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Determination of minimal inhibitory concentration of kanamycin as selective agents and marker genes for use in *Agrobacterium* mediated transformation in sugarcane

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Abstract
Efficient genetic transformation of sugarcane requires an effective dose of selective antibiotics/agents for differentiating actively transformed cells from non-transformed ones. The objective of the present study was to optimize and establish lethal minimal inhibitory concentration of kanamycin that could be used to select true transformed shoots of sugarcane. A step-wise increase doses of kanamycin antibiotic from 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and then to 120 mg L^{-1} as a selection agent were evaluated for their effectiveness in transformation of sugarcane elite variety CoS 96268. *In vitro* regenerated shoots were inoculated on MS medium containing 0.8 mg L^{-1} BAP + 0.1 mg L^{-1} NAA with various concentrations of kanamycin. At lower concentrations of kanamycin (10-40 mg L^{-1}), the growth of shoots were unaffected and the survival rates were substantially high (100%). Chlorosis starts at a kanamycin concentration of 50 mg L^{-1}. Survival percentage of the non-transformed shoots was decreasing gradually with the step-wise increase of kanamycin concentration. The most effective concentration of kanamycin was found to be 100 mg L^{-1} at which all the non-transformed (susceptible) shoots were died after four weeks of inoculation. Maximum transformed shoots were obtained at 100 mg L^{-1} kanamycin for sugarcane with a death rate of more than 95% of non-transformed shoots on selection medium. Among the different antibiotics doses tested for selection of transformants, kanamycin at 100 mg L^{-1} was found to be optimum. So worked out for the selection of transformed shoots of CoS 96268 could be used as selective *npt II* marker in other sugarcane transformation practices.

Keywords: Kanamycin, *npt II* gene, *Agrobacterium* mediated genetic transformation, sugarcane

Introduction
Sugarcane is used primarily as a raw material for sugar, Jaggery and ethanol production in India as well as in tropical and sub-tropical parts of the world. It is also an important source for several others by-products other industrially important products including; molasage, vinegar, paper, plywood, cork, bio-fertilizers and power are generated (Singh *et al.* 2017) [20]. Crop improvement through genetic transformation has been identified as a useful tool for sugarcane because breeding advances by conventional crossing programs are too slow (Singh *et al.* 2011) [21]. Transformation is now become an acceptable technique due to various reasons; ever-increasing world population, need to fulfill the requirement of food security for foreseeable future, low productivity in changing climate scenario. The increasing prospective of biotechnology in the field of crop development is now adopting for food crops in many developed and developing countries (James *et al.* 2014) [8]. The adoption rate of genetically modified crops like corn, brinjal, cotton, alfalfa, sugar beet, papaya, canolla, and soybean in the world was 90% during 2014 (Klumper and Qaim., 2014). The commercial cultivation of transgenic crops has started since 1996 with insect resistance and herbicide tolerance coupled with selectable marker gene. Sugarcane has been successfully transformed using several techniques (Lakshmanan *et al*. 2005) [14] and the most commonly used methods like particle bombardment (Bower and Birch 1992; Setamou *et al*. 2002; Vickers *et al*. 2005a; Christy *et al*. 2009; Weng *et al*. 2011) [3, 19, 23, 7, 24] and Agrobacterium (Arencibia *et al*. 1998; Enriquez *et al*. 2000; Manickavasagam *et al*. 2004; Arvinth *et al*. 2010; Khan *et al*. 2013; Dong *et al*. 2014) [7, 11, 16, 2, 13, 10]. The success of the genetic transformation is depending on the three crucial steps *viz*; DNA integration, protein expression and transmission of the transgene into its next generation progenies. Basically, during the process of genetic transformation, desired gene is transferred into target tissue, which contains thousands of cells. Only a few cells will become transgenic or will have the transgene integrated into its genome. Subsequently, it is very important to identify the transformed cells from non-transformed ones. It requires a competent identification or selection system (selectable marker genes)

which visibly discriminates transformed and non-transformed cells. The most frequently used selectable marker gene is the neomycin II phosphotransferase (*npt II*) genes which gives resistance to amino-glycoside antibiotics kanamycin and neomycin.

