DIRECT SHOOT REGENERATION AND TRANSFORMATION OF CUCUMBER (*Cucumis sativus* L.) WITH GUS GENE

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DEPARTMENT OF PLANT BIOTECHNOLOGY

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DEDICATED TO MY BELOVED PARENTS, BROTHER, SISTER & JIJU

DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
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CERTIFICATE

This is to certify that the thesis entitled, **"Direct Shoot Regeneration and Transformation of Cucumber (*Cucumis sativus* L.) with Gus Gene.",** submitted in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture)** in **PLANT BIOTECHNOLOGY** of the University of Agricultural Sciences, GKVK, Bangalore is a bonafide record of research work done by **Ms. RINI PAKARLA, ID No. PALB 1231,** during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

BANGALORE
JULY, 2013

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DIRECT SHOOT REGENERATION AND TRANSFORMATION OF CUCUMBER (*Cucumber sativus* L.) WITH GUS GENE

Thesis Abstract

Cucumber (*Cucumis sativus* L.) is one of the major vegetable crops of India occupying second position in the global production. Cucumber (*Cucumis sativus* L.) cv. Green Long was tested for direct shoot induction and regeneration on MS medium treated with different concentrations of Plant Growth Regulators (PGR) using cotyledonary explants from ten day old *in-vitro* grown seedlings. Shoots and roots were obtained in the shooting media. Number of days taken for shoot initiation was significantly less (7.0) in 2.0 mg/L kinetin and the average length of roots was significantly high (13.4 cm) in a combination of kinetin and IBA at 2.5 and 2.0 mg/L respectively. Kinetin at 2.0 mg/L gave the best results for other parameters, namely maximum numbers of shoots (2.6) and maximum number of roots (2.0) among all the treatments. Rooting was enhanced in all the treatments with IBA at 2.0 mg/L. Maximum number of roots (5.6) was recorded with kinetin at 2.0 mg/L and IBA at 2.0 mg/L. Cotyledonary explants were transformed with *Gus* gene and confirmation was done with X-gluc substrate which gave blue color for transformed cotyledons. In recent years, the production of cucumber is greatly affected by a number of pests and diseases which seriously limit crop production. Genetic transformation is one of the ways to manage this issue. This study is an initial attempt towards this end.

Signature of Student Signature of Major Advisor

ಗಸ್ ಅನುವಂಶಿಕ ಧಾತುವಿನೊಂದಿಗೆ ಸೌತೆಕಾಯಿ (*ಕುಕುಮೀಸ್ ಸಟೈವಸ್*. ಎಲ್) ಯ ಕಾಂಡದ ನೇರ ಪುನರುತ್ಪಾದನೆ ಮತ್ತು ರೂಪಾಂತರ

ರಿನಿ ಪಾಕರ್ಲಾ

ಪ್ರಬಂಧದ ಅಮೂರ್ತ

ಸೌತೆಕಾಯಿ (*ಕುಕುಮೀಸ್ ಸಟೈವಸ್*. ಎಲ್)ಯು ಭಾರತದ ಪ್ರಮುಖ ತರಕಾರಿ ಬೆಳೆಯಾಗಿದ್ದು ಜಾಗತಿಕ ಉತ್ಪಾದನೆಯಲ್ಲಿ ಎರಡನೇ ಸ್ಥಾನವನ್ನು ಕಾಯ್ದುಕೊಂಡಿದೆ, ಸೌತೆಕಾಯಿ (*ಕುಕುಮೀಸ್ ಸಟೈವಸ್*. ಎಲ್)ಯನ್ನು ಕಾಂಡದ ನೇರ ಪುನರುತ್ಪಾದನೆ ಮತ್ತು ರೂಪಾಂತರಕ್ಕಾಗಿ ಎಂ ಎಸ್ ಬೆಳವಣಿಗೆ ಮಾಧ್ಯಮದಲ್ಲಿ ವಿವಿಧ ಪರಿಮಾಣದ ಸಸ್ಯ ಬೆಳವಣಿಗೆ ನಿಯಂತ್ರಕಗಳ ಜೊತೆಗೆ ೧೦ ದಿನ ಹಳೆಯ ದಳೀಯ ಪ್ರಭಾರೀ ಸಸ್ಯಗಳ ಮೊಳಕೆಗಳನ್ನು ಉಪಯೋಗಿಸಿಕೊಂಡು ಬೆಳೆಸಲಾಯಿತು. ಬೇರು ಮತ್ತು ಕಾಂಡವನ್ನು ಬೆಳೆವಣಿಗೆ ಮಾಧ್ಯಮದಿಂದ ಪಡೆಯಲಾಯಿತು.ಬೇರಿನ ಬೆಳವಣಿಗೆ ಆರಂಭವು(೭.೦) ೨.೦ ಎಂಜಿ/ಕೆಜಿ ಕೈನೆಟಿನ್ ನೊಂದಿಗೆ ಗಮನಾರ್ಹವಾಗಿ ಕಡಿಮೆ ಕಂಡಿತು ಮತ್ತು ಆದರೇ ಸರಾಸರಿ ಬೇರಿನ ಉದ್ದವು(೧೩.೪) ೨.೫ ಎಂಜಿ/ಕೆಜಿ ಕೈನೆಟಿನ್ ಮತ್ತು ೨.೦ ಎಂಜಿ/ಲೀ ಐಬಿಎ ನೊಂದಿಗೆ ಗಮನಾರ್ಹವಾಗಿ ಅಧಿಕ ಕಂಡಿತು. ೨.೦ ಎಂಜಿ/ಕೆಜಿ ಕೈನೆಟಿನ್ನ ಎಲ್ಲಾ ಉಪಚರಣೆಗಳಲ್ಲಿ ಕೆಲವು ಮಾನದಂಡ ಗಳಾದ ಅತ್ಯಧಿಕ ಕಾಂಡದ ಸಂಖ್ಯೆ (೨.೬) ಮತ್ತು ಅತ್ಯಧಿಕ ಬೇರಿನ ಸಂಖ್ಯೆ(೨.೦)ಯನ್ನು ತೋರಿದವು. ೨.೦ ಎಂಜಿ/ಲೀ ಐಬಿಎ ನ ಎಲ್ಲಾ ಉಪಚರಣೆಗಳಲ್ಲಿ ಬೇರುಗಳು ಅಧಿಕ ವರ್ಧಿತವಾಗಿದೆ. ಅತ್ಯಧಿಕ ಬೇರುಗಳ ಸಂಖ್ಯೆಯನ್ನು ೨.೦ ಎಂಜಿ/ಕೆಜಿ ಕೈನೆಟಿನ್ ಮತ್ತು ೨.೦ ಎಂಜಿ/ಲೀ ಐಬಿಎ ನಲ್ಲಿ ಧಾಖಲಿಸಲಾಯಿತು. ದಳೀಯ ಪ್ರಭಾರೀ ಸಸ್ಯಗಳನ್ನು ಗಸ್ ಅನುವಮ್ಮಿಯ ಧಾತುವಿನೊಂದಿಗೆ ಪರಿವರ್ತಿಸಲಾಯಿತು. ದೃಢೀಕರಣಕ್ಕಾಗಿ ಪರಿವರ್ತಿತ ದಳಕ್ಕಾಗಿ ನೀಲಿ ಬಣ್ಣ ಕೊಡುವ ಎಕ್ಸ್ ಗ್ಲುಕ್ ನ್ನು ಪದಾರ್ಥವಾಗಿ ಬಳಸಲಾಯಿತು, ಈಗಿನ ವರ್ಷಗಳಲ್ಲಿ ಸೌತೆಕಾಯಿ ಬೆಳೆಯ ಉತ್ಪಾದನೆಯು ಕೀಟ ಹಾಗೂ ರೋಗಗಳಿಂದ ಕುಂಟಿತಗೊಂಡಿದೆ, ಅನುವಂಶಿಕ ರೂಪಾಂತರವು ಈ ಈ ಬೆಳೆಯಲ್ಲಿನ ನಷ್ಟವನ್ನು ತಡೆಯಲು ಒಂದು ಸೂಕ್ತ ಮಾರ್ಗವಾಗಿದೆ.

ವಿದ್ಯಾರ್ಥಿನಿಯ ಸಹಿ

ಪ್ರಧಾನ ಸಲಹೆಗಾರರ ಸಹಿ

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Introduction

I. INTRODUCTION

Cucumber (*Cucumis sativus* L.) is a member of the family Cucurbitaceae, which comprises 90 genera and 750 species. Besides *Cucumis sativus* L., the genus *Cucumis* comprises of about 40 different species. It is an important crop in the tropics, subtropics and in the minor portions of the temperate zone (Jelaska, 1986).

Cucumbers are cultivated either for fresh consumption, or for preservation as pickling cucumber, marinated with vinegar, salt and other spices. It has a cooling effect and prevents constipation apart from its use for treatment of jaundice. Its seeds are diuretic, tonic and refrigerant. The odorous principle of *Cucumis sativus* L. is extractable with alcohol and is used in certain bouquet perfumes. Fruits are also used in folk medicine and for cosmetic means. Cucumbers are mainly eaten in the unripe green form. The ripe yellow form normally becomes too bitter and sour (Pandey, 2000).

Cucumber (*Cucumis sativus* L.), a popular vegetable crop of the family Cucurbitaceae, is rich in phosphorus, potassium, iron and oxalic acid and is popularly used in salads. It contains 95-98% water, up to 2.2% carbohydrates and fibers, about 0.5% of cindery substances, carotene, pertinacious substances, organic acids, vitamin B and is a good source of vitamin C. The high water content makes cucumbers a diuretic and it also has a cleansing action within the body by removing accumulated pockets of old waste material and chemical toxins. Cucumbers help eliminate uric acid which is beneficial for those who have arthritis, and its fiber-rich skin and high levels of potassium and magnesium helps regulate blood pressure and help promote nutrient functions. The magnesium content in cucumbers also relaxes nerves and muscles and keeps blood circulating smoothly.

A good micropropagation protocol could reduce the cost of hybrid seed production, which can account for 30% of the total seedling cost (Pandey, 2000).

Different diseases and pests cause significant crop loss in cucumber. Conventional plant breeding technique is generally used to obtain a high yielding plant variety, which is resistant to some diseases and pests. The introduction of desirable genes by conventional plant breeding technique requires much time and is often accompanied by changes in other desirable traits and has other problems such as sterility barriers between species. Cross-incompatibility and narrow genetic base in cucumber pose limitations to improvement by classical breeding.

Cucumber is an important vegetable crop which suffers from a number of viral diseases including the cucumber mosaic virus, watermelon mosaic (papaya ring spot virus) and cucumber green mottle mosaic virus which seriously limit the crop production. Transformation techniques provide the potential to introduce virus resistance into cucumber and improve crop yield and quality (Den, 1984).

