Kanamycin spray as an effective method of screening transgenic cotton in field

Abstract

Transgenic technology has become the most favoured technology in cotton for the development of biotic and abiotic stress resistant varieties. Npt-II is the most commonly used selectable marker gene in transgenics that expresses gene product resistant to Kanamycin. Since the regeneration capability of cotton is very minimal and there are very few plants regenerated from callus, selection for Kanamycin resistance needs to be very precise. Kanamycin selection is one of the methods that can be effectively used to screen true positives, false-positives and true negative plants. The present study reports the systemic effects of kanamycin on seedlings of cotton and also a simple low cost method for screening transgenics. The soaking of cotton seedlings in 0.01g/L kanamycin and spraying of 1g/L kanamycin on 15 days old seedlings in field has been an effective method for differentiating transgenic and non-transgenic cotton plants.

Introduction: Cotton has been a well known cash crop especially due to the presence of transgenic varieties developed for pest resistance. Scientific community has been working intensively on the development of transgenic cotton for enhancement of pest resistance (Oliveira et al., 2016). Many selectable markers like *nptII* and *hptII* genes conferring resistance to antibiotics are used for the development of transgenics. Different concentrations of kanamycin and hygromycin are used in MS media for rapid identification of transformed plants (Miki et al., 2004). Techniques like PCR, ELISA/lateral flow strip test and Bioassays are used to screen for transgenic plants. The first two techniques are cost intensive and the third is labour intensive and thus there has been search for simpler methods of transgenic selection. The regeneration capability of cotton is very minimal and there are very few plants regenerated from callus, selection for Kanamycin resistance needs to be very precise. Kanamycin selection is one of the methods that can be effectively used to screen true positives, false-positives and true negative plants.

Attempt has been made to develop simpler and low cost methods that can identify transgenic cotton plants from their non-transgenic counterparts. Kan-resistant plants were found to germinate at 0.75g/l kanamycin containing medium. When kanamycin solution of concentration 4.0g/l was painted on cotyledons, they remained green in kan-resistant (BT) plants. In another method pouring of kanamycin solution (2.0g/l) into a pore of cotyledon screened out the kan-resistant plants very easily (Patent: WO 2005069744 A2).

Being a aminoglycoside antibiotic, kanamycin combines with 30S subunit of ribosome in chloroplast and mitochondria where it disturbs the protein synthesis and this results in etiolation and death of non-transgenic plants (Napj Bijvoet 1998, Yang 2002, Chen 2005). Kanamycin has been shown to have profound effect on the growth and development of main and lateral roots of non-transgenic *Arabidopsis thaliana* seedlings and also the meristematic

zone of root tip is diminished. Thus, kanamycin affects photosynthesis and supply of nutrients by influencing growth of seedling leaves and also restrains growth and development of main and lateral roots (Duan et al., 2009) in non-transgenics. In transgenic plants the gene neomycin phosphotransferase gene product degrades the kanamycin in the plant and keeps the plant healthy.

With the above background of study on kanamycin as an selective agent for transgenic and non-transgenic cotton plants, an experiment was conducted to optimize kanamycin concentration for selection of true transformants.

Materials and Method:

The experiment was performed using *Gossypium hirsutum* transgenic Akka Bt hybrid (-Bollgard) harbouring nptII gene as selection marker and non-transgenic seeds of *Gossypium hirsutum* cv.Suraj (Non Bt) respectively.

Experimental methods

1. Seed treatment: The transgenic and non-transgenic seeds (10 seeds per replication with two replicates) were soaked in 0.5g/L, 1.0g/L, 1.5g/L and 2g/L kanamycin along with water control in two sets i.e., for 4hrs and overnight. The seeds were sown in black cotton soil with FYM.
2. Seedling treatment A:The transgenic and non- transgenic seeds (10 seeds per replication with two replicates) were soaked in water overnight and the seeds were put onto paper towels for germination. The two days old germinated seedlings were soaked in 1g/L and 4g/L kanamycin with water control for overnight. The treated seedlings were put onto paper towels to monitor their growth behaviour.
3. Seedling treatment B: The transgenic and non- transgenic seeds (10 seeds per replication with two replicates) were soaked in water overnight and the seeds were put onto paper towels for germination. The four day old germinated seedlings were soaked in 0.01g/L, 0.1g/L,0.5g/L,1.0g/L and 2g/L kanamycin with water control in two sets i.e., for 1hr and overnight. The treated seedlings were put back onto paper towels for monitoring growth.
4. Spray on field grown seedlings: The transgenic and non- transgenic seeds were sown in field in alternative lines at two locations representing two replicates and the 15 days old seedlings were sprayed with 1g/L and 4g/L kanamycin with water control.

Note: The solutions for the above experiments were prepared using water as solvent of different concentrations of kanamycin sulphate

Results and Discussion

Transgenic cotton available commercially comes with some marker gene either an antibiotic or an herbicide. These marker genes can be used as a tool for screening regenerated plants for true positives, false positives and true negatives. In the present study, Kanamycin is used for selection of transgenic and non-transgenic seedlings. The results have been given as per the experiments performed.

