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STUDY OF FACTORS INFLUENCING *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION IN *VIGNA UNGUICULATA*.

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ABSTRACT
Cowpeas are important legume grown primarily in the semi-arid tropics providing great source of protein, vitamins and highly digestible energy. The demand for cowpeas is high but yield remain critically low, largely due to insect pests like other crop plants. As the germplasm of cowpea contains little or no resistance to major insect pests hence a gene technology approach for insect protection traits is now a high priority. The present work was aimed to study various factors affecting *Agrobacterium tumefacience* mediated transformation of *Vigna unguiculata*. *Agrobacterium* strain EHA 101 having kanamycin resistant gene as selectable marker and β-glucuronidase (GUS) as a reporter gene was used for transformation. Factors affecting transformation efficiency such as type and age of explant, effect of sucrose concentration in co-cultivation medium were studied. Histochemical analysis was performed to determine the activity of the GUS gene. Results concluded that the transgene was transmitted efficiently to the cowpea explants.

KEYWORDS: *Agrobacterium tumifaciens*, EHA 101, GUS gene, *Vigna unguiculata*.

INTRODUCTION

Cowpea (*Vigna unguiculata* L.), widely grown in Africa, Latin America, Southeast Asia and south western regions of North America, is a major source of high-quality dietary protein (18-35%) and energy for local people (FAOSTAT 2005). It plays a critical role in the lives of millions of people in developing countries of Africa and Asia. In spite of the great importance of this crop, its productivity is low, which is mainly limited by the damage caused by biotic constraints like virus, bacteria, fungus, insects, plants, and nematodes, as well as abiotic stresses such as heat and drought (Singh *et al*. 1997). Plant molecular biology and genetic engineering approaches offer alternative ways of overcoming these stresses. Genetic transformation techniques can be used to answer many basic questions pertaining to cowpea biology such as understanding of gene function and regulation of physiological and developmental processes (Gelvin *et al*. 1998). Although legumes are considered "Recalcitrant" to regeneration and transformation, routine protocols for obtaining stable transformants are available for major legumes. *Agrobacterium* mediated transformation method is widely used in this area in cowpea recent years. The first report on *Agrobacterium* mediated transformation was based on the tobacco leaf disc transformation method (Horsch *et al*., 1985) and the first production of transgenic cowpea plants was reported by Muthukumar *et al*., (1996). Earlier findings reported that transformation efficiency of *Agrobacterium* decreases as age of explants increases (Vergauwe *et al*., 1996 and Nin *et al*., 1997).

The transgenic cowpeas that transmit the transgenes to their progeny can be recovered at a frequency of transformation of 0.05 to 0.15% (Popelka *et al*., 2006). The present work was aimed to study various factors affecting *Agrobacterium tumefacience* mediated transformation of *Vigna unguiculata*. *Agrobacterium* strain EHA 101 having kanamycin resistant gene as selectable marker and β-glucuronidase (GUS) as a reporter gene was used for transformation. Factors affecting transformation efficiency such as type and age of explant, effect of sucrose concentration in co-cultivation medium were studied.

MATERIALS AND METHOD

Two related experiments were conducted. One was set up to determine the effect of age and type of explants in cowpea transformation and second was to determine the effect of sucrose concentration in co-cultivation medium on cowpea transformation embryos.

Agrobacterium strain

Agrobacterium tumefaciens EHA 101 strain having kanamycin resistant gene as selectable marker and β-glucuronidase (GUS) as a reporter gene was provided by MCC (Microbial Collection Center) VSBT, Baramati. The pure culture maintained at 4˚C with regular subcultures after every 15 days.

Plant material

The seeds of cowpea (*Vigna unguiculata*) were rinsed with 70% ethanol and surface sterilized by 0.1% mercuric chloride for 10 min and these seeds were washed 3 times in sterile distilled water. Seeds were inoculated on MS0 medium (pH 5.8) in bottle. These bottles were incubated under dark for 3-4 days and then transferred to light for the germination of seeds in the tissue culture incubation room. After particular time interval such as 10, 15 and 20 days *in vitro* grown seedlings were taken out from the bottle and explants were prepared by excising leaves with the forceps and scalpel. Embryonic axes of surface sterilized seeds soaked for 24 hr, 48 hr and 72hr were dissected.