Different plant tissue culture techniques are useful for propagation of important plants and for the production of transgenic plants. Large scale somaclonal variation in tissue culture derived regenerants was found when plants were regenerated indirectly through somatic embryogenesis, cell suspensions or isolated protoplast culture. Indirect shoot regeneration is also time consuming. In contrast, direct shoot regeneration (organogenesis) is less time consuming with less abnormality observed in the regenerants (Lorz and Brown, 1986).

Methods are available for the direct plantlet formation from cucumber tissue explant or developmentally advanced structures. Direct plantlet regeneration from axillary bud explants, differentiation from

hypocotyl, cotyledon, and root segments, differentiation of meristemoids and embryos from anther culture have been reported. Plantlet regeneration from leaf callus and hypocotyl callus has also been reported. Recently protoplast cultures in cucumber were established, but very low frequency plant regeneration was reported. The regeneration capacity also depends on the genotype (variety) of the plant (Kim *et al*., 1988).

Tissue culture method for production of multiple shoots in cucumber using cotyledon explants was described by Gambley and Dodd (1990).

Standardization of the concentration of plant growth regulators is the most important step in the establishment of regeneration protocols for crop plants. However, in cucumber (*Cucumis sativus* L.) variety Green Long studies on tissue culture in general and transformation in particular are very limited. In the present study, a reliable regeneration protocol and transformation work was conducted for this cultivar with the following objectives.

1. Standardization of growth hormone concentration for direct shoot regeneration of cucumber.
2. Transformation of cucumber cotyledons with *GUS* gene using Gene Gun method.
3. Histochemical assay of the GUS gene expression in the transgenic plant.

Review of Literature

II. REVIEW OF LITERATURE

There is a tremendous increase in crop improvement with the advancement of tissue culture and gene delivery techniques. These approaches have increased efficiency over traditional breeding methods to increase the yield and also the crop productivity. Production of transgenic plants with more advantageous genes will lead to the biotechnological revolution. In this chapter work done by the various scientists on various aspects of regeneration and gene delivery in cucumber (*Cucumis sativus* L.) has been reviewed under the following sub-headings.

2.1 Choice of explants for tissue culture

2.2 Use of plant growth regulators in regeneration of cucumber

2.3 Genetic transformation through particle bombardment system

2.1 Choice of explants for tissue culture

Wehener and Locy (1981) noticed that when hypocotyl and cotyledon explants taken from seven day-old seedlings were grown on Murashige and Skoog (MS) medium containing 1mg/L each of benzyl amino purine (BAP) and NAA, 28 out of 85 lines formed shoots from the cotyledon. While shoots were not formed from the hypocotyl explants, but roots were initiated from both the explants.

The use of hypocotyls as targets for transformation is assumed to be advantageous over the use of intact embryos, because hypocotyls, once detached from cotyledons, start multiple secondary embryogenesis (Kott and Coventry, 1988).

Niedz *et al*. (1989) reported that cotyledonary explants of four-day old *Cucumis melo in-vitro* seedling, showed maximum initiation of shoot

buds when cultured on MS medium supplemented with 5µM each of IAA and BAP. They also found that the presence of abscisic acid (ABA) significantly increased the number of explants producing shoot buds initiation which was affected by genotype, seedling age, light intensity and temperature.

Trojanowska and Malepszy (1989) induced shoot regeneration directly in hybrid *Cucurbita maxima* X *Cucurbita pepo* L. from abnormal embryos with spongy cotyledons without an intervening callus phase on MS medium without hormones. Rooting was induced in the same medium containing 1.5 mg/L of IAA. Average of 65 plants was obtained per embryo.

Custers and Verstappen (1989) obtained normal plants in cucumber, when shoots tips and nodal explants, taken from *in-vitro* seedlings were cultured on MS medium.

Singh *et al*. (1990) reported that hypocotyl segments and cotyledon halves of *Cucumis melo* cv. Pusa Madhura cultured *in-vitro* on basal MS media with 1µM BAP produced a large number of shoots either directly or indirectly with an intervening stage of nodule formation, and the shoots rooted easily on medium containing 1µM IBA.

Cade *et al*. (1990) reported that shoot production was highest (60%), when 6 day-old cucumber cotyledons were cultured on MS medium having 0.3 mg/L of BAP and no NAA. Root production was also influenced by NAA and BAP, with many short roots being formed in media having NAA but lacking BAP.

Ali *et al*. (1991a) obtained cucumber plants when cotyledons from old cucumber seeds were cultured on MS medium containing 1mg/L each of NAA and BAP.

High frequency shoot formation from internode explants of a hybrid (*Cucurbita maxima x C. moschata*) squash was achieved on MS medium. Explanted internodal sections were induced to develop multiple shoots through direct regeneration without the intervening callus phase (Rahman *et al*., 1993).

A single regeneration procedure using cotyledon explants effectively regenerated five commercially grown muskmelon cultivars (Gonslaves *et al.,* 1994).

Hooymans *et al*. (1994) have described regeneration of shoots from cotyledons of three to five day-old seedlings of cucumber which ranged up to 100% response and the regenerated plants had normal morphology.

Compton and Gray (1994) found that two day-old cotyledon explants of watermelon cultured on MS medium produced large number of shoots compared to the cotyledon explants taken from 4, 6, 7, or 10 day-old seedlings.

Cotyledon halves (proximal) of *Cucumis melo L*. cv. Pusa Madhura cultured on MS medium with 1.0µM BAP resulted in direct induction of shoot buds from petiolar end with a regeneration frequency of 100 per cent. Rooting of shoots was achieved on MS medium with 1.0µM IBA. Further, the plants were successfully hardened by planting in a mixture of sand and garden soil in greenhouse (Singh *et al*., 1996).

Shoot induction in muskmelon was better from cotyledon petiole explants compared to the apex or leaf explants. BAP (0.5 mg/L) was better than Zeatin (ZT) or Kinetin, and LS medium was better compared to MS medium (Dong *et al*., 1996).

Sarowar *et al*. (2003) developed an efficient *in-vitro* micropropagation protocol for direct shoot growth of interspecific Cucurbita hybrid variety using shoot–tips of 5-day-old explants.

Hypocotyl explants of three cultivars of melon (*Cucumis melo* L.) (cvs Revigal, Topmark and Kirkagac), and a cucumber (*C. sativus* L. cv. Taoz) rapidly directly regenerated multiple shoots on MS medium. Regeneration from the hypocotyl resulted in nearly 100% diploid shoots, whereas regeneration from the cotyledons resulted in 40 – 70 % of polyploid regenerants (Curuk *et al*., 2003).

Using cotyledon explants excised from seedlings germinated *in-vitro*, an efficient plant regeneration system via organogenesis was established for bottle gourd (*Lagenaria siceraria* Standl.). Maximum shoot regeneration was obtained when the proximal parts of cotyledons from 4-day-old seedlings were cultured on MS medium with 3 mg/L BA and 0.5 mg/L $AgNO_3$ under a 16-h photoperiod. After 3–4 weeks of culture, 21.9–80.7% of explants from the five cultivars regenerated shoots (Han *et al*., 2004).

Krug *et al*. (2005) studied the *in-vitro* organogenesis of water melon (*Citrullus lanatus)*, by the induction of adventitious buds in cotyledon segments cultured in medium supplemented with cytokinin. Explants were collected from one, three and five-day-old *in-vitro* germinated seedlings, considering the distal and proximal cotyledon regions. Their data showed that *in-vitro* organogenesis of watermelon occurred with higher efficiency, when cotyledon segments from the proximal region collected from three-day-old seedlings were cultivated in MS medium, supplemented with BAP (1 mg/L) and coconut water (10%).

Mohiuddin *et al*. (2005) initiated significantly better shoot regeneration and induced higher number of shoots per explant in two

cultivars of *Cucumis sativus* L. viz., Spring Swallow and Tasty Green from proximal cotyledon and hypocotyl explants using $AgNO_3$ at 30µM and 50 µM in the seed germination medium and regeneration medium when compared to control (without $AgNO_3$).

Embryonal axis explants from 2-day-old *in vitro*-germinated seeds were used to induce multiple shoot production. The combination of 4.44 µM BA and 1.59 µM NAA in MS medium triggered the initiation of adventitious shoot buds. The explants with shoot buds produced maximum number of shoots per explant (10.6) in MS medium supplemented with 4.44 µM BA and 0.065 mM L-glutamine in three successive transfers. The elongated shoots were rooted on MS medium with 4.92 µM IBA. Rooted plants were transferred to soil with a survival rate of 65 % (Vasudevan *et al*., 2007).

Efficient plant regeneration via organogenesis was established for two summer squash (*Cucurbita pepo* L.) cultivars viz. Bulum and Rumbo, using hypocotyl and cotyledon derived calli. Seeds were surface sterilized in 0.1% $HgCl_2$ for 5 min, and germinated *in-vitro* in plant growth regulator free MS medium. The maximum morphogenic callus induction rate (86%) was observed from a hypocotyl explant by culturing in MS medium supplemented with 2.5 mg/L 2,4-D. Calli size and fresh weight increased substantially through sub-culturing (Pal *et al*., 2007).

Shoot regeneration using cotyledons derived from seedlings of diploid and triploid yellow water melon (cultivars Hwang Fong Yellow Queen, Round Dragon and Chin San seedless) was investigated. Multiple shoots from auxillary meristems were obtained without adventitious shoot. Shoot regeneration system for water melon was successfully established from cotyledon sections of 4 to 5 day-old *in-vitro* seedling. The explants were collected from the proximal cotyledon with hypocotyl segments (Suratman *et al*., 2009).

Chovelon *et al*. (2011) reported that cucumber cotyledons and young leaves from 4 and 13 day-old seedlings, respectively were used as explants for shoot regeneration. They were cut transversely into four equal pieces and placed on two different regeneration media viz., MS medium with 0.2 mg/L BAP + 0.2 mg/L 2-iP and MS medium with 1.12 mg/L BAP + 0.88 mg/L IAA + 0.26 mg/L abscisic acid (ABA). Very low regeneration rates were obtained with different explants sources of both genotypes placed on MS medium supplemented with 1.12 mg/L BAP, 0.88 mg/L IAA and 0.26 mg/L ABA. The highest regeneration rates were obtained from cotyledon explants cultured on MS medium supplemented with 0.2 mg/L BAP and 0.2 mg/L 2-iP.

