The regenerated calli were subcultured onto the fresh culture medium after every 4 weeks. After 2-3 subcultures, the calli were aseptically shifted to regeneration medium in test tubes under aseptic conditions. Regeneration medium composed of MS medium (macro, micro elements and vitamins) supplemented with 30 g L^{-1} sucrose and 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA, and solidified with 7 g L^{-1} agar. The cultures were incubated at 25 ± 2 °C under 16 hr photoperiod (2,000 lux) with white fluorescent tube lights.

Transformation vector and preparation of bacterial cultures

Agrobacterium tumefaciens strain LBA4404 harboring vector pBI121 was used for transformation. The Chitinase gene (for fungal resistance) and GUS gene (for phenotypic expression) were introduced in the embryogenic calli developed from the leaf explants of grapes (*Vitis vinifera* L.) cv. King's Ruby. T-DNA regions of pBI121 derived binary vector was cloned with hygromycin phosphotransferase (*hptII*) gene conferring resistance to hygromycin and neomycin phosphotransferase (*nptII*) gene conferring resistance to kanamycin as selectable markers. Bacterial suspension was grown in 50 ml YEP medium (An *et al.,* 1988) containing 50 mg L^{-1} kanamycin, 50 mg L^{-1} rifampicin. About 1-2 µL bacterial culture adjusted to $O.D_{600nm} = 0.6$ was further cultured for 24-48 hours in dark in an incubator on shaker at 28 °C at 105 rpm before using in transformation experiments.

Inoculation time and co-cultivation period

The bacterial cultures were taken and centrifuged for 10 minutes at 10,000 rpm. Supernatants were discarded and pellet cells were re-suspended in plain MS medium. Embryogenic calli were infected with bacterial suspension for 10, 20 or 30 minutes and transferred to co-cultivation medium (containing 50 µM acetosryingone) for 1, 2 or 3 days.

Selection of putative transgenic calli

After co-cultivation, excess of *Agrobacterium* was removed by washing the calli with antibiotic cefotaxime with three different concentrations 150, 300 and 500 mg L^{-1}. The calli were first washed with sterilized distilled water 3-4 times for about 45 min. The calli were dried out on sterilized filter paper for 10 min and shifted to petri plates containing MS medium supplemented with an antibiotic. Independent experiments with different concentrations of each hygromycin (0, 5, 10 and 15 mg L^{-1}) and kanamycin (100, 200 and 300 mg L^{-1}) were carried out for selection of transformed cells with cefotaxime 200 mg L^{-1} and each experiment was repeated thrice. Hygromycin and kanamycin resistant calli were counted and transformation efficiencies were determined. Transformation efficiencies were calculated by percentage of resistant calli separately for hygromycin and kanamycin.

Histochemical GUS assay

Expression of introduced GUS gene in the embryogenic calli was analysed by the X-Gluc (5-bromo-4-chloro-3-indolylglucuronide) histochemical assay (Rueb *et al.,* 1989). For this purpose, infected explants were treated with X-Gluc solution {1M Na_2HPO_4, 1M NaH_2PO_4, 0.5M EDTA, 1% Triton X-100, X-Gluc (dissolved in dimethyl sulfoxide / dimethyl foramide), methanol and ultrapure water} in petri plates for 24 hours at 37 °C in water bath and then left for one week so that blue staining become distinct. The transient GUS expression was scored on the basis of blue spots and colour developed by GUS staining on each explant. Transformation efficiency (%) was calculated as the total number of blue spots

Plate 1. Co-cultivation with *Agrobacterium tumefaciens* having (a) Chitinase gene and (b) GUS gene