Transformation

Leaf discs (10, 15 and 20 days) and Embryonic axes (24 hr, 48 hr and 72hr) were infected with *Agrobacterium tumefaciens* EHA 101 strain containing 100µM acetosyringone for 30 min and after removal of excess bacterium by blotting, were co-cultivated in co-cultivation medium with different sucrose concentration (1%,2%,3% and 4%) for 72 hrs at 28^0C in dark condition .Co-cultivated explants were rinsed in sterile distilled water and 5 minutes in sterile distilled water having cefotaxime (200mg/l).Then the explants were blotted on sterile blotting paper to remove excess bacterium and moisture and then transferred to regeneration medium. Leaf disc explants were transferred to regeneration medium (MS-Basal) containing 1 mg/l 2, 4-D with kanamycin (100 mg/l) and 100mg/l cefotaxime for callus generation whereas embryonic axis explants were transferred to regeneration medium (MS-Basal) containing 100mg/l cefotaxime and kanamycin (30 mg/l) for plantlets generation. The cultures were maintained at 28^0C with photoperiod 16h.

Analysis of GUS expression

Transient expression of the Gus A gene was tested by histochemical staining of the tissues 48 hr after bombardment using X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronidase), procedure outline by Jefferson *et al.,* (1987).

RESULTS

- **Evaluation of transformation factors**

In this study, he susceptibility response of cowpea explant to *Agrobacterium tumefaciens* infection was determined by scoring the GUS activity 24 hrs after infection resulting in blue colour spots development on leaf disc callus and plantlets of embryonic axis. None of the control plants expressed GUS activity.

- **Effect of types and ages of explant:**

Leaf Disc transformation:

Frequency of callus induction of leaf explants by *Agrobacterium tumefaciens* 75%. Transformation indicated by swelling at the ends of infected explants along with curling of explants on themselves. Swellings at edges denote a localized increase in cell division and cell elongation further lead to formation of tumor (Figure 1).

Embryonic axis transformation:

Shoot initiation was seen as small green buds after10 days (Figure 2). Frequency of plantlet regeneration of embryonic axis by *Agrobacterium tumefaciens* was 64 %.

Conformation by GUS assay: Gus staining gave prominent blue spots on explants, which indicates greater frequency of transformation (Figure 3). But due to clumping of cells to count the exact number of spots was difficult.