Shrivastava and Roy (2011) reported that the nodal explant of *Citrullus colocynthis* (cucurbitaceae) when cultured on MS medium supplemented with BAP and IBA generated best response in terms of regeneration of complete plantlet. The shoots were then aseptically transferred to shoot proliferating medium having treatment of cytokinin, i.e., BAP (0.5 mg/L - 2.5 mg/L) and kinetin (0.5 mg/L - 2.5 mg/L). Among different concentrations used, maximum multiple shoots (12.1±0.5) were obtained on MS medium supplemented with BAP (2.0 mg/L) and they proved that BAP (2.0 mg/L) and kinetin (2.0 mg/L) at higher concentration are suitable for shoot multiplication as well as shoot elongation.

Cotyledon explants of watermelon (*Citrullus lanatus*) were excised from six day-old seedlings and were cut into proximal and distal halves. Proximal cotyledons were used as explants (Li *et al*., 2012).

2.2 Use of plant growth regulators in regeneration of cucumber

The pioneering work on *in-vitro* propagation of cucumber was carried out by Handley and Chambliss (1979). They cultured axillary

buds of gynoecious cucumber on MS medium supplemented with 0.1mg/L of NAA and kinetin to get both shoots and roots, and the plants were successfully weaned in greenhouse.

Barnes (1979) stimulated an average of 10.3 axillary shoots, by culturing the shoot tips of watermelon on Linsmaier and Skoog (LS) medium supplemented with 9.3µM of kinetin and 0.28µM of IAA. Rooting of these shoots was carried out in a medium containing 11.5µM IAA, and hardened in greenhouse in 1:1 peat and sand medium for 3 weeks.

Pumpkin (*Cucurbita pepo* L.) can be rapidly multiplied by culturing the meristem tips of seedling on MS medium containing 2.56 mg/L of kinetin and 8 mg/L of IAA. Maximum shoot proliferation can be achieved on MS medium with 1mg/L of BAP, while rooting can be accomplished in medium containing 8 mg/L of IAA (Pink and Walkey, 1984). Shoots were produced in 12 days from each bud, when young main and lateral apices of cucumber were cultured on agar medium containing 20-30 ppm 2-ip. Further, both elongation and rooting was achieved on a medium containing 1.0 ppm each of IAA and 2-ip and 0.025 ppm Gibberellic Acid (GA3).

Kathal *et al*. (1988) noticed maximum shoot differentiation when leaf explants of *Cucumis melo* were cultured on MS medium containing BAP and 2-ip at 1µM each. These shoots were elongated in MS medium with 1µM BAP and rooted in 0.5µM IAA, and they observed that the regeneration potential of leaves declined with the increase in size of the leaves and age of the donor seedling.

Cotyledonary explants of four day old *Cucumis melo* cv. Hales Best Jumbo *in-vitro* seedlings showed maximum initiation of shoot buds cultured on a revised Murashige & Skoog medium when supplemented with 5µm Indole-3-Acetic acid and 5 µm Benzylaminopurine and cultured

at 25-29^0 C under low light intensity (5-30 µm/m^2/s). Subculture of the shoot buds on the same medium without auxin and supplemeted with 3µm BAP caused the development of the shoots from 30 % of the buds. The presence of ABA significantly increased the number of explants producing shoot buds (Niedz *et al*., 1989).

Tabei and Kanno (1989) reported that organogenesis of cucumber depends on the type and concentration of auxins 2,4-dichlorophenoxy acetic acid (2, 4-D) at 0.1 mg/L and NAA at 1 mg/L induced organogenesis, and IAA at a concentration of 1-100 mg/L induced adventitious shoots. Further, IAA was found to be more effective than 2, 4-D in induction of morphogenesis.

Hisajima *et al*. (1989) induced multiple shoot buds in cucumber when seeds were cultured *in-vitro* with 2.5 to 5µM BAP, and these shoots were continuously multiplied using a combination of BAP and IBA. Plant regeneration was achieved by rooting single shoots in IBA, wherein the rooting rate was 100 per cent.

A technique is described for the production of cucumber (*Cucumis sativus* L.) shoots in the presence of cytokinins using cotyledon explants. Shoots which arose from adventitious buds and not from enhanced axillary branching are confined to the specific region at the base of the cotyledon. Cytokinins BAP, Kinetin and 2-ip at concentration of 4 mg/L or less are effective in producing adventitious buds. It is possible to achieve a yield of 23 shoots per cotyledon by removal of the axillary bud (Rhonda and William, 1990).

Two studies were conducted to test the effects of various tissue culture media on somatic embryogenesis from cotyledon tissue of cucumber (*Cucumis sativus* L.). The two best media for embryo initiation were Murashige and Skoog (MS) salts and vitamins containing either 1.0

or 2.0 mg/L 2, 4-D and 0.5 mg/L kinetin. In the second study, embryos developed more normally. More plantlets developed when tissue was removed from the initiation medium after 3 weeks and transferred to MS containing 1 mg/L NAA and 0.5 mg/L kinetin for 3 weeks, rather than leaving the embryos on a medium containing 2, 4-D (Cade *et al.,* 1990).

Gambley and Dodd (1991) reported that in cucumber, explants including at least part of the cotyledon, a short section of hypocotyls, and the apical bud are capable of producing multiple axillary buds from the seedling apex and adventitious shoot from the hypocotyls base, in a medium containing 2.0 mg/L of kinetin.

Dong and Jia (1991) on culturing five day-old cotyledons of watermelon on MS medium with 5.0 mg/L of IAA noticed high frequency of shoot regeneration (60-92%). Elongation of multiple shoots was achieved on a medium with 0.2 mg/L kinetin, and rooting was accomplished on MS medium with 0.1 mg/L of NAA.

Ali *et al*. (1991b) obtained shoot regeneration in *Cucumis* from cotyledon explants cultured on MS medium containing 4 mg/L of BAP and 0.2 mg/L of NAA. Further, rooting was accomplished on medium containing 1.5 mg/L of NAA.

Chee (1991) obtained shoot regeneration in *Cucumis melo* by culturing the cotyledons on MS medium with NAA at 0.01 mg/L.

Compton and Gray (1992) developed a protocol for micro propagating watermelon by culturing the shoot tip explants in MS medium with 1µM BAP, wherein 2.3 to 5.2 shoots could be obtained per explant, which can be rooted *in-vitro* and acclimatized in greenhouse for one week.

Hooymans *et al*. (1994) regenerated plants from cucumber (*Cucumis sativus* L.) cotyledonary explants within six weeks, which included the induction of buds on MS-medium supplemented with 40 g/L sucrose, 500 mg/L tryptone L 42, 50µM IAA and 0.1µM kinetin. The induced buds developed into plants on MS-medium supplemented with 20 g/L sucrose, 500 mg/L tryptone L42, 0.5µM kinetin and 0.1µM IAA.

Leaf explants cultured on 5µM BAP taken from 14 day-old cucumber seedlings gave good shoot differentiation, which were rooted on MS medium with 1.0µM of IBA (Misra and Bhatnagar, 1995).

A melon leaf of 3-4cm diameter excised from greenhouse plants, cultured on MS medium with 5µM each of IAA and BAP + 1µM of ABA, with 30µM $AgNO_3$ and 2.6 g/L of phytagel gave the best regeneration with 10-100 shoots per explant (Yadav *et al*., 1996).

Zhang and Cui (2001) studied the effect of four kinds of cytokinins viz., kinetin, 6-benzyladenine (BA), Zeatin (ZT) and thidiazuron (TDZ)] on direct shoot regeneration from cotyledons of 5-day-old seedlings of *Cucumis sativus* L. inbred line Jin431 in order to establish a regeneration system for gene transformation. They found that ZT had a highest efficiency among the four cytokinins, and its regeneration frequency was 85% with multiple shoots which may be useful for gene transformation while TDZ had the highest activation, and the optimal concentration of TDZ effect was considerably lower than that of the other three cytokinins. An important finding of their study was that shoots as well as roots were induced by TDZ.

Sarowar *et al*. (2003) excised shoot–tips and cultured on MS medium containing two plant growth regulators 6-BA and NAA with various combinations and concentrations for the study of shoot induction. They got best condition for shoot growth with 3 mg/L 6-BA in

MS medium. The shooting frequency was 84% and five shoots were obtained from each explant after 30 days of culture.

Vasudevan *et al.* (2004) cultured shoot tip explants of cucumber cv. Poinsett 76 *in-vitro* on MS medium with L-glutamine, ammonium nitrate, adenine sulphate, asparagine, ammonium succinate, potassium nitrate and sodium nitrate as the nitrogen sources along with optimal concentration of 0.044 mM BA to study their effects on *in-vitro* morphogenesis. The explants grown with 0.068 mM L-glutamine displayed the highest culture response (74.6 %) and greatest shoot numbers per explant (13.6) at the end of two subcultures. The explants cultured with other nitrogen sources resulted in low culture frequency and low number of shoots per explant accompanied by basal callusing and necrosis.

Mohiuddin *et al*. (2005) obtained high shoot regeneration of 96% and 92% in proximal cotyledon of Spring Swallow and Tasty Green cultivars, respectively with $AgNO_3$ at 30μM combined with 1.0 mg/L BAP. Shoot regeneration from proximal hypocotyl explants of SS (72%) was also found in the same treatment. They also proved that $AgNO_3$ at 50 μM combined with 2.0 mg/L BAP was the best treatment for shoot regeneration from proximal hypocotyl (72%) of Tasty Green.

Mahzabin *et al.* (2008) achieved micropropagation of pumpkin (*Cucurbita maxima Duch*.) using shoot tip of *in-vitro* grown seed derived plants of two cultivars namely, Bikrompuri and Baromasi of Bangladesh. They cultured the excised shoot tips on MS medium containing kinetin, BA, and NAA at various concentrations and combinations for shoot induction and proliferation, and best response was found at 3.0 mg/L of BA. Shoots were rooted most effectively in half-MS medium supplemented with 1.0 mg/L IBA.

Melara and Arias (2009) induced the highest average of shoots from cotyledonary explants of Costa Rican melon (*Cucumis melo L*.) on the shoot induction medium supplemented with 0.5 mg/L BAP and 0.05 mg/L IAA or 1.0 mg/L BAP and without IAA.

Mendi *et al*. (2010) also cultured explants consisting of the proximal part of cotyledons from seven day-old seedlings, germinated under dark and light conditions on MS media containing different concentrations of BA (0, 0.5, 1.0, 2.0 mg/L) and IAA (0, 0.25, 0.5, 1.0 mg/L). The highest frequency of adventitious shoot regeneration obtained (42.8 %) on MS medium with 1.0 mg/L BA+ 0.25 mg/L IAA from the seeds germinated under dark condition while from cotyledon explants of snake melon (*Cucumis melo* var. Flexousus) seeds germinated under light conditions, the maximum frequency of adventitious shoot regeneration achieved (2.8 %) on MS medium with 1.0 mg/L BA+ 0.25 mg/L IAA.