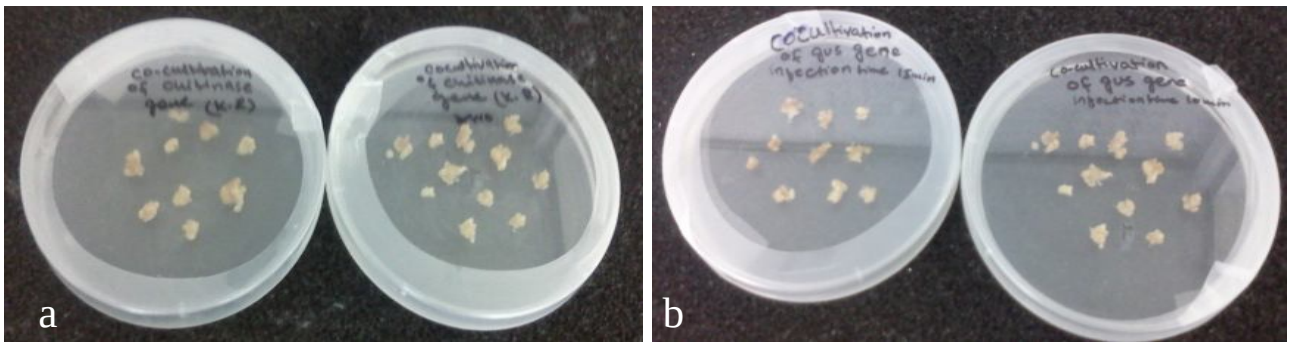
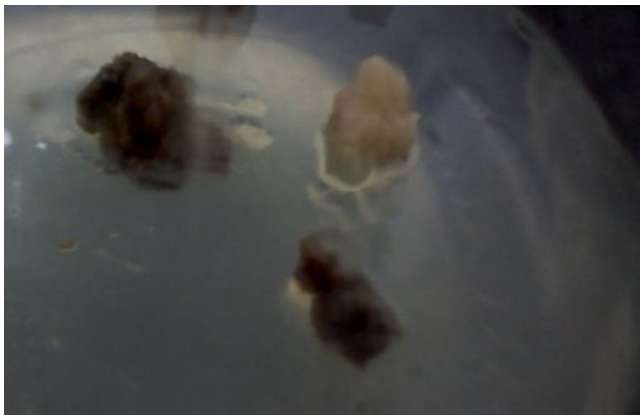


Plate 2. Transformed calli cultured on selection media after one month containing 10 mg L^{-1} hygromycin.



per total number of explants infected.

Result and Discussion

Establishment of embryonic cultures

For establishment of *in vitro* culture, apical meristems of grape (*Vitis vinifera* L.) cv. King's Ruby were taken from field grown plants. Explants were inoculated in culture jars containing shoot proliferation medium i.e. half strength MS medium supplemented with 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} GA_3. Developed shoots were multiplied through periodic subcultures. Callus was induced from leaf disc explants taken from *in vitro* shoot cultures on MS medium supplemented with 2.0 mg L^{-1} 2,4-D + 0.3 mg L^{-1} BAP + 0.2 mg L^{-1} NAA and maintained through subculturing on the fresh medium of same composition. Shoots were also successfully regenerated by transferring the calli on MS medium containing 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA.

Effect of inoculation time on transformation efficiency

In the process of transformation, inoculation in bacterial suspension and co-cultivation are the essential steps, as conditions at the time of infection and co-cultivation may differ and considerably affect transformation efficiency (Svabova and Griga 2008). Data regarding transformation efficiency (%) illustrated significant differences among the inoculation times used (Fig 1). The maximum transformation efficiency (2.83%) was achieved when inoculation time was 10 minutes for Chitinase gene. This was followed by transformation efficiency of 0.91% with inoculation time of 15 minutes, whereas performance concerning transformation efficiency percentage remained very low i.e. 0.25 for inoculation period of 20 minutes. Present findings indicated that transformation efficiencyis directly influenced by inoculation duration, as the duration was increased, transformation efficiency was decreased sharply. Further, it was noticed that due to increased inoculation time, there was appearance of calli necrosis.

The highest transformation efficiency (2.5%) was also obtained when inoculation time kept for 10 minutes for GUS gene. This was followed by transformation efficiencyof 0.75% with inoculation time of 15 minutes, whereas very low transformation efficiency (0.16%) was recorded for inoculation time of 20 minutes (Fig 1).

The results depicted that inoculation with *Agrobacterium* for 10 min was better for embryogenic calli which were cultured on half strength MS medium. The effectiveness of inoculation time was determined for *Agrobacterium* strain used for transformation method (Gelvinand Liu 1994). Calli of grapes were too sensitive to the inoculation with *Agrobacterium* that is due to natural host/pathogen interaction between *Agrobacterium* and grape cells. Plant cells respond to microbial infection by a defense mechanism that involves the generation of super active oxygen species, the accumulation of which leads to cell death and tissue necrosis (Bolwell and Wojtaszek 1997).