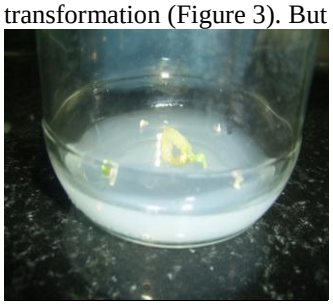


Figure 1

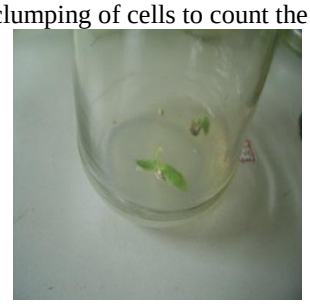


Figure 2

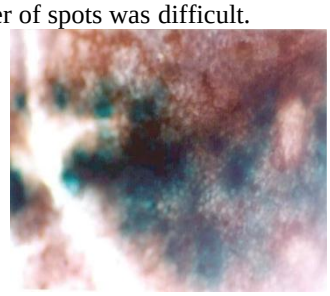
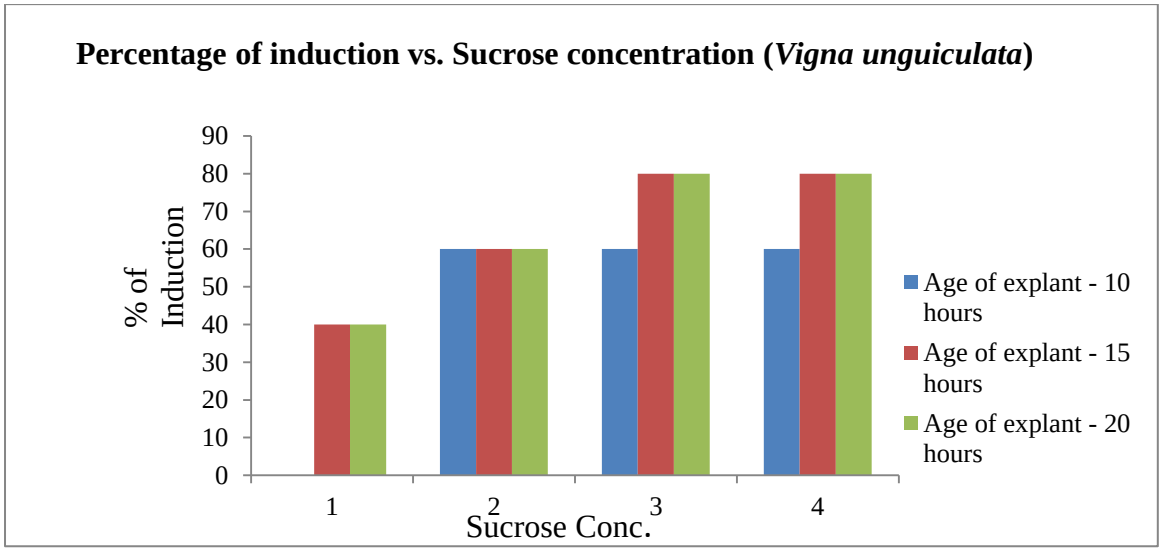
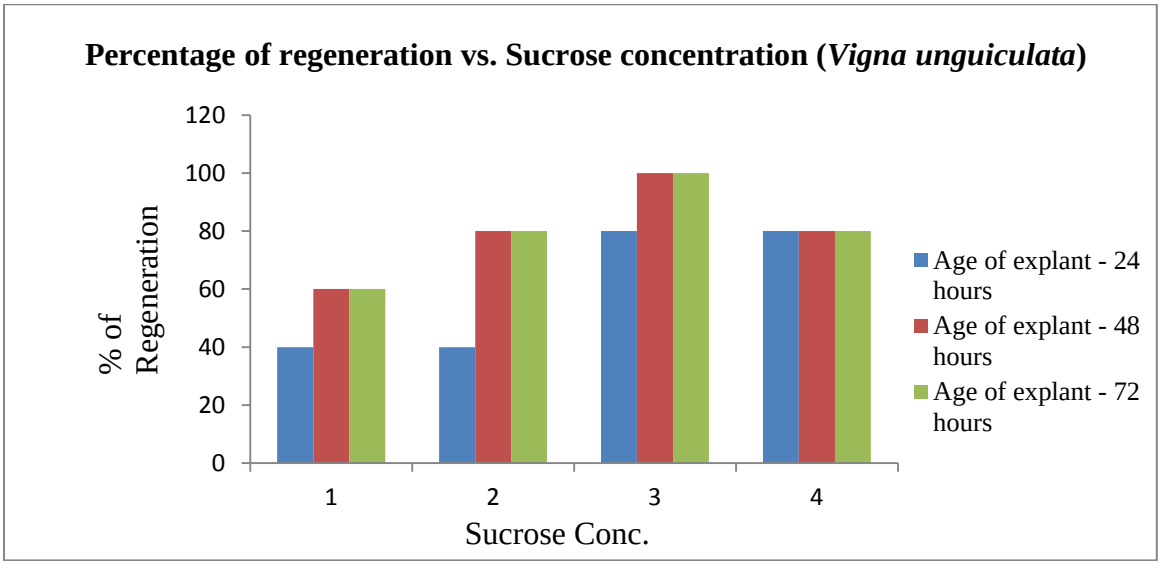


Figure 3

(Figure 1: Callus formation; Figure 2: Regeneration of transformed embryonic axis; Figure 3:GUS expression in transformed leaf explant).



Graph 1: Response of *Vigna unguiculata* leaf explants to infection by *Agrobacterium tumefaciens* (Leaf Disc transformation)



Graph 2: Response of *Vigna unguiculata* embryonic axis to infection by *Agrobacterium tumefaciens*.