In-vitro plantlet regeneration has been obtained from 15-20 day old seedling cotyledon and hypocotyl segments of *Cucumis sativus* L. They were examined using various phytohormones individually and in combination on MS semi-solid medium supplemented with BAP (1.0-5.0 mg/L), kinetin (1.0-5.0 mg/L), IAA (0.5 mg/L)+BAP (1.0-5.0 mg/L) and IAA (0.5 mg/L)+kinetin (1.0-5.0 mg/L) for shoot proliferation. IAA (0.5 mg/L)+BAP (3.0 mg/L) was proved to be best for induction of shoots for cotyledon and hypocotyl explants. All regenerated plantlets were rooted on MS medium supplemented with (1.0 mg/L) IAA and the regenerated plants grew normally in the green house (Ugandhar *et al*., 2011).

In-vitro plantlet regeneration has been obtained from 20-25 days-old seedling shoot tip segments of *Cucumis melo* (L.). They were examined using various phytohormones individually and in combination on MS semi-solid medium supplemented with BAP (1.0-3.0 mg/L), kinetin (1.0-3.0 mg/L), IAA (0.5 mg/L)+ BAP (1.0-3.0 mg/L) and IAA (0.5

mg/L)+kinetin (1.0-3.0 mg/L) for multiple shoots proliferation. IAA (0.5 mg/L)+BAP (2.0 mg/L) was proved to be best for induction of shoots for shoot tip explants. All regenerated plantlets were rooted on MS medium supplemented with IAA (1.0 mg/L) and the regenerated plants grew normally in the green house (Venkateshwarlu, 2012).

The maximum percentage establishment of cotyledonary node explants was observed on MS medium + 1.0 mg/L 6-BAP + 0.5 mg/L NAA. However, MS medium fortified with 1.0 mg/L BAP + 1.0 mg/L kinetin + 200 mg/L activated charcoal exhibited maximum multiplication rate for the first two subcultures. The maximum frequency of multiple shoots in cotyledonary explants (86.33 %) was observed on treatment MS + 1.0 mg/L BAP + 1.0 mg/L kinetin + 200 mg/L activated charcoal (Singh *et al*., 2013).

2.3 Genetic transformation of Cucumber (*Cucumis sativus* L.)

2.3.1 Agrobacterium mediated transformation

Vasudevan *et al.* (2002) developed a transformation and regeneration protocol for the monoecious cucumber cultivar 'Green long' using the proximal end of the cotyledon explants with *Agrobacterium tumefaciens* strain EHA 105. The explants were dipped in bacterial suspension containing acetosyringone (20µl/L) for 10 min and co-cultivation on MS basal medium free of any PGR in the dark for 48h. Then, they transferred explants into shoot regeneration medium containing MS salts, 1 mg/L BA, 25 mg/L kanamycin and 300 mg/L cefotaxime, and grown for 15 days. Thereafter, they selected transgenic cucumber shoots from the regenerated shoots *in-vitro* on PGR free MS medium containing 100 mg/L kanamycin and 300 mg/L cefotaxime. Finally, they had confirmed the transformed cucumber by GUS assay and PCR analysis and obtained 12% of transformed shoots.

The generation of transgenic *Cucumis sativus* cv. Green long plants resistant to phosphinothricin (PPT) was obtained using *Agrobacterium tumefaciens* mediated gene transfer. Cotyledons were inoculated with the strain EHA105 harboring the neomycin phosphotransferase II (*npt II*), and phosphinothricin resistance (*bar*) genes conferring resistance to kanamycin and PPT. Putative transformants were confirmed for transgene insertion through PCR and Southern analysis (Vengadesan *et al*., 2005).

Vasudevan *et al.* (2007) developed an efficient transformation protocol for cucumber cv. Poinsett 76 using *Agrobacterium* strain EHA 105 using five-day-old mature cotyledon explants. They co-cultivated the infected explants for 2 days in MS medium containing BA (1.0 mg/L). Then, they carried out selection of transformed shoots in MS medium fortified with BA (1.0 mg/L), Cefotaxime (300 mg/L) and PPT (2.0 mg/L). The transformed shoots were elongated in MS medium containing BA (1.0 mg/L), Cefotaxime (300 mg/L), PPT (2.0 mg/L) along with GA3 (0.5 mg/L). The rooting of elongated shoots was achieved in MS medium with BA (1.0 mg/L), Cefotaxime (300 mg/L), PPT (2.0 mg/L) and IBA (0.6 mg/L). The transient Gus expression assay and leaf disc assay were carried out in order to find transformed shoots. The molecular confirmation of transformed shoots revealed the foreign gene integration into cucumber genome and about 6.6% transformation efficiency.

2.3.2 Gene gun mediated transformation

Particle bombardment was first described by Klein and co-workers in 1987 and this process involves the delivery of DNA into plant cells by coating the DNA onto metal micro carriers that are then driven into plant cells by gas acceleration using a particle gun or biolistic transformation system. Plant tissues are subsequently cultured and transformants are

selected using various selectable markers as described in the previous section.

This technology (biolistic = biology + balistic) was conceived by John Sanford (Sanford *et al.*, 1987) and allows the delivery of naked DNA into intact plant cells. It involves the acceleration of dense DNA-coated microparticles (tungsten or gold) by means of an explosion (i.e. gun powder or instantaneous water vaporisation) or a burst of gas (i.e. carbon dioxide, nitrogen or helium), up to a sufficient velocity to penetrate the plant cell wall. Moreover, bombardments can even be performed using desiccated bacteria (as microprojectiles) containing the gene to be transferred (Rasmussen *et al.,* 1994).

Chee and Slightom (1992) used the microprojectile bombardment method to transfer DNA into embryogenic callus of cucumber (*Cucumis sativus*), and stably transformed cucumber plant lines were obtained.

Schulze *et al*. (1995) developed transgenic cucumber (*Cucumis sativus* L.) plants by biolistic transformation of a highly embryogenic cell suspension culture using the neomycin phosphotransferase gene (*nptII*) and beta-glucuronidase (uidA) gene. Functional expression of the genes in transgenic plants was determined by neomycin phosphotransferase and β-glucuronidase enzyme assays.

Microprojectile bombardment mediated transformation is one of the most promising gene transfer techniques even for those plants which have proved recalcitrant to genetic transformation by any other procedure. Due to acceleration, the microcarriers cross the cell wall/membrane barrier, deliver the foreign DNA inside the cell, and transformants are then regenerated under selection. The transgenic nature of the plants is confirmed by assays of transgenic expression, molecular analysis, and inheritance of the introduced gene in subsequent generations (Luthra *et al*., 1997).

Material and Methods

III. MATERIALS AND METHODS

An efficient regeneration protocol is the basic prerequisite for genetic transformation in plants. The present study was conducted in the plant tissue culture laboratory of the Department of Plant Biotechnology, University of Agricultural Sciences (B), G.K.V.K. Campus, Bangalore, India during the year 2012-2013.

3.1 TISSUE CULTURE OF CUCUMBER

3.1.1 Glasswares and chemicals

Glasswares like culture tubes, conical flasks, bottles, petriplates, beakers etc., were of Borosil make. All the chemicals, plant growth regulators, vitamins, agar, were of analytical grade and were procured from Himedia, Co., Mumbai and Sigma Aldrich Co., USA. Microcentrifuge tubes, micropipette tips and micro test plates were from Tarsons (India).

3.1.2 Cleaning and sterilization of glassware

Glasswares like culture tubes, conical flasks, petriplates, beakers, bottles were washed with detergent, rinsed with distilled water and dried. Cleaned glasswares were rinsed in double distilled water and dried in oven at 80^0 C. Petriplates and all other glassware were placed in autoclavable cover, instruments like forceps, blade holders, wrapped in aluminium foil and autoclaved at 15 PSI pressure for 15 minutes at 121^0 C temperature.

3.2 Equipments

Refrigerated high speed centrifuge, Kubota, Japan

Single pan digital balance, Mettler, AK-100, Switzerland

Bio-Rad Power pack, Bio-Rad, India

Water bath, Heto, Denmark

Gel documentation system, Bio-Rad, India

Air displacement pipettes P_2, P_{20}, P_{1000} -Eppendorf, Germany

LG refrigerator, India, -20°C low freezer, Blue Star, India

Microwave oven intello wave, LG electronics Ltd, India

pH meter, ECIL India

Electronic shaking incubator, Innova-4000, Brunswick Scientific, USA

Horizontal laminar air flow, Klenzaids, India

Vertical laminar air flow, Clean Air, India

Polyacrylamide gel electrophoresis apparatus, Bangalore Genei, India

Nano drop (Eppendorf)

Gene gun (Bio Rad)

3.3 CHEMICALS

3.3.1 MS media composition

The basal medium used for the experiment was MS medium (Murashige and Skoog, 1962).

Stock solutions

Stock solutions were prepared by dissolving the chemicals by dissolving the analytical grade chemicals in required quantities in volumetric flasks using double distilled water and transferred to reagent bottles. These stock solution reagent bottles were stored in Corning reagent bottles in refrigerator at 4^0C for further use. The composition of the basal medium used is given in Table 1.

All the chemicals were mixed using magnetic stirrer and the volume was made up to one litre using sterile distilled water, sterilized at 15 PSI pressure for 15 minutes at 121^0 C temperature and refrigerated at 4^0 C. Iron EDTA stored in brown bottles at 4^0 C.

Table 1: Composition of Murashige and Skoog (1962) medium

Ingredients	Salt Concentration in stock solutions (g/L)	Aliquot taken for 1 lit of medium (ml)	Final Concentration of salt in 1 lit of medium (mg/L)	Group
Macro Nutrients stock (10X)				
NH_4NO_3	33	25	1650	I
KNO_3	38		1900	
$MgSO_4.7H_2O$	7.4		370	
KH_2PO_4	3.4		170	
$CaCl_2.2H_2O$	8.8	10	440	II
Iron stock (200X)				
$Na_2.EDTA.2H_2O$	7.46	5	37.30	IV
$FeSO_4.7H_2O$	5.56		27.30	
Micro Nutrients (100X)				
H_3BO_3	1.24	10	6.2	III
$MnSO_4.H_2O$	4.46		22.3	
$ZnSO_4.7H_2O$	1.72		8.6	
$CuSO_4.5H_2O$	0.005		0.025	
KI	0.166		0.83	
$CoCl_2.6H_2O$	0.005		0.025	
$Na_2MoO4.2H_2O$	0.05		0.25	
Vitamin stock (1000X)				
Glycine		10	2.0	V
Myo-Inositol			100	
Thiamine HCl			0.50	
Pyridoxine HCl			0.50	
Nicotinic acid			0.50	
Sucrose			30000	
Agar			8000	
pH			5.8	

Kinetin stock solution (1 mg/L)

100 mg Kinetin was weighed and dissolved in 1.0 ml of 0.2 N HCl and the volume was made upto 100 ml with sterile distilled water and stored at 4^0 C.