Effect of co-cultivation period on transformation efficiency

After inoculation of calli with *Agrobacterium* strain LBA4404 (vector pBI121 and selectable marker *hptII*), calli were cultured on co-cultivation medium for one, two and three days (Plate 1a). Co-cultivation period of two days showed normal bacterial growth which performed better in selection and showed the highest (2.75%) transformation efficiency of Chitinase gene. While for co-cultivation period of one day,

Plate 3. Transformed calli cultured on selection media after one month containing 100 mg L^{-1} kanamycin.

Plate 4. Transient GUS expression in calli of King's Ruby. GUS assay was done after 24 hours of transformation. Blue spots and diffused blue colour were observed after GUS staining.

bacterial growth was not enough with low transformation efficiency (1.25%). In co-cultivation period for three days, only 0.75% of transformation efficiency was observed (Fig 2).

After inoculation of calli with *Agrobacterium* strain LBA4404 (vector pBI121 and selectable marker *nptII*), calli were also placed on co-cultivation medium for one, two and three days (Plate 1b). Co-cultivation for two days resulted in normal bacterial growth and gave the maximum (3.25%) transformation efficiency of GUS gene. However, in co-cultivation for one day, bacterial growth was not sufficient for transformation effectively (1.41%). Co-cultivation for three days showed only 0.5% transformation efficiency (Fig. 2). During co-cultivation, a major hurdle was necrotic response of calli. Leaf derived grapes callus proved sensitive to *Agrobacterium* infection, died after co-cultivation and restricted the improvement in transformation efficiency level. The study showed rapid and localized necrotic response of plant cells at the site where *Agrobacterium* was used, oxidative browning and tissue shrinkage was also noticed.

Achieved results were nearly similar to the investigation of Bornhoff *et al.* (2005) who stated that after infecting with *Agrobacterium*, co -cultivating the calli for two days and then transferring to the selection medium containing kanamycin were more effective. Different periods of co-cultivation have been reported in literature for different plant species. Ho *et al.* (1998) found two days co-cultivation time as optimum for transformation of *Eucalyptus camaldulensis*, while Ahad *et al.* (2014) reported as one day. The difference was probably due to different strains of *Agrobacterium* used for the transformation.

Effect of different doses of cefotaxime on elimination of bacteria

After co-cultivation for the given periods, calli were washed at least three times by using different concentrations of cefotaxime in MS liquid medium to kill the bacteria from surface of the calli. The results revealed that washing of calli treated with *Agrobacterium* having Chitinase gene with cefotaxime at the rate of 300 mg L^{-1} exhibited the highest (3.16%) survival, followed by cefotaxime at the concentration of 500 mg L^{-1} with 1.08% survival (Fig 3). The optimum concentration of cefotaxime for washing was standardized as 300 mg L^{-1}. When washing was done with cefotaxime 150 mg L^{-1}, bacterial growth was difficult to control. However, bacterial growth was completely controlled when washing was done with 500 mg L^{-1} cefotaxime, besides this browning of calli was also noted during the treatment. Washing of calli treated with *Agrobacterium* having GUS gene with cefotaxime 300 mg L^{-1} also exhibited the maximum (2.5%) survival rate, followed by cefotaxime concentration of 500 mg L^{-1} with 1.25% survival (Fig 3). The results of the present study are in line with the investigation of Wang *et al.* (2005), who stated that the calli were transferred to selection media after three times washing with 50 mL liquid medium supplemented with 300 mg L^{-1} cefotaxime for removal of bacteria which gave the maximum survival percentage.

Effect of hygromycin on transformation efficiency

The most commonly used selectable marker gene is *hptII* that is resistant to hygromycin (Rao and Rohini, 2003). In the present investigation, hygromycin was used for selection of calli treated with the Chitinase gene inserted in *Agrobacterium tumefaciens* strain LBA4404 containing plasmid pBI121 and selectable marker gene *hptII*. During selection, hygromycin was used in selection medium for selection of transformed and growing calli (Plate 2). It helps in differentiating resistant and growing calli. As for response of various treatments of hygromycin for the

Fig 1. Effect of inoculation time on transformation efficiency of King's Ruby treated with *Agrobacterium tumefaciens* possessing Chitinase and GUS genes.