In genetic transformation these antibiotics are use in variable concentrations, inhibiting the growth of untransformed cells by hampering the chlorophyll and proteins bio-synthesis in chloroplast and mitochondria (Brasileiro and Aragon, 2001; Chen *et al*. 2005) [4, 6]. The kanamycin resistant gene (npt II) is one of the most frequently used selectable marker genes for the development of transgenic plants in dicots and some of the monocots for an instances wheat (Cui *et al*. 2011) [9], sugarcane (Manickavasagam *et al*. 2004; Mustafa *et al*. 2012; Kaur *et al*. 2012; Tanween *et al.* 2014) [16, 18, 12, 22]. Zhangsun *et al*. (2007) was used the nptII gene as one of the most efficient selectable marker for the selection of Agrobacterium-mediated transformed sugarcane calli. Suitable concentration/lethal dose of kanamycin would be able to kill all the non-transformed cells and allow only transformed cells to survive in culture media. To find out the optimum lethal dose of selective agents for the screening of transformed shoots from non-transformed shoots on selection media, is utmost important steps for the development of transgenic plants. This can be achieved by using an eco-friendly dose of selective agent like kanamycin antibiotics. The objective of the present study was to optimize and establish lethal minimal inhibitory concentration of kanamycin that could be used to select true transformed shoots of sugarcane commercial variety CoS 96268 to obtain transgenic sugarcane resistant to neomycin phosphotransferase (nptII) genes.

Materials and Method

Kanamycin solution

The stock solution of amino-glycoside antibiotic kanamycin (10 mg/ml) was made by dissolving kanamycin sulphate monohydrate (Duchefa Biochemie) and was filter sterilized. It was added into the autoclaved and cooled MS media at 40-50°C in step-wise increasing concentrations from 0 mg/l to the 100 mg/l.

Sugarcane shoots tolerance to kanamycin

In order to tests the sensitivity of sugarcane shoots to kanamycin, a propagule containing three secondary shoots (3-4 cm long) obtained from primary shoots of sugarcane elite variety CoS 96268 were inoculated on MS medium (Murashige and Skoog 1962) [17] supplemented with (MS salts + 0.1 mg/l NAA + 0.8 mg/l BAP + various doses of kanamycin). Kanamycin was added at different concentrations via serial dilution *viz*; 0 mg/l (control), 10, 20, 30, 40, 50, 60, 80, 90, 100, 110 and 120 mg/l in the medium (Fig.1). Kanamycin free media served as positive control. The cultures were incubated for 4 weeks at 25±2 °C under 16 hrs light and 8 hrs dark in growth room. After 4 weeks of culture, the sensitivity of kanamycin to non-transformed primary and secondary shoots was evaluated based on necrosis and albinism of shoots. Data were recorded on percent survival of non-transformed shoots and percent albinism of shoots (Fig. 2 & 3).

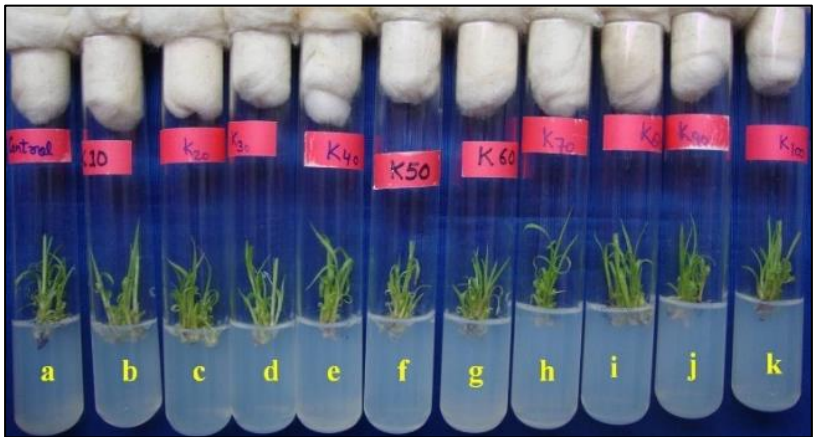

Fig 1: Kanamycin treated plant at different concentrations via serial dilution *viz.*, 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/1 in MS medium at 0 hr.

Fig 2: *In vitro* regenerated shoots were growing on various treatments of kanamycin: A-control. B-M6+10mg/ kanamycin, C-M6+20mg/l kanamycin, D-M6+30mg/l kanamycin, E-M6+40mg/l kanamycin, E-M6+40mg/l kanamycin, F-M6+50mg/l kanamycin, G-M6+60mg/l kanamycin, H-M6+70mg/l kanamycin, I-M6+80mg/l kanamycin, J-M6+90mg/l kanamycin, K-M6+100mg/l kanamycin