- **Effect of Sucrose concentration**

Higher sucrose concentration showed enhanced callus induction indicating higher transformation frequency as compared to less sucrose concentration (Graph 1 and 2). Different sucrose concentration indicates change in plantlet regeneration optimum concentration would be 3%.

DISCUSSION

The results obtained from this work showed that we have established conditions in *Agrobacterium tumefaciens* mediated transformation in cowpea *Vigna unguiculata*. *Agrobacterium tumefaciens* mediated transformation to a number of plant species is routine work but parameters for improvement in transformation efficiency and regeneration of plant from infected explant are important to be studied.

Type and age of explant: the present study suggested that leaf disc was found to be more efficient for transformation than embryonic axes which were confirmed after induction of callus and regeneration of plantlets on selectable medium

(kanamycin) and prominent appearance of blue spots after GUS staining. The plant regeneration is largely depending up on the appropriate choice of the explants. Both explants the age and types of explant like epicotyl, cotyledon and hypocotyl have great influence on regeneration in cowpea (Amitha and Reddy 1996). Higher sucrose concentration showed enhanced callus induction indicating higher transformation frequency as compared to less sucrose concentration. Different sucrose concentration indicates change in plantlet regeneration optimum concentration would be 3%. According to previous studies it indicates that sucrose improved T-DNA delivery into immature embryos rice. This treatment was extensively used to produce large numbers of transgenic plants for various projects (Ye *et al.*, 2000; Lucca *et al.*, 2001).

Abbreviations

MS, Murashige and Skoog (1962) medium; **MS0**, Murashige and Skoog (1962) medium (contains no sucrose)**; MS basal**, Murashige and Skoog (1962) salts and gamborg b5 vitamins (1968) medium; **2,4-D**, (2,4- dichlorphenoxy) acetic acid; **GUS**, β-glucuronidase; **X-Gluc** (5-bromo-4-chloro-3-indolyl-β-D-glucuronidase.

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REFERENCES

Amitha K. and Reddy T.P. (1996). Regeneration of plantlets from different explants and callus cultures of cowpea (*Vigna unguiculata* L.). *Phytomorphology*. 46(3):207-211.

FAO (Food and Agriculture Organisation). (2005). Cowpea production data base for Nigeria 1990-2004.

Gelvin S.B. (1998). *Agrobacterium* VIrE2 proteins can form a complex T strands in the plant cytoplasm, *J. Bacteriol.* 180 (16): 4300-4302.

Horsch R.B., Fraley R.T., Rogers S.G., Sanders P.R., Lloyd A. and Hoffman N. (1984). Inheritance of functional foreign genes in plants. *Science*. 223, 496-498

Jefferson R.A., Kavanagh T.A. and Bevan M.W. (1987). GUS fusion: ß-glucuronidase as a sensitive and versatile gene marker in higher plants. *EMBO J*. 6: 3901-3907.

Lucca P., Ye X. and Potrykus I. (2001). Effective selection and regeneration of transgenic rice plants with mannose as selective agent. *Mol. Breed*. 7: 43-49.

Muthukumar B., Mariamma M., Veluthambi K. and Gnanam A. (1996). Genetic transformation of cotyledon explants of cowpea (*Vigna unguiculata* L Walp) using *Agrobacterium tumefaciens*. *Plant Cell Rep*. 15: 980–985.

Popelka J.C., Gollasch S., Moore A., Molvig L. and Higgins T.J.V. (2006). Genetic transformation of cowpea (*Vigna unguiculata* L.) and stable transmission of the transgenes to progeny. *Plant Cell Rep*. 25:304-312.

Singh B.B., Chambliss O.L. and Sharma B. (1997). Recent advances in cowpea breeding. *Advances in Cowpea Research*, Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Center for Agricultural Sciences (JIRCAS). IITA, Ibadan, Nigeria, pp 30-50.

Vergauwe A., VanGeldre Inze D., VanMontagu M. and Vanden E. (1998). Factors influencing *Agrobacterium tumefaciens* mediated transformation of *Artemisia annua* L. *Plant Cell Rep*.18: 105-110.

Ye X., Al-Babili S., Kloti A., Zhang J., Lucca P., Beyer P., and Potrykus I. (2000). Engineering the provitamin. A (b-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science*. 287: 303-305.