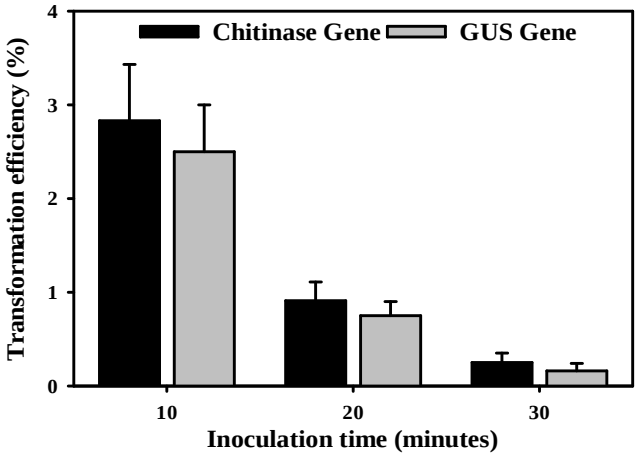
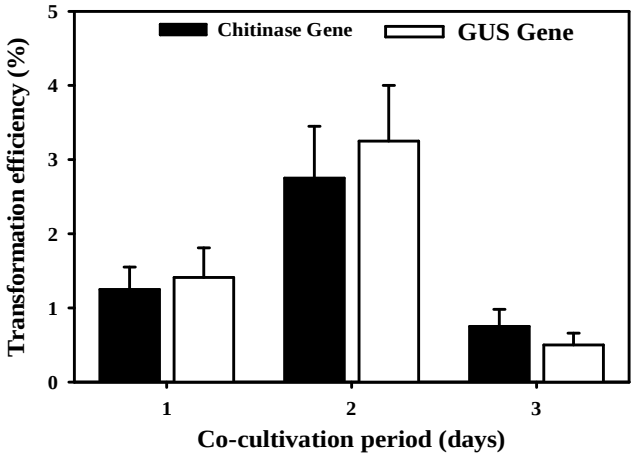


Fig 2. Effect of co-cultivation period on transformation efficiency of King's Ruby treated with *Agrobacterium tumefaciens* possessing Chitinase and GUS genes.



transformation efficiency rate is concerned, hygromycin 10 mg L^{-l} showed competitively better transformation rate (2.83%), followed by 5 mg L^{-l} (1.66%) and 15 mg L^{-l} showed the lowest transformation efficiency (0.66%) (Fig 4). On the other hand, no response regarding transformation was observed on calli cultured without hygromycin treatment (control). Further, calli transferred to the medium containing higher levels of hygromycin (15 mg L^{-l}), became yellowish and were discarded. Besides this, at medium level of hygromycin (10 mg L^{-l}), all non-transformed calli were suppressed and showed necrosis due to increased concentration of hygromycin and changed into brownish black in color. However, when hygromycin was used at the low level (5 mg L^{-l}), callus growth was more but transformation efficiency was low as compared to medium level (10 mg L^{-l}) of hygromycin. Minimum level of selective agent plays important role in the suppression of non-transformed calli completely (Yang *et al.* 2006). A concentration of antibiotic as a selective agent greater than required rate leads to reduction of transformation efficiency. These results were in confirmation with the findings of Nirala *et al.* (2010) that 10 mg L^{-l} of hygromycin resulted in high number of transformed somatic embryos.

Effect of kanamycin on transformation efficiency

It is highly unpredictable whether the tissues of grapevine are sensitive to the increased level of kanamycin or not. It is therefore, essential to determine the sensitivity of the particular tissues of explants to kanamycin for the development of new transformation system (Lopez-Perez *et al.*, 2008). In the present study, different concentrations of kanamycin were used to study their effects on selection of transformants. Kanamycin used at the rate of 100 mg

Fig 3. Effect of different doses of cefotaxime for elimination of excessive bacteria on survival rate of calli treated with *Agrobacterium tumefaciens* possessing Chitinase and GUS genes.