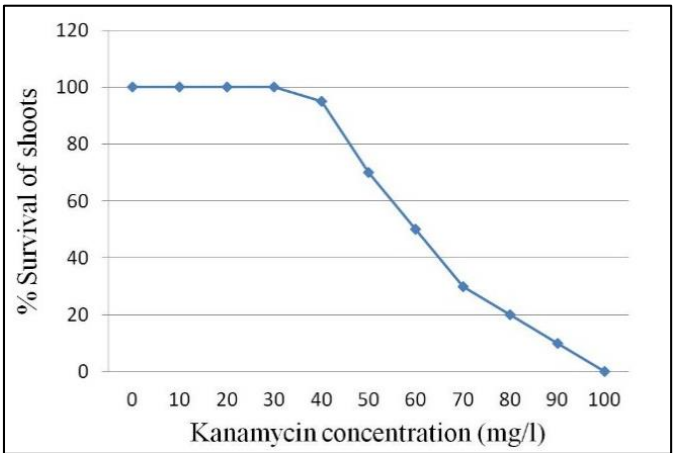

Fig 3: Effect of kanamycin on percent survival of non-transformed shoots of CoS 96268. Date were recorded after 4 weeks

Experimental design and statistical analysis

The experiment was repeated three times to compare the regeneration efficiency on kanamycin. Data were analyzed using one-way analysis of variance (ANOVA). A completely randomized design was used for the experiment.

Results and Discussion

The sensitivity of sugarcane shoots to kanamycin was evaluated by inoculating the shoots without co-cultivation with *A. tumefaciens* on selection medium contained different concentrations of kanamycin such as 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 mg/l. In control (kanamycin free MS media) excellent shoot growth were observed. The same growth pattern was observed in kanamycin at 10, 20 and 30 mg/l as compared to control. After four weeks in culture the mean shoot length of non-transgenic sugarcane (variety CoS 96268) was observed (11.467 ± 0.267 to 10.200 ± 0.306) with 100 % survival rate of shoot up to kanamycin concentration 30 mg/l. At 40 mg/l kanamycin concentration 90 % survival rate was found with mean shoot length of (5.8 ± 0.252). Degeneration of cholorophyll was started at kanamycin concentration of 50 mg/l (Fig. 2) and complete necrosis and cholorosis or albinism of primary and secondary shoots was gradually increase with increasing concentration of kanamycin 50 to 120 mg/l (Fig. 2). However, chlorophyll degradation and bleaching was observed directly proportional to the gradually increased with the concentration of kanamycin. Thus, chlorosis appeared to be the most fundamental factor in the development of etiolated shoots, which makes a visible difference among the transformed and untransformed plants (Larkin *et al.*, 1996) [15]. Even, there was no sign of regeneration of transformed shoots at higher kanamycin concentration 110 mg/l and 120 mg/l. Complete cell death and etiolation of non-transformed shoots was found to be optimum @ 100 mg/l kanamycin with average shoot length 3.900 ± 0.058 (Table). 100 mg/l kanamycin restricted chlorophyll development in the leaves of normal shoots, and thus found suitable for use during selection. The addition of lower than 100 mg/l concentration of kanamycinin to the selection medium of cultivar CoS 96268 resulted in too many "escapes" (growth of non- transformed plants which escape the antibiotic selection system). Cervera *et al.* (1998) [5] however, emphasized that smaller the antibiotic selection pressure, the greater the probability of the emergence of escape plants probably due to the protection of untransformed cells by neighboring transformed cell. The appropriate doses of kanamycin @100 mg/l, so worked out for the selection of transformed shoots of CoS 096268 could be used as selective npt II marker in other sugarcane varieties.

The concentration 150 mg/l of kanamycin was reported as the minimal lethal dose in a genetic transformation study (Manickavasagam *et al.* 2004 and Mustafa *et al.* 2012) [16, 18]. In some earlier analogous studies dose of kanamycin at 50 mg/l was found significant while 100 mg/l kanamycin caused complete cessation of shoot growth, followed by death within 21-28 days (Tanween *et al.* 2014; Kaur *et al.* 2012) [22, 12]. Kanamycin at 1,250 mg/l was used for the selection of transformed calli of sugarcane, there were no escapes for the selection of non-transgenic shoot at this concentration and this dose was found to be optimum for selection of transformed shoots (Khamrit *et al.* (2013). In the present study the best selection was found over the application of 100 mg/l, which was applied over the non-transformed shoot propagules (3-4 cm long) derived from primary shoots of sugarcane (Table). On the basis of the results of the optimization protocol, the concentration of the kanamycin antibiotic at 100 mg/l acts as an optimum dose for the selection of the genetically transformed shoots of sugarcane.

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Compliance with ethical standards

Conflict of Interest

The authors declare that they have no conflict of interest directly or indirectly and informed consent to publish this study.

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