Chapter 3

Establishment of Regeneration and Transformation System in Sweet Potato

3.1 Introduction

Sweet potato (*Ipomoea batata* L.) is the world's seventh most important food crop and it is cultivated in more than hundred countries worldwide covering tropical, subtropical and temperate zones, as an inevitable source of food. It is a high energy food crop propagated in the tropical and subtropical regions [2]. Regardless of the profound breeding emphasis on enhancing productivity and imparting resistance to diseases and insect pests, advancement through conventional breeding is impeded due to the major constrains like incompatibility and sterility associated with hexaploid nature of this crop [323]. The capacity to regenerate an entire plant from explants which have been genetically transformed predicates most plant transformation systems. Therefore, plant regeneration via tissue culture approach has been the fundamental part of transformation strategies, and can often prove to be the most challenging and critical aspects of a sweet potato transformation approach. A reliable, efficient, robust and reproducible method is the key for efficient production of plants in tissue culture. An efficient plant regeneration and transformation system is very important and highly desirable for the successful application of genetic engineering to sweet potato improvement. Sweet potato is found to be relatively easy to micro-propagate; however, it is quite recalcitrant to transform [155]. In addition, the regeneration frequency in sweet potato is observed to be highly genotype-dependent [171]. In most of the cases, regeneration at a high frequency has been restricted to one or a few genotypes of sweet potato. Therefore, there is a great amount of justification in exploring the possibility towards developing an efficient system of plant regeneration for a wide range of sweet potato genotypes. In present study, as the preliminary step is to obtain transgenic plants, an approach was considered to establish a simple, robust and efficient plant regeneration system from the culture of internodal segments. The seed storage protein AmA1 was selected as a promising candidate for nutritional improvement of sweet potato by genetic engineering. This improvement strategy may prove to be more acceptable to the general public than currently used genetically modified crops since AmA1 is an edible crop derived sequence. The preliminary aim of this study was to develop an efficient protocol for genotype independent regeneration and transformation system for sweet potato.

3.2 Materials and methods

3.2.1 Explant source

Six genotypes of sweet potato (cv. SP-1, SP-6, SP-9, SP-11, SP-12 and SP-17) obtained from the Central Tuber Crop Research Institute (CTCRI), India, were utilized for obtaining the explants for the experiment. The micropropagation medium (MM) *i.e.* MS medium supplemented with IAA was used for rooting and micropropagation of sweet potato plants for their maintenance with pH adjusted between 5.6 -5.8.

3.2.2 Bacterial strains and constructs

The prime objective was to introduce *AmA1* cDNA into sweet potato plants in such a way so that expression would be effected in both constitutive and tuber-specific manner. For tuber-specific expression, *CaMV-35S* promoter in pSB8 (AmA1 plasmid; **Chakraborty et al., 2000** [112]) was replaced by *β-amylase* promoter of sweet potato and the construct thus resulted has been named as pSB8ß. A reporter gene construct pBI121 containing *uidA* and *nptII* genes, was also been used in this study (**Fig. 3.1**). *Agrobacterium tumefaciens* strain EHA105 and helper plasmid HB101:: pRK 2013 were used in order to mobilize the constructs.

3.2.3 Media and solutions

YEP (yeast extract and peptone), Linsmaier and Skoog (LS), Murashige and Skoog (MS) and Gamborg's B-5 Basal Medium was used in this study. Chemicals used were of analytical or molecular biology grades, mostly from Sigma, unless mentioned otherwise and the solutions and media were made in Milli-Q or Milli-RO water. All the solutions were sterilized, either by autoclaving at 15 pounds/sq inch at 121°C or by filter sterilization (0.22 μm) whichever was applicable. A list of media and solutions is provided in Appendix.

3.2.4 Mobilization of Expression Plasmid in *Agrobacterium*

Successes in transformation of plants widely vary depending on the cultivar, *Agrobacterium* strain and antibiotic marker. The *Agrobacterium* EHA105 strain was used to transform sweet potato. The strain contains L, L-succinamopine Ti plasmid which makes it hyper virulent. Constructs were mobilized into the strain by triparental mating.