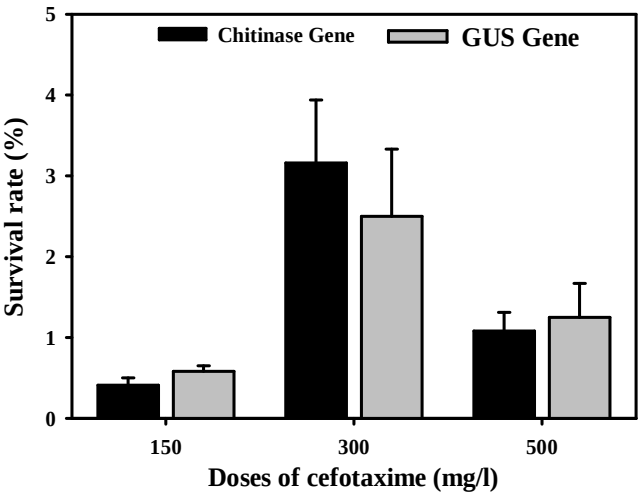


Fig 4. Effect of various doses of hygromycin on transformation efficiency of King's Ruby treated with *Agrobacterium tumefaciens* possessing Chitinase gene.

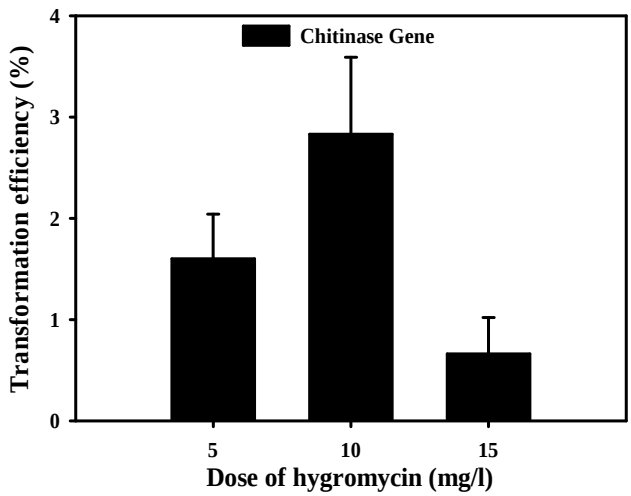
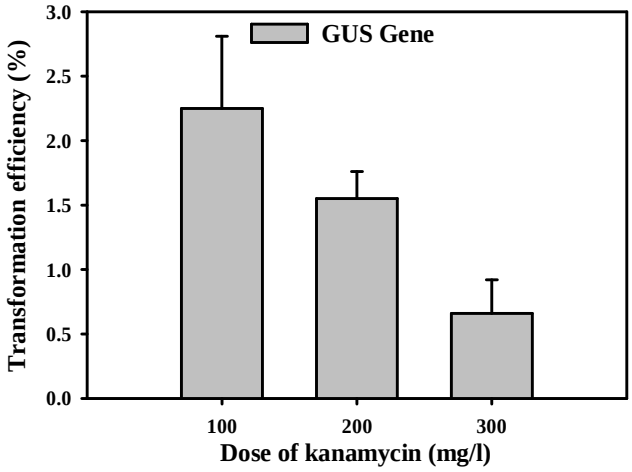


Fig 5. Effect of kanamycin on transformation efficiency of King's Ruby treated with *Agrobacterium tumefaciens* containing GUS Gene.



L^{-1} exhibited the highest transformation efficiency (2.25%), effectively suppressing the non-transformed calli, followed by 200 mg L^{-1} kanamycin which showed 1.55% transformation efficiency. When the antibiotic was applied at the rate of 300 mg L^{-1}, it resulted in lower transformation efficiency i.e. 0.66% (Fig 5).

Kanamycin at the level 100 mg L^{-1} was standardized as optimum because when concentration of kanamycin exceeded from this dose, high necrosis rate was observed (Plate 3). It was also noticed that high levels of kanamycin damaged the non-transformed calli and also resulted in progressive necrosis of the tissue. Grapevine calli were proved to be very sensitive to kanamycin, which led to questions concerning efficiency of kanamycin as a selection agent. However, both Motioike *et al.* (2002) and Das *et al.* (2002) have used kanamycin to recover viable transformed calli of *V. labruscana* and *V. vinifera,* respectively; but none of them observed the kanamycin insensitivity problem as reported by Perl *et al.* (1996). However, transformation in grapevine explants was achieved by using kanamycin for selection and the cultures were effectively transformed using the *nptII* resistance gene (Gray *et al.*, 2005). In grapevine (*Vitis spp.*), somatic embryogenesis is the most frequently used regeneration system adopted for genetic engineering (Gambino *et al.*, 2007). However, transformation procedure of grapes depends on the induction of embryos and their selection (Iocco *et al.* 2001).