3.3.2 Preparation of culture media

Composition of MS basal medium (per litre)

Double distilled water	800 mL
10X MS Macro nutrient stock	100 mL
1000X MS Micro nutrient stock	1.0 mL
2000X MS Iron stock	5.0 mL
1000X MS Vitamin stock	1.0 mL
Myo-inositol	100 mg
Sucrose	30.0 g
Agar	8.0 g

All the chemicals mentioned in Table 1 were dissolved and the final volume was made up to 1000 ml. Sucrose (30g/L), Myo-inositol was weighed and added afresh each time. Required concentrations of the growth regulators were added and the pH was adjusted to 5.8 using 0.1 N NaOH and 0.1 N HCl. The required quantity of agar (0.8%) was added and the mixture was then heated for dissolving the agar. The media was then transferred to conical flasks. The flasks were plugged with cotton tightly and autoclaved at 15 PSI pressure for 15 min. at 121^0 C temperature. Flasks were not filled to more than half to ensure proper autoclaving.

3.4 Tissue culture methods

3.4.1 *In vitro* seed germination

Cucumber seeds of the cv. Green long (obtained from MAHYCO Pvt. Ltd.,) were sterilized as follows:

1. Seeds were washed thrice in tap water.
2. Seeds were rewashed in distilled water for twice.
3. These seeds were treated with 1% Mercuric chloride for 10 min.
4. Treated seeds were washed thoroughly with distilled water twice.
5. The seeds were then left in sterile water for some time and dried on autoclaved filter paper.
6. The dried seeds were taken transferred in to bottles containing basal MS media in a Laminar Air Flow (LAF) chamber.
7. These bottles were kept in culture room having 16/8 h photoperiod at 25^0 C and 2000 lux of light.

3.4.2 Standardization of *in-vitro* shoot regeneration from cotyledon as explant

Seeds of cucumber were sown in basal MS media. After 5 days of sowing cotyledons were excised with the basal portion intact and transferred into bottles containing MS media and different concentrations of Kinetin ranging from 1.8 mg/L to 2.0 mg/L and incubated in the culture room.

Treatment details for direct shoot regeneration in cucumber (*Cucumis sativus*) cv. Green long are given in Table 2.

Five replications were maintained per treatment. Observations were recorded once in a week for the following parameters:

1. Number of days taken for shoot initiation,
2. Number of shoots per explant,
3. Number of days taken for root initiation, and
4. Number of roots per explant.

The data were statistically analysed as per completely randomized design.

3.4.3 Standardization of *in-vitro* root regeneration from cotyledon as explant

Shoots were transferred to fresh media after they reached a height of 5 cm. The plants were later transferred to rooting media (MS+2 mg/L IBA) after sufficient leaves had developed.

3.4.4 Acclimatization

Washing of rooted shoots

After proper development of the roots, plants were carefully taken out of the culture tubes without damaging the delicate root system. These plants roots were washed under gentle flow of tap water for 4 h, to remove the adhering agar completely. Plants were treated with fungicide (Bavistin 0.8 %) for about 10 min to avoid the fungal contamination and then transferred to potting mixture.

3.4.5 Planting in potting mixture

Before the plants were transferred to potting mixture caps of the bottles were removed and covered with polybags having holes in them for air circulation to maintain high relative humidity, after 2 days they were transferred to potting mixture.

Coco peat, vermiculite, sand and perlite were taken in ¼ : ¼ : ¼ : ¼ ratio in disposable paper cups. Plants were taken from IBA medium

Table 2: Treatment details for direct shoot regeneration in cucumber (*Cucumis sativus*) cv. Green long.

Treatments	Kinetin (mg/L)
T_0	-
T_1	1.8
T_2	2.0
T_3	2.5

and transferred into the cups for good root growth and acclimatization. The potting mixture was watered with half MS media till it gets wet. These plants were kept in glass house at about 20-22^0 C temperature. Plants were irrigated as per the requirement.

3.5 Transformation studies

3.5.1 Biolistic/ Gene gun method of transformation (Plate 1)

3.5.2 Preparation of explants

Pre-culture

Cotyledons were prepared for transformation and placed as a circle in the centre of the petriplate for the convenient bombarding.

Osmotic treatment

Cotyledonary explants were transferred to osmotic medium (MS_0 medium with 30 g/L of mannitol) four hours before transformation

3.5.3 Microcarrier preparation

- 30 mg of tungsten particles (Bio-Rad) was taken in a 1.5 mL eppendorf tube.
- To this 0.5 mL of absolute ethanol was added and vortexed for 1 min and centrifuged at 10,000 rpm for 1 minute.
- Supernatant was discarded and same step repeated for two times.
- Tungsten particles were washed by resuspending in 0.5 mL sterile distilled water, centrifuged and supernatant was discarded. This step was repeated for three times.
- Finally washed tungsten particles were resuspended in 0.5 mL sterile distilled water and stored at 4^{0}C.

3.5.4 Plasmid construct (Plate 2)

The plasmid construct pABC with GUS gene under the control of a modified CaMV35S promoter called as a super promoter which gives the constitutive expression was used. This is to enhance the expression levels of the gene. The other selectable marker present in the construct is *npt II* gene.

3.5.5 Plasmid DNA coating on to Tungsten particles

- To a 50 µL aliquot of tungsten particles following were added in sequence under continuous vertexing.
    - 5 µL of plasmid DNA (1 µg/ml)
    - 50 µL of 2.5 M $CaCl_2$
    - 20µL of 0.1 M spermidine
- Vortexed continuously for 3 minutes and incubated at room temperature for 10 minutes.
- Centrifuged at 13,000 rpm for 10 sec. and supernatant was discarded.
- Pellet was washed with 250 µL of absolute ethanol by vortexing for 2 minutes and centrifuged at 13,000 rpm for one minute.
- Supernatant was discarded; finally tungsten particles coated with DNA were resuspended in 60 µL of absolute ethanol.

3.5.6 Sterilization of consumables

Macrocarriers, stopping screen, rupture discs (1100 psi) were soaked in 70% ethanol for 15 minutes and dried (inside LAF) on a sterile blotting paper.

3.6 Gene gun operation (Plate 1)

1. 10 µL of DNA coated micro carriers (tungsten particles) were pipetted on to center of each macro carrier and dried for 5 minutes.
2. Helium valve was opened and adjusted to 1100 psi.
3. PDS machine and vacuum pump were switched on.
4. Rupture disc was loaded onto microchamber.
5. Microcarriers were placed at the center of macrocarrier, installed into macrocarrier holder and loaded into launch assembly.
6. Four hours osmotic treated explants plates were positioned at T2-Level 3 (9 cm from the macrocarrier assembly) and chamber was closed.
7. VACUUM switch was turned onto VAC position.
8. After vacuum created in the chamber, the FIRE switch was held on until the pressure reached 1100 psi and rupture disc was ruptured.
9. Immediately FIRE switch was released and VACUUM switch was turned to VENT position.
10. Bombarded samples were removed out of the chamber, stopping screen, macrocarrier and rupture disc were discarded.

3.7 Selection and regeneration

1. The bombarded samples were incubated in dark for overnight at 26^0C.
2. Initially cotyledonary explants were placed on shoot induction medium of 2 mg/L kinetin and without any selection agent i.e. kanamycin.

3. Two to three weeks later they were transferred to multiple shoot induction medium containing 50 mg/L of kanamycin concentration and incubated in dark at 25^0C for 2-3 weeks.
4. Two weeks later resistant explants were sub-cultured on fresh multiple shoot induction medium containing kanamycin for selection.
5. Well grown shoots were transferred to MS medium containing 0.2 mg/L IBA for root initiation.
6. Rooted plants were hardened in green house by transferring to pots.

3.8 Confirmation of GUS gene by histochemical staining assay

The expression of the GUS gene was tested by histochemical staining of bombarded calli. The best substrate for histochemical localization of β-glucuronidase activity in callus is 5-Bromo- 4-chloro-3-indolyl glucuronide (X-gluc). The substrate gives a blue precipitate at the site of enzymatic activity. The product of glucuronidase action on X-gluc is not coloured. The indoxyl derivative produced must undergo an oxidative dimerization to form the insoluble and highly coloured indigo dye.

3.8.1. Staining solution

1. 1mg of X-gluc was dissolved in 100µl of methanol
2. 1.87ml of 50mM sodium phosphate buffer (pH 7) was added.
3. 10µl of 0.1M potassium ferrocyanide and 10µl of 0.01M potassium ferricyanide were also added.
4. Finally 10µl of 10% (w/v) Triton-X-100 was added and total 2ml staining solution was made.

The cotyledon explants bombarded with GUS gene and control one were immersed with Gus staining solution of 400µl separately in five 2 ml micro tubes and incubated overnight at 37^0C. Then next day the cotyledons bombarded with Gus gene and the control cotyledons were observed for blue color formation.