1. The seeds of transgenic and non-transgenic soaked in 0.5g/L, 1.0g/L, 1.5g/L and 2g/L kanamycin for 4 hrs and overnight showed no difference in root growth response as compared to the control. All the plants remained healthy with green cotyledonary leaves as these were transferred into soil with FYM. Seeds having a protective seed coat would have hindered the absorbance of kanamycin. As it is known that kanamycin affects the 30 s ribosomal proteins of chloroplast (Zhang et al., 2001), the seeds having no or minimal number of chloroplast show no or minimal kanamycin response. Among the different tested concentrations of kanamycin, none could be used to differentiate the transgenic from non-transgenic.
2. To further investigate the optimum concentration of kanamycin the seeds were first germinated on paper towels for two days and then seedlings were treated with kanamycin sulphate overnight. The seedlings thus treated on 1g/L and 4 g/L kanamycin showed reduced root growth and also absence of lateral roots (Figure 1). The tip of the roots was also affected. Kanamycin is known to affect cell division and cell elongation and this has been evident in root development process (Table 1). As the kanamycin concentration increases there is hindrance in root elongation and also inhibition of lateral roots (Figure 2). It was observed that the transgenic seedlings performed better than non-transgenic seedlings at 1g/l of kanamycin where as growth was inhibited in both transgenic and non-transgenic at 4g/L.
3. Since 1g/L of kanamycin showed growth inhibition in transgenic seedlings as well, A set of lower concentrations of kanamycin were tried for optimisation. With increase in kanamycin concentration the seedling growth reduced significantly, and another important observation is browning and drying of the roots and also blunting of the root tip affecting the root cap. The transgenic and Non transgenic could be easily identified at concentrations 0.01g/L for both 1hr and overnight incubations. With increase in incubation time the lethal effect of kanamycin on the seedlings also enhanced (Table 2). The seedling growth is reduced significantly and the leaves

showed chlorosis. Leaf is the main organ involved in photosynthesis, transpiration and absorption (Franko et al., 2007). Application of kanamycin is known to affect not only leaf growth and chlorosis but also affect the process of photosynthsis due to which the energy required for growth of roots in the seedlings is affected (Duan et al., 2008). Another interesting observation was that the seedlings were infected by fungus in a increasing manner with corresponding increase in kanamycin concentration (Figure 3). The reason for this succeptibility might be due to slow growth rate or arrest of cell division that makes the plant tissue as an inactive dead matter (Figure 4).

4. On application of 1g/l and 4g/l spray, the 15 days old transgenic seedlings appeared green and healthy with no or meagre chlorotic and burnt symptoms whereas the non-transgenic plant leaves showed significant chlorotic zones with burnt symptoms. Both the concentrations were differentiating the transgenic BT and non-BT plants but since kanamycin is a costlier chemical we can go with the lower concentrations.Thus a 1g/L kanamycin solution can be effectively used for screening the cotton plants for true transformants or transgenics. This would be a rapid and easy method of screening the transgenic materials.

Conclusion

The optimum concentration of kanamycin on cotton plants has been well studied and a simple rapid on-field as well as in vitro screening method has been developed to assist identification and selection of true transformed plants. From the above experiments it can be concluded that 0.01g/L kan can be used for selection of transgenic seeds at 2 to 4 days old seedling stage with 1 hr incubation and 1g/L kan can be effectively used for selection of 15 days old transgenic and non- transgenic seedlings on field.

References

1. de Oliveira, R. S., Oliveira-Neto, O. B., Moura, H. F., de Macedo, L. L., Arraes, F. B., Lucena, W. A., ... & Grossi-de-Sa, M. F. (2016). Transgenic Cotton Plants Expressing Cry1Ia12 Toxin Confer Resistance to Fall Armyworm (Spodoptera

frugiperda) and Cotton Boll Weevil (Anthonomus grandis). *Frontiers in plant science*, *7*.

2. Miki, B., & McHugh, S. (2004). Selectable marker genes in transgenic plants: applications, alternatives and biosafety. *Journal of Biotechnology*, *107*(3), 193-232.
3. Parthasarathi, B., & Ramachandran, E. (2005). A Simple low cost method and product for screening transgenic and non-transgenic plants from a large population either in field or green house. Patent, WO 2005069744 A2.
4. Zhang, B. H., Liu, F., Liu, Z. H., Wang, H. M., & Yao, C. B. (2001). Effects of kanamycin on tissue culture and somatic embryogenesis in cotton. *Plant growth regulation*, *33*(2), 137-149.
5. Franco, A. C., Matsubara, S., Orthen, B,, 2007. *Tree Physiol*, 27(5), 717-725.
6. Yang, G. D., Zhu, Z., Li, Y.E., Zhu, Z. J., 2002. Journal of Agricultural Biotechnology, 10(2), 127-132.
7. Nap, J. P., Bijvoet, J., Stiekema, W. J., 1992. Prophyta, 46(1), 48-51.
8. Chen, X. B., Xu, C. B., Luo, Y.L., Li, B. J., 2005. Seed, 24(7), 29-31.
9. Duan, H., Ding, X., Song, J., Duan, Z., Zhou, Y., & Zhou, C. (2009). Effects of kanamycin on growth and development of Arabidopsis thaliana seedling, cotyledon and leaf. Pakistan Journal. Bot, 41(4), 1611-1618.