It has been observed that regeneration efficiency of transformed grape calli is greatly affected by the presence of necrotic areas on them (Perl *et al.* 1996). Grapevine has been found to be very sensitive to the presence of kanamycin in the medium (Baribault *et al.* 1990). Sensitivity is dependent on the kind of explant tissue that was placed on medium containing selective agent. In fact, it is very hard to maintain stability between the high level of kanamycin that is required and its inhibitory effect on explants. Findings of the current investigation are in accordance with outcome of Bornhoff *et al.* (2005), who co-cultivated the explants with *Agrobacterium* and transferred to MS medium containing 100 mg L^{-1} kanamycin. Similar results were also reported by Dhekney *et al.* (2008) that kanamycin at 100 mg L^{-1} in the culture medium was efficient for inhibition of non-resistant tissues, allowing the progressive growth and development of transformed cells.

Histochemical GUS assay

GUS gene is most widely used reporter gene in plants because of its easy to assay, both qualitatively and quantatively. Histochemical GUS assay was performed for confirmation of transformation in grapes calli. After one week of treating the infected explants with staining (X-Gluc) solution, the transient GUS expression was scored on the basis of blue spots and colour developed on each explant. The results showed that out of eight calli, five showed positive GUS expressions (Plate 4), so transformation efficiency recorded was 62.5%. Golles *et al.* (1997) also observed a strong GUS-activity in histochemical assays when somatic embryos of *Vitis sp.* were transformed with the GUS gene and selected for kanamycin resistance, confirming that the developed transformation system was suitable to transform grapevine. Histochemical GUS assay at different developmental stages of transgenic explants of *Vitis vinifera* showed a strong uniform blue colour (Harst *et al.* 2000). The GUS expression has also been reported by Das *et al.* (2002) and Wang *et al.* (2005).

References

Agrios GN (2005). Plant Pathology. Academic Press. New York. Pp. 633.

Aguero CB, Meredith CP and Dandekar AM (2006) Genetic transformation of *Vitis vinifera* L. cvs. 'Thompson Seedless' and 'Chardonnay' with the pear PGIP and GFP encoding genes. Vitis 45: 1-8.

Alves AC, Quecini VM and Vieira MLC (1999) Plant transformation: advances and perspectives. Sci Agric 56: 1-8.

An G, Ebert PP, Mitra A and Ha SB (1988) Binary vector. In: Gelvin, S.B., Schilperoort, R.A. and Verma, D.P.S. (eds.). Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht, Pp. 1-19.

Barlass M and Skene KGM (1978) In vitro propa-

gation of grapevine (*Vitis vinifera* L.) from fragmented shoot apices. Vitis 17: 335-340.

Baribault TJ, Skene KGM, Cain PA and Steele Scott N (1990) Transgenic grapevines: Regeneration of shoots expressing a-glucuronidase. J Exp Bot 41: 1045-1049.

Bolwell GP and Wojtaszek P (1997) Mechanisms for the generation of reactive oxygen species in plant defence-a broad perspective. Physiol Mol Plant Pathol 51: 347-366.

Bornhoff BA, Harst M, Zyparian E and Topfer R (2005) Transgenic plants of *Vitis vinifera* Seyval blanc. Plant Cell Rep 24: 433-438.

Das DK, Reddy MK, Upadhyaya KC and Sopory SK (2002) An efficient leaf disc culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). Plant Cell Rep 20: 999-1005.

Dhekney SA, Li ZT, Dutt M and Gray DJ (2008) Agrobacterium-mediated transformation of embryogenic cultures and plant regeneration in *Vitis rotundifolia* Michx. (muscadine grape). Plant Cell Rep 27: 865-872.

Fan C, Pu N, Wang X, Wang Y, Fang L, Xu W and Zhang J (2008) Agrobacterium-mediated genetic transformation of grapevine (*Vitis vinifera* L.) with a novel stilbene synthase gene from chinese wild Vitis Pseudoreticulata. Plant Cell Tiss Org Cult 92: 197-206.

Gambino G, Grebaudo I, leopold S, Schartl A and Laimer M (2005) Molecular Characterization of grapevine plants transformed with GPLV resistance genes: I. Plant Cell Rep 24: 655-662.