Plate 1: PDS He 1000 Biolistic Gene gun

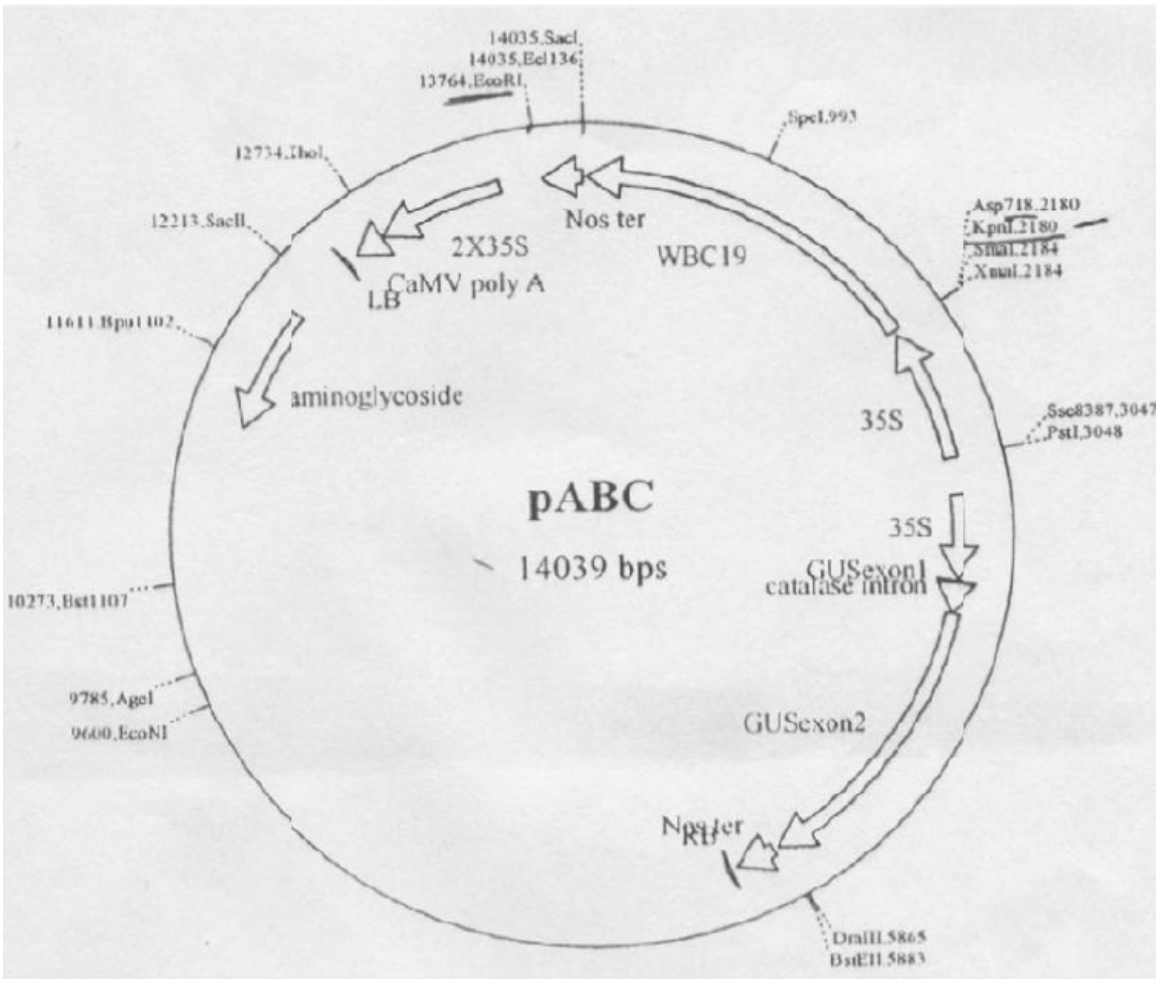


Plate 2. Gene map of plasmid pABC containing GUS gene

Experimental Results

IV. EXPERIMENTAL RESULTS

The aim of the present experiment was to standardize the concentration of the growth regulators for good regeneration of cucumber as an efficient regeneration protocol, which is an important prerequisite for efficient plant transformation. In this study, direct shoot regeneration of cucumber (*Cucumis sativus*) was standardized and it was transformed with the *gus* gene by particle bombardment method (gene gun). The results of the experiment for the present study are presented below.

4.1 Standardization of *in-vitro* regeneration of cucumber from cotyledon explants.

Seeds were sown in the MS (basal) medium. After 10 days, cotyledons were excised from the embryo and transferred onto MS basal containing Kinetin at various concentrations like 0 (T_1), 1.8 (T_2), 2.0 (T_3) and 2.5 mg/L (T_4) with five replications per treatment.

4.1.1 Regeneration on shooting medium.

4.1.1.1 Number of days taken for shoot initiation

Cotyledonary explants responded well for shoot initiation on kinetin. The number of days taken for shoot initiation was significantly less in T_1 (1.8 mg kinetin/L) (7.0 days) compared to T_3 (2.5 mg/L) in which it took 10.8 days for shoot initiation. T_3 was also significantly different from T_2 (7.6 days) (Table 3).

4.1.1.2 Number of shoots

The number of shoots per explant did not reveal significant difference among the treatments. Maximum average number of shoots per explant was observed in T_2 (2.6) i.e. where kinetin was used at 2.0 mg/L. Shoot regeneration was not recorded in T_0.

4.1.1.3 Number of days taken for root initiation

Roots emergence was observed when plants were placed in the shooting medium. Significant difference in average number of days taken for root initiation after the formation of shoots was not observed. The number however was the highest in T_2 (10.4) (2.0 mg/L kinetin) (Table 3).

4.1.1.4 Number of roots

The number of roots emerging from regenerated shoots also did differ significantly between the treatments. Average number of 2.0 roots per plant was observed in the treatment T_2 (2.0 mg/L kinetin) (Table 3, Plate 3).

4.1.2 Root induction on rooting medium

4.1.2 a. Number of roots

Nineteen day-old shoots that emerged from the cotyledonary explants which were approximately 8 cm in height were transferred to rooting medium containing IBA at the concentration of 2.0 mg/L to observe if rooting would be better. There was no significant difference in the number of roots between the treatments (Table 4 and Plate 4), however the numbers were more compared to the treatments in which IBA was not used (Table 3 and Plate 3). Maximum number of shoots were observed in T_2 (5.6) (2.0 mg/L kinetin + 2.0 mg/L IBA).

4.1.2 b. Length of shoots and roots of regenerated plants in rooting media

The average length of shoots recorded on the 44th day after the initiation of the experiment on regeneration in cucumber, did not vary significantly between the treatments (Table 5). However maximum length was found in T_2 (10.3 cm) (2.0 mg/L kinetin + 2.0 mg/L IBA). The average length of roots was significantly high in T_3 (13.14cm) (2.5 mg/L

kinetin + 2.0 mg/L IBA) (Plate 4). Root regeneration was not recorded in $T_{0.}$

4.1.3 Acclimatization and hardening

The rooted plants obtained from the above experiment were carefully removed from the culture bottles. The roots were washed under gentle flow of running water and dipped in 0.8 % bavistin solution and transferred into plastic cups containing vermiculite, perlite and peat on which half strength MS media was added to the potting mixture to avoid transplantation stress to the plants (Plate 5).

Flow chart of the regeneration protocol is shown in plate 7. Shoot initiation was observed by the seventh day, multiple shoot regeneration by fifteenth day and root regeneration nine to ten days later involving a total of twenty five days.

4.2 Transformation of cucumber with p^{ABC} plasmid construct through Biolistic method

Transformation of cucumber cotyledonary explants was done through the biolistic method by bombarding with p^{ABC} plasmid with GUS gene under CaMV35S promoter. The efficiency of transformation was 43.3 (13 out of 30 cotyledons) percent. The explants were then transferred to media containing 2.0 mg/L KI and 50 mg/L kanamycin.

4.2.1 GUS histochemical assay

The cotyledon explants samples bombarded with GUS gene and control were immersed with GUS staining solution of 400µl separately in five 2ml micro tubes and incubated overnight at 37^0C. The following day, cotyledons were sectioned and observed under the microscope. Transformed cotyledon explant sections showed blue color which was not observed in the control explant (Plate 6).

Table 3: Effect of various concentrations of Kinetin on multiple shoot induction from cotyledons of Green long cultivar of cucumber

Treatment	Average number of days taken for shoot initiation	Average number of shoots/ explant	Average number of days taken for root initiation	Average number of roots/ explant
$T_0(MS_0)$	-	0.00	-	0.00
T_1(MS+kinetin @1.8mg/L)	7.6	1.8	9.0	1.8
T_2(MS+kinetin @2.0mg/L)	7.0	2.6	10.4	2.0
T_3(MS+kinetin @2.5mg/L)	10.8	1.4	9.6	1.6
SEM ±	0.80	0.34	0.63	0.25
CD (1%)	3.45	1.49	2.73	1.11
CV (%)	22.17	40.06	14.04	32.07

Note: The number of shoots and roots per explant were recorded on 19th day.

Table 4: Effect of IBA on rooting of regenerated plants derived from cotyledons of Green long cultivar of cucumber

Treatment	Average number of enhanced roots/plant
T_0 (MS_0)	0.00
T_1 (1.8 mg/L kinetin + 2.0 mg/L IBA)	3.6
T_2 (2.0 mg/L kinetin+ 2.0 mg/L IBA)	5.6
T_3 (2.5 mg/L kinetin + 2.0 mg/L IBA)	3.8
SEM ±	0.84
CD (1%)	3.66
CV (%)	43.78

Table 5: Shoot and root lengths of plantlets of Green long cultivar of cucumber on rooting media

Treatment	Average length of the shoots (cm)	Average length of the roots (cm)
T_0 (MS_0)	0.00	0.00
T_1(1.8 mg/L kinetin + 2.0 mg/L IBA)	8.68	6.6
T_2(2.0 mg/L kinetin + 2.0 mg/L IBA)	10.3	9.0
T_3(2.5 mg/L kinetin + 2.0 mg/L IBA)	9.78	13.14
SEM ±	0.90	1.38
CD (1%)	3.89	5.97
CV (%)	21.03	32.29