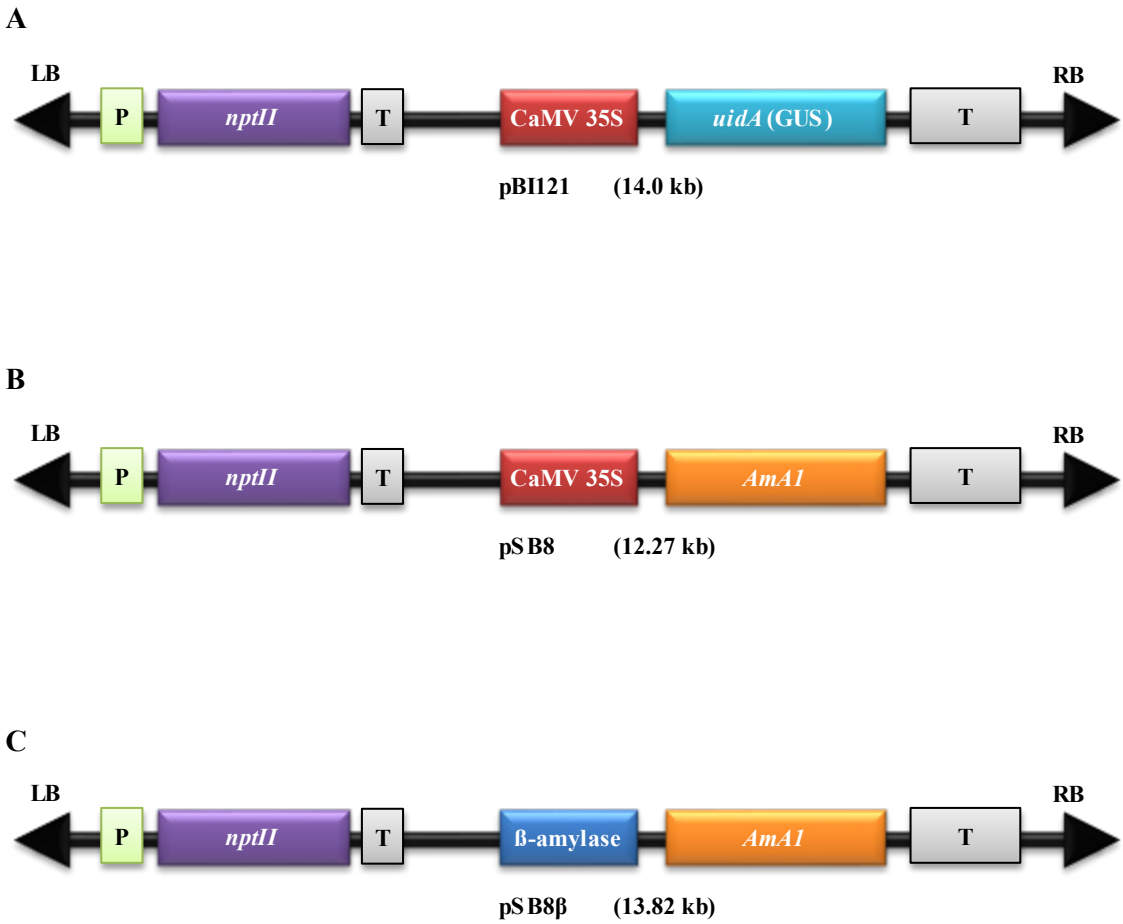

Fig. 3.1. Schematic representation of the plant expression vectors. pBI121 containing *nptII* and *uidA*, (encoding neomycin phosphotransferase II and *GUS*, respectively) (**A**) pSB8 containing AmA1 under the control of *CaMV 35S* promoter (**B**) and (**C**) pSB8β containing AmA1 under the control of *β-Amylase* promoter. LB, T-DNA left border; P, NOS promoter; T, NOS terminator; RB, T-DNA right border.