Gelvin SB and Liu CN (1994) Genetic manipulation of *Agrobacterium tumefaciens* strains to improve transformation of recalcitrant plant species. In: Gelvin S.B. and Schilperoot, R.A. (eds.). Plant Molecular Biology Manual. Kluwer Academic Publishers, Belgium. Pp 1-13.

Gray DJ, Jayasankar S and Li Z (2005) Vitaceae (Grape Vitis spp.). *In*: Litz RE (ed.). Biotechnology of Fruit and Nut Crops. CAB International Wallingford, Oxford, UK. Pp. 672-706.

Gray DJ (1995) Somatic embryogenesis in grape. In: Jain, S.M., Gupta, P.K. and Newton, R.J. (eds.). Somatic Embryogenesis in Woody Plants. Vol. 2. Kluwer Academic Press, Dordrecht, Netherlands, Pp. 191-218.

Gray DJ and Benton CM (1991) *In vitro* micropropagation and plant establishment of muscadine grape cultivars (*Vitis rotundifolia*). Plant Cell Tiss. Org Cult 27: 7-14.

Harst M, Bornhoff BA, Zyprian E and Topper R (2000) Influence of culture technique and genotype on the efficiency of *Agrobacterium*-mediated transformation of somatic embryos (*Vitis vinifera*) and their conversion to transgenic plants. Vitis 39: 99-102.

Iocco P, Franks TM and Thomas R (2001) Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. Transgen Res 10: 105-112.

Lopez-Perez JL, Velasco LM, Navarro PM and Dabauza M (2008) Development of highly efficient genetic transformation protocols for table grape Sugraone and Crimson Seedless at low Agrobacterium density. Plant Cell Tiss Org Cult 94: 189-199.

Motioike SYR, Skirvin RM, Norton MA and Otferbacher AG (2002) Development of methods to genetically transform American grape (Vitis x labruscana L.H. Bailey). J Hort Sci Biotech 77: 691 -696.

Nirala NK, Das DK, Srivastava PS, Sopory SK and Upadhyaya KC (2010) Expression of a rice chitinase gene enhances antifungal potential in transgenic grapevine (*Vitis vinifera* L.). Vitis 49: 181-187.

Perl A, Lotan O, Abu-Abied M and Holland D (1996) Establishment of an Agrobacterium mediated transformation system for grape (*Vitis vinifera* L.): the role of antioxidants during grape-*Agrobacterium* interactions. Nat Biotech 14: 624-628.

Pearson R and Goheen A (1988) Compendium of Grape Diseases. Amer Pathol Soc Press, St. Paul. 93: 7-8.

Rao KS and Rohini VK (2003) Plant transformation and genetic markers. Foundation for Biotechnology Awareness and Education, Indian Institute of Science, Bangalore. India.

Sajid MG, Ilyas MK and Anwar R (2006) Effect of diverse hormonal regimes on *in vitro* growth of grape germplasm. Pak J Bot 38: 385-391.

Svabova L and Griga M (2008) The effect of cocultivation treatments on transformation efficiency in pea (*Pisum sativum* L.) Plant Cell Tiss Org Cult 95: 293-304.

Taylor NJ and Fauqet CM (2002) Microparticle bombardment as a tool in plant science and agricultural biotechnology. DNA Cell Biol 21: 963-

977.

Torregrosa L, Locc P and Thomas MR (2002) Influence of Agrobacterium strain, culture medium, and cultivar on the transformation efficiency of *Vitis vinifera* L. Amer J Enol Vitic 53: 183-190.

Wang Q, Li P, Hanania U, Sahar N, Mawassi M, Gafny R, Sela I, Tanne E and Perl A (2005) Improvement of Agrobacterium-mediated transformation efficiency and transgenic plant regeneration of *Vitis vinifera* L. by optimizing selection regimes and utilizing cryopreserved cell suspensions. Plant Sci 168: 565-571.

Yamamoto T, Iketani H, Ieki H, Nishizawa Y, Notuska K, Hibi T, Hayashi T and Matsuta N (2000) Transgenic grapevine plants experessing a rice chitinase with enchanced resistance to fungal pathogens. Plant Cell Rep 19: 639-646.

Yang XM, An LZ, Wang YM and Li S (2006) Somatic embryogenesis and analysis of the genetic stability of regeneration system in grapevine 'Sinsaut'. Acta Hort Sinica 33: 1317-1320.