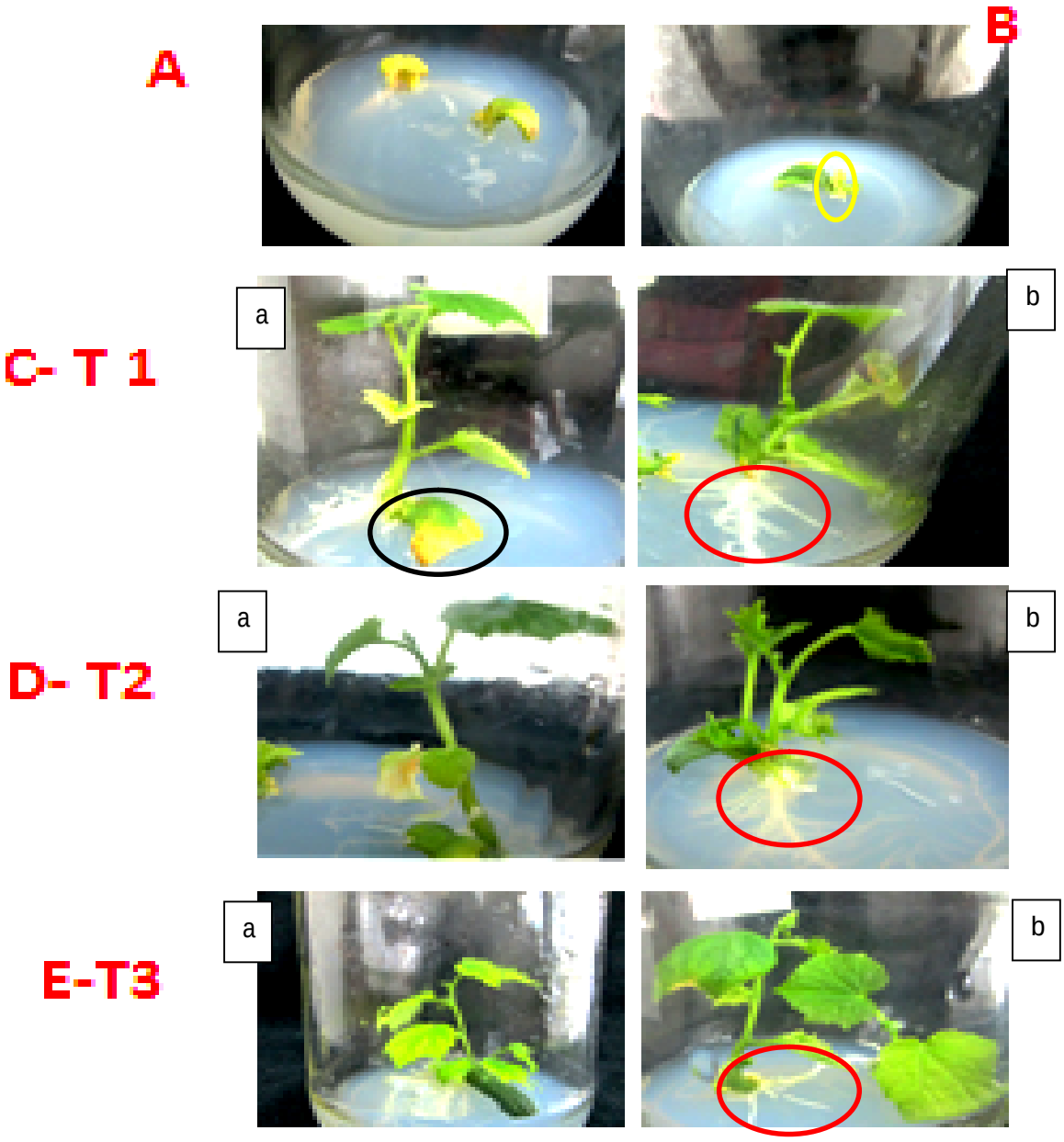


Plate 3: Different treatments of kinetin showing direct shoot regeneration from cotyledonary explants of cucumber

A- Explant cut into half (with the basal portion intact) placed on media.

B-Three days old explant showing bulging at the cut portion.

C-T_1: MS + 1.8 mg/L kinetin;

D-T_2: MS + 2.0 mg/L kinetin;

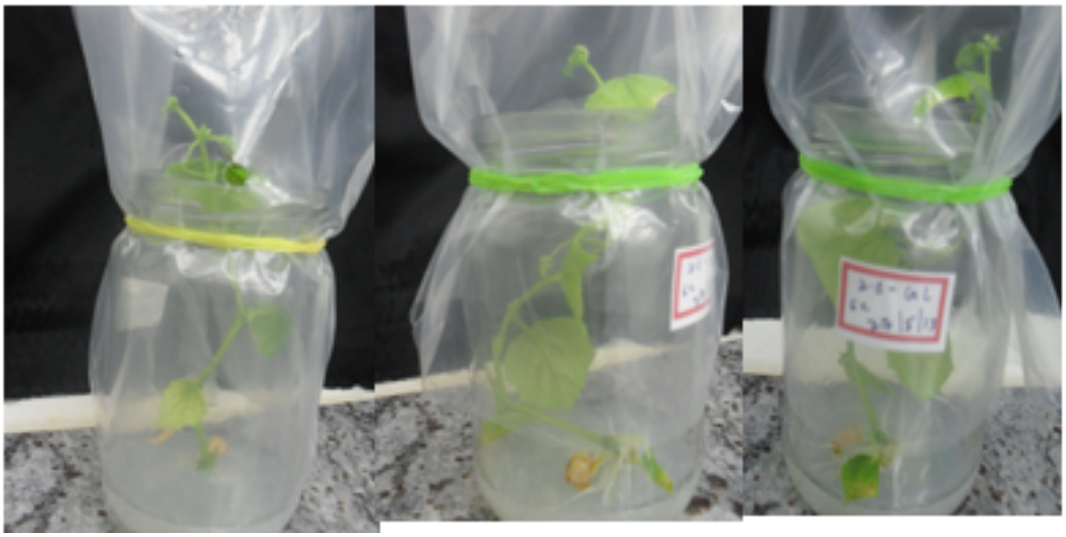
E-T_3: MS + 2.5 mg/L kinetin.

NOTE: In every treatment 'b' indicates multiple shoots with roots

PLATE 4: Induction of rooting on rooting media.

T_0 (MS_0); T_1 (MS + kinetin 1.8 mg/L+ IBA 2.0 mg/L); T_2 (MS + kinetin 2.0 mg/L+ IBA 2.0 mg/L); T_3 (MS + kinetin 2.5 mg/L+ IBA 2.0 mg/L).

A



B

PLATE 5: Plantlets in a containment facility for hardening

A: In culture room

B: In green house

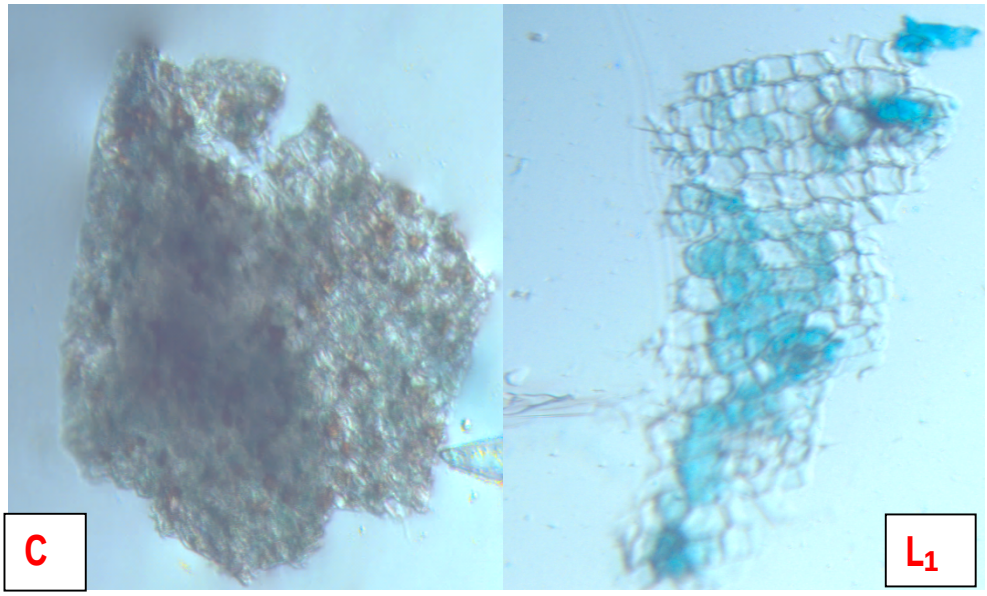


Plate 6: Histochemical assay for transgene expression (Gus) in putative transgenic lines of cucumber (*Cucumis sativus* L.)

C: Control (untransformed) – GUS negative

L_1: Transformant shows GUS positive

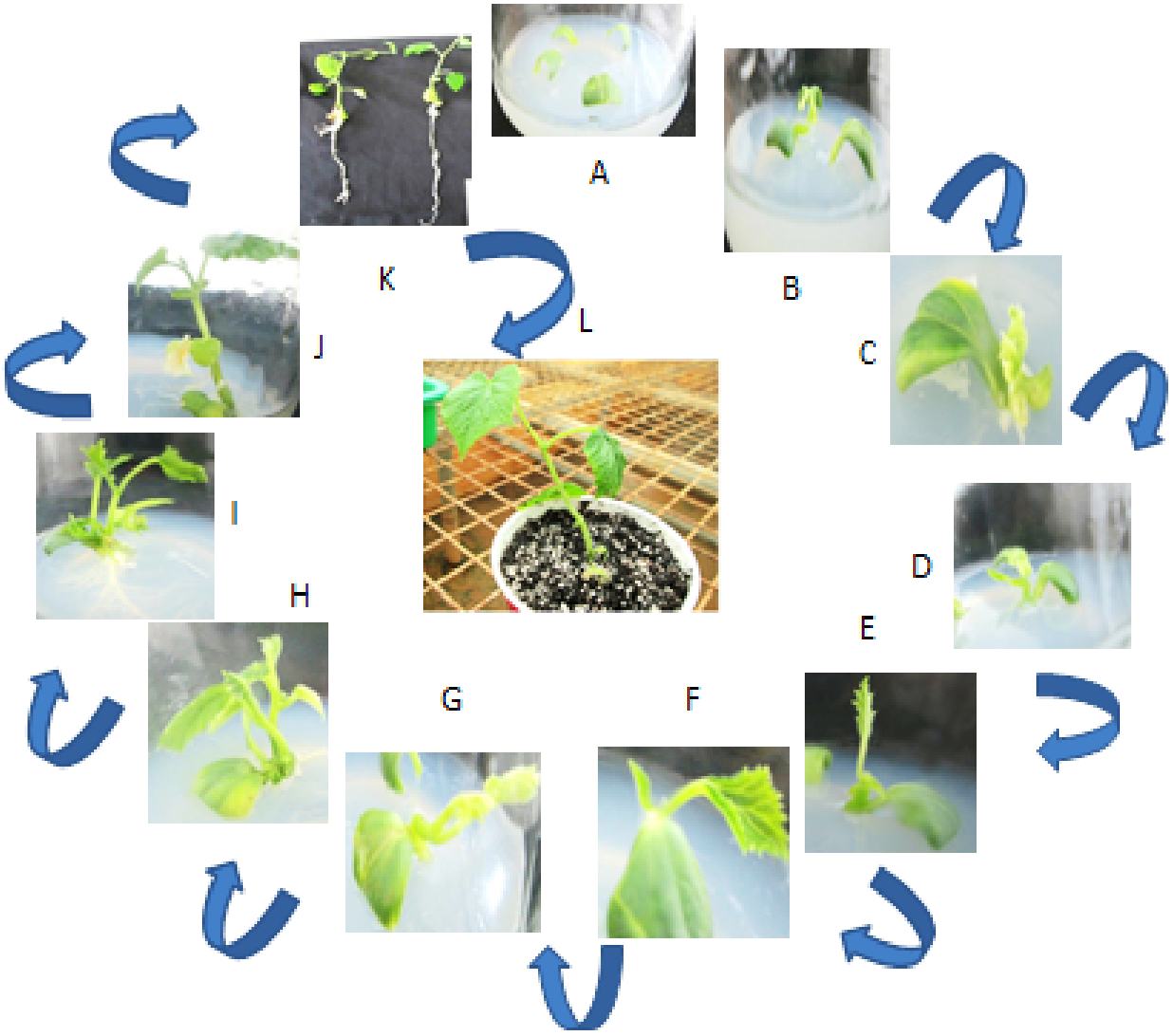


PLATE 7: Flow chart depicting standardized protocol for *in-vitro* regeneration of Green long cultivar of cucumber

LEGEND:

A = Ten day-old cotyledon placed in MS media with kinetin
B = Bulging at the cut portion after three days
C = Direct Regeneration at the cut portion
D = Leaf primordia from the basal portion of explant
E, F = Emergence of leaves
G, H = Induction of multiple shoots
I = Multiple shoots with roots
J = Increase in length of shoot
K = Enhanced rooting after placing in media with IBA
L = Regenerated plants transferred to potting mixture

Discussion

V. DISCUSSION

Cucumber is a popular vegetable crop, rich in minerals like phosphorous, potassium, iron and vitamins like ascorbic acid. It has number of medicinal properties and is beneficial for patients suffering from arthritis and blood pressure. It suffers from number of diseases and pests which reduce the crop yields to a great extent. Transformation techniques either *Agrobacterium* mediated or biolistic method provides the potential to produce viral resistant transgenic plants. Regeneration protocol is a must for developing transgenic plants. Direct shoot regeneration has been proven to be the best among the various regeneration protocols, since it takes less time and helps one to overcome the problem of somaclonal variation which is a serious limitation of callus mediated regeneration.