3.2.4.1 Triparental mating

E. coli DH5α containing the constructs and *E.coli* HB101 harboring the helper plasmid pRK2013 were streaked on LB kanamycin plate and incubated overnight (O/N) at 37°C, and *A. tumefaciens* strain EHA105 was streaked on YEP plate supplemented with rifampicin (50 mg/ml), incubated at 28°C for 48 h. Thereafter, single colonies of *E. coli* and *A. tumefaciens* were inoculated independently in 2 ml of LB and YEP broth supplemented with appropriate antibiotics. The cultures were grown O/N at their respective optimum temperature. 100 µl of each culture was mixed in tube and incubated for 5 min. The mix was spotted onto YEP agar plate and incubated at 28°C for 48 h. The spotted cell were then streaked on to selection YEP agar plate containing 50 µg/ml rifampicin and 50 µg/ml kanamycin for double selection, and incubated at 28°C for 48 h. The presence of intact plasmids in *A. tumefaciens* EHA105 was confirmed by PCR as well as by colony hybridization.

3.2.4.2 PCR Confirmation of recombinant clone

Mobilization was confirmed by the amplification using gene specific primers of *nptII* and *AmA1* as described in the next Chapter and a list of primers used in this study is provided in Appendix.

3.2.4.3 Colony hybridization

For colony hybridization, colonies were transferred to nylon membrane. The membrane was placed facing the colony side of the agar plate for 1-2 min. With a sterile needle, asymmetric orientation marks were made by piercing it into the membrane and agar**.** The needle holes were marked to on the membrane as well as on the plates. Membranes were then treated with denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 2 min at room temperature (RT) with colony side up such that the solution did not run over the colony side. Thereafter, the membranes were lifted from the solution and excess solution was allowed to drain on Whatman #3. The membrane was then transferred to the neutralization solution (1.5 M NaCl and 0.5 Tris-HCl, pH 8.0) for 5 min at RT in the same way as earlier. The membrane was washed with washing buffer (2X SSC and 0.2 M Tris-HCl, pH 7.5) for 5 min at RT. The membrane discs were blotted on filter paper and allowed to air dry, and the DNA on the membrane was cross linked using UV cross-linker (Strata-linker) for 30 sec at 1200 joules/cm.

3.2.4.4 Probe preparation and nucleic acid hybridization

Around 25 ng amplified fragments of *nptII* and *AmA1* were used for preparing probes using random primer labeling by α^{32} CTP. The DNA was denatured at 95°C for 5 min and then chilled on ice for 1 min. The reaction mix contained DNA, labelling buffer (1X), dNTPs (-dCTP) (25 µM each), α^{32} CTP (10 µCi) and Klenow DNA polymerase enzyme (5U). Denatured DNA was added to the reaction mix and incubated at 37°C for 1 h. The probe was purified on Sephadex G-50 column equilibrated in TE (pH 8.0) [324]. Sheared salmon sperm DNA (200 µg/ml) was added to the purified probe and the mixture was denatured by incubation at 100°C for 5 min followed by chilling on ice for another 5 min. This was added to the pre-hybridization solution (50% deionized formamide, 10% dextran sulphate, 1% SDS and 1X SSC). Hybridization was carried out for 16-18 h at 42°C in hybridization oven with 0.035 ml of pre-hybridization solution /cm^2 of the membrane, in a hybridization bottle.

3.2.4.5 Post hybridization washing and autoradiography

The blot was washed with 1X SSC and 0.1% SDS for 15 min at RT. High stringent washing was given twice using 0.5X SSC and 0.5% SDS at 42°C for 5 min each. After washing, the membranes were briefly blotted on dry filter paper and was exposed to Kodak X-ray films (GE Biosciences) using intensifying screens at -80°C and autoradiographed subsequently after 24h.

3.2.4.6 Retrieval of positive clones and its maintenance

The asymmetric marks on the filter were aligned with the film orientation marks and were matched with the respective colonies, and the positive colonies representing the mobilized plasmids were picked up and streaked on the selection YEP agar plate supplemented with 50 µg/ml rifampicin and 50 µg/ml kanamycin. For long term storage, overnight grown bacterial cultures were stored in glycerol. Bacterial cultures were grown overnight and sterile glycerol was added to a final concentration of 15% and stored at -80ºC.

3.2.5 Genetic transformation of sweet potato

3.2.5.1 Maintenance of different sweet potato cultivars and preparation of explants

Sweet potato cultivars were micropropogated in micropropagation medium [(MS supplemented with IAA) (**Table 3.1**)] and maintained at 22 ±2ºC with 270 µmol/m^2/s light intensity under 16 hrs photoperiod. Different explants like leaf, petiole, roots and internodes were used from four-week-old micropropogated plants to establish a robust regeneration method in sweet potato.

3.2.5.2 Preparation of *Agrobacterium* culture for transformation

Agrobacterium tumefaciens strain EHA105 harboring binary plasmid pBI121, pSB8 and pSB8ß was used for genetic transformation of different cultivars and explants of sweet potato. The *Agrobacterium* strain containing the gene of interest was first grown in solid YEP plates containing kanamycin (50 mg/l) and rifampicin (50 mg/l). The suspension for co-cultivation was prepared by picking a single *Agrobacterium* colony and inoculated in liquid medium (YEP) containing the same antibiotics for primary inoculums and allowed to grow for 28°C for 16-18 h. Further, one fiftieth volume of the primary culture was inoculated and allowed to grow at 28°C till the optical density (OD600) reached 0.6-0.8. The *Agrobacterium* culture was pelleted at 5000 g for 5 min at ambient temperature (~22°C) by centrifugation and resuspended in same volume of MS liquid medium.

3.2.5.3 Co-cultivation

Explants were incubated for 45 min in a saturated culture of *Agrobacterium tumefaciens* with occasional swirling. The explants were retrieved from the culture and blotted on sterile Whatman filter paper to drain away extra inocula. The explants were then transferred to callus induction medium (CIM) [(Gamborg's B-5 basal medium supplemented with NAA (0.4 mg/l) (**Table 3.1**)] with 0.8% agar for callusing and incubated for co-cultivation for 48 hrs.

3.2.5.4 Selection and regeneration

Explants were transferred into the shoot induction medium (SIM) (**Table 3.1**) [Gamborg's B-5 basal medium supplemented with varying concentration of NAA (0.1-0.4 mg/l)]

with 0.8% agar for shooting and regeneration. The concentration of NAA was gradually reduced in subsequent subculturing after the initiation of callusing and rooting. Bacteriostatic agents such as cefotaxime (250 mg/l) and kanamycin (100 mg/l) for selection were added to the media for selection. All the cultures were maintained under a 16 h photoperiod and the temperature was maintained between 22-24°C. Regular subculturing was done after every 7-10 days. After regeneration, the putative transformants were transferred to micropropagation media *i.e.* MS containing IAA (0.1 mg/l) and the required antibiotics.

3.3 Results

3.3.1 Mobilization of the Recombinant Plasmids

The plant transformation binary vectors carrying the gene of interest are mobilized into *Agrobacterium* either by conjugation or electroporation because *Agrobacteria* are recalcitrant to standard $CaCl_2$ mediated direct DNA transformation. The conjugation required to transfer the plasmid of interest from *E. coli* to *Agrobacterium* is most commonly carried out by triparental mating. This interspecies transfer of plasmid is efficiently promoted by broad host range plasmid that encodes transfer functions able to act in *trans* to mobilize other plasmid present within the same bacterium. Most of the binary system cloning vectors have wide host range RK2 replicon and are usually conjugated using helper functions supplied by pRK2013 [325]. Mobilization is achieved by either two biparental mating or more commonly a single triparental mating [326].

The expression plasmids pBI 121, pSB8 and pSB8ß were mobilized in *Agrobacterium* strain EHA105 by triparental mating. The mobilization of recombinant plasmids were confirmed by PCR using the gene specific primers of *nptII* and *AmA1* as well as by colony hybridization using *nptII* and *AmA1* amplified fragments as probe (**Fig. 3.2 A-C**).

3.3.2 Establishment of regeneration system via the expression of *GUS*

In the present investigation, *in vitro* regeneration experiments were carried out using leaf, petiole, roots and internodal explants. Different genotypes of sweet potato were also tested for their ability for regeneration and genetic transformation. To introduce the expression plasmid into the sweet potato genotypes, diverse media and hormone combinations were examined but different growth media had different effects on the development of the explants. Also the

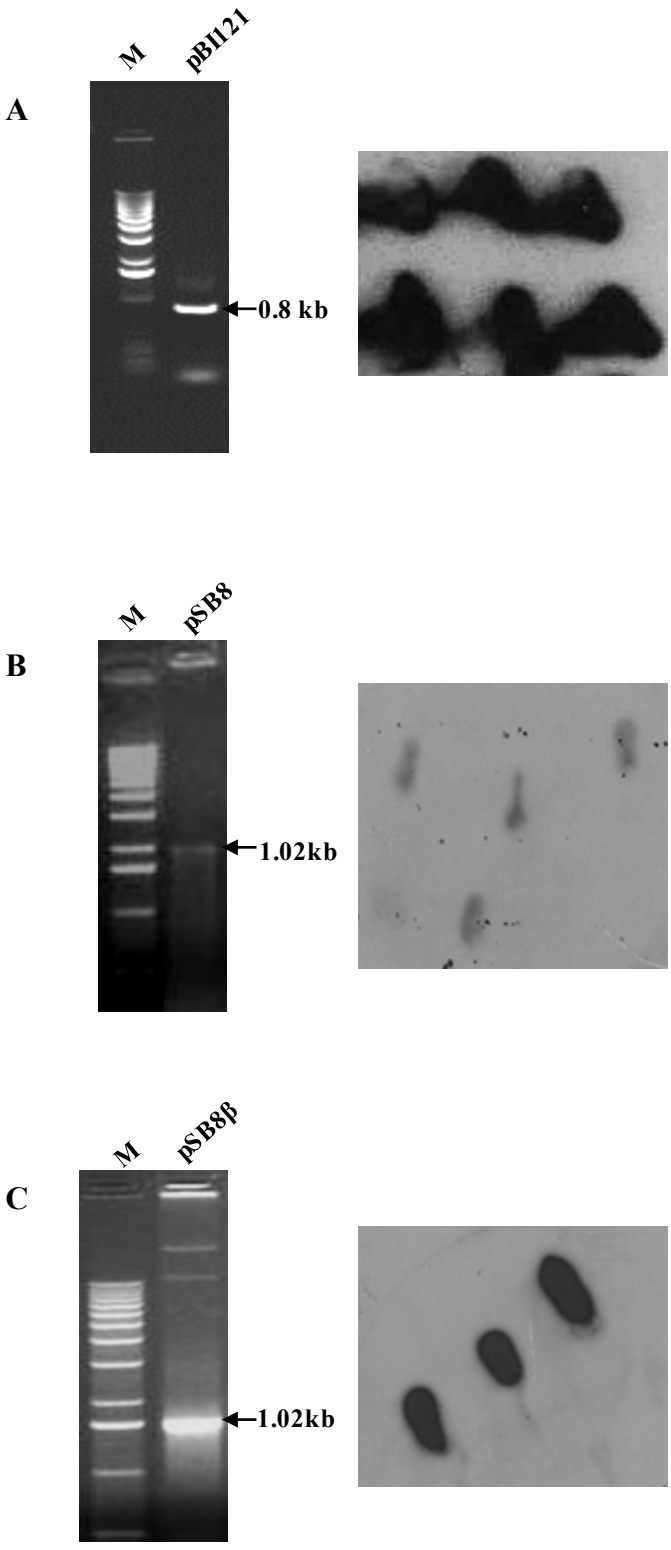

Fig. 3.2. Mobilization of recombinant plasmids in *Agrobacterium*. The expression plasmids pBI 121, pSB8 and pSB8β were mobilized in *Agrobacterium* and the mobilization of recombinant plasmids were confirmed by PCR (left panel) as well as by colony hybridization (right panel). Confirmation of mobilized pBI 121 (**A**) pSB8 (**B**) and pSB8β expression plasmids (