5.1 Standardization of *in-vitro* regeneration of cucumber from cotyledon explants

Micropropagation and shoot regeneration protocols for cucumber are required to decrease the cost of hybrid seed production which is usually higher than 30% of total seedling cost (Konstas and Kintzios, 2003). Regeneration of cucumber plants has been reported with limited success either directly or indirectly on various explants (Ziv, 1992). These include anthers (Lazarte and Sasser, 1982), cotyledons (Trulson and Shahin, 1986), leaves (Malepszy and Nadolska-Ocyzyk, 1983), protoplasts (Jia *et al*., 1986), axillary bud explants (Handley and Chambliss, 1979), shoot tip culture (Vasudevan *et al*., 2004; 2008), nodal segments (Ahmad and Anis, 2005) and embryonal axis (Vasudevan *et al*., 2007).

The present investigation on cucumber (*Cucumis sativus* L.) cv. Green long was taken up to induce shoots from cotyledonary explants

using different concentrations of kinetin (1.8, 2.0 and 2.5 mg/L) followed by treatments with IBA for rooting. Significantly less number of days for shoot initiation was found in T_2 (2.0, mg/L kinetin) and the average length of roots was significantly high (13.14 cm) in a combination of kinetin 2.5 mg/L and IBA at 2.0 mg/L. Other parameters like average number of shoots per plant, average number of days taken for root initiation, average number of roots per plant in the regenerated plants on shooting media and the number of roots per plant and average shoot and root length of regenerated plants in media containing kinetin and IBA did not reveal any significant difference.

Rauf *et al*. (2004) reported that cotyledonary nodes produced maximum number of shoots (3.43 shoots/explant) when cultured on MS medium supplemented with 0.25 mg/L kinetin in cucumber. Highest percentage (93.3 %) of root development and root length (5.85 cm) was obtained when shoots were cultured on MS medium supplemented with 0.5 mg/L napthalene acetic acid (NAA) and 0.1 mg/L kinetin.

Ugandhar *et al*. (2011) found that when the seedling excised explants (cotyledon and hypocotyls) were placed on MS medium containing 3% w/v sucrose with various concentrations of cytokinin BAP(1.0-5.0 mg/L), kinetin (1.0-5.0 mg/L) alone and also in combination with auxin IAA (0.5mg/L) + BAP (1.0-5.0 mg/L), and IAA (0.5mg/L) + kinetin (1.0-5.0 mg/L). The shoot induction was enhanced in all the concentrations of cytokinin tested.

In the present study a single cytokinin (Kinetin) was used at different concentrations and found that kinetin was responsible for establishment of both roots and shoots.

Hesar *et al*. (2011) presented a simple and reliable strategy for micropropagation of *Matthiola incana,* an ornamental plant, in the

presence of the single growth regulator, kinetin, which enables the production of stock plants. *In-vitro* single nodes of *Matthiola incana* were cultured in MS basal medium with different concentrations of kinetin to produce shoots and roots. Multiple shoots containing roots simultaneously obtained on MS medium only with supplementation of 0.5-2 mg/L kinetin. The best shoot length (11.72 mm) and the most number of nodes (4.64) were obtained when 2 mg/L of kinetin was used. The largest number (3.40) and the longest length of roots (54.0 mm) were achieved using 1 mg/L kinetin. Data analysis showed that the effect of kinetin was significant on the length of shoot and root, and the number of node and root.

Saha *et al*. (2007) reported that in direct shoot regeneration of bottle gourd (*Lagenaria siceraria*), use of BA or kinetin separately or in synergistic combination led to a characteristic brownish color of the explants at the bud formation stage due to ethylene production. In the cotyledon explants cultured on MS media supplemented with kinetin, the production of ethylene was lower compared to using BA or the combination of BA and kinetin. Shoot elongation was more pronounced on MS medium containing kinetin compared with BA or a synergistic combination of kinetin and BA. The longest shoot (3.70 cm) was observed on MS media supplemented with 1 mg/L of kinetin.

Shooted plants of cucumber were transplanted to rooting media containing IBA @ 2 mg/L which produced good number of roots in the present investigation. Sarma *et al.* (2011) tried i*n-vitro* rooting of the microshoots of *C. reticulate* L. Blanco on MS medium incorporated with various concentrations and combinations of BAP, NAA and IBA. IBA was found to be superior to other auxins for *in-vitro* root induction. Eighty eight percent of the regenerated shoots developed roots when transferred to MS medium with 2 mg/L IBA.

5.2 Transformation of cucumber cotyledons

The present experiment was done to transform the cotyledonary tissue of cucumber cv. Green long with GUS gene and the transformed cotyledons were subjected to GUS assay. The blue color in transformed cotyledons was observed in less than 24 hr.

One distinct advantage of bombardment-mediated transformation is the ability to deliver DNA into intact cells and tissues. The transient expression of the introduced genes can be assayed 24-48 h after bombardment, thus providing a quick means to determine the activity of gene constructs in the recipient cells (Bruce *et al*., 1989 and Norris *et al*., 1993).

Gray *et al*. (1994) reported transient expression of pBI 221 (GUS) in *Cucumis melo* L. cotyledonary basal quarters of explants.

Schulze *et al*. (1995) reported that the generation of transgenic cucumber (*Cucumis sativus* L.) plants was achieved by biolistic transformation of a highly embryogenic cell suspension culture using the neomycin phosphotransferase (*npt II*) and *uidA* gene. Functional expression of the genes in transgenic plants was determined by neomycin phosphotransferase and ß-glucuronidase enzyme assays. Southern analysis of DNA isolated from kanamycin-resistant plants confirmed stable integration of the genes as well as multicopy integration and rearrangements. A study of gene expression showed activity of the *uidA* gene in plants regenerated from kanamycin-resistant calli about one year after bombardment, indicating a high stability of the nonselectable gene.

The generation of transgenic *Cucumis sativus* cv. Green Long plants resistant to phosphinothricin (PPT) was obtained using *Agrobacterium tumefaciens* mediated gene transfer. Cotyledons were

inoculated with the strain EHA105 harboring the neomycin phosphotransferase II (*npt II*), and phosphinothricin resistance (*bar*) genes conferring resistance to kanamycin and PPT. Putative transformants were confirmed for transgene insertion through PCR and Southern analysis (Vengadesan *et al*., 2005).

Gonslaves *et al*. (1994) has done a single regeneration procedure using cotyledonary explants of five commercially grown muskmelon cultivars. This regeneration scheme was used to facilitate gene transfer of neomycin phosphotransferase II *(npt II*), ß-glucuronidase (GUS),and the CMV–WL coat protein (CP) from the T-DNA region of the binary vector plasmid pGA482GG/cp cucumber mosaic virus–white leaf strain (CMV–WL)using either *Agrobacterium tumefaciens* (using 'Burpee Hybrid' and 'Hales Best Jumbo') or microprojectile bombardment (using 'Topmark') methods. Their comparisons of *A. tumefaciens*- and microprojectile -mediated gene transfer procedures showed that both methods effectively produce nearly the same percentage of transgenic plants. R_0 plants were first tested for GUS or *npt II* expression, then the polymerase chain reaction (PCR) and other tests were used to verify the transfer of the *npt II*, GUS, and CMV–WL CP genes.

Tola (2011) transformed cucumber callus with gus gene by the particle bombardment method and confirmed the presence of the gene in the callus using the histochemical assay.

Summary

VI. SUMMARY

Cucumber (*Cucumis sativus* L.) is a widely cultivated plant in the gourd family Cucurbitaceae. It is mainly a salad crop which has several medicinal properties too. The crop is prone to different diseases like cucumber mosaic virus, watermelon mosaic (papaya ring spot virus) and cucumber green mottle mosaic virus and pests like caterpillars, fruit borers cause significant crop loss in cucumber and seriously limit the crop production. Conventional plant breeding technique is used generally to obtain a high yielding new plant variety, which is resistant to some diseases and pests but the introduction of desirable genes by this technique requires much time and is often accompanied by changes in other desirable traits and has other problems such as sterility barriers between species.

Transformation techniques provide the potential to introduce disease and pest resistance into cucumber and improve crop yield and quality.

The present work was done to develop a suitable protocol for *in - vitro* regeneration and transformation of cucumber. The highlights of the investigation have been summarized below.

The induction of direct shoot regeneration was done using cotyledonary explants, which were cultured on MS medium supplemented with different concentrations of kinetin i.e. 1.8 mg/L, 2.0 mg/L, and 2.5 mg/L. The highest number of multiple shoots were obtained with KI 2.0 mg/L. The average number of shoots recorded per explant was 2.6. Rooting of the regenerants was obtained using IBA 2.0 mg/L.

In the present work, only a single growth regulator used namely kinetin (cytokinin) has induced both shoots and roots in the cultivar Green long.

Gene gun method was used for transformation of *GUS* gene. The molecular characterization was carried out using GUS histochemical assay.

Future line of work:

1. In this study for the first time robust *in-vitro* regeneration protocol has been developed and standardized in cucumber variety Green long. Field performance of these tissue culture plants are to be evaluated.
2. Although transformation protocol has been developed in the study, there is a need to standardize generation of large number of transgenic lines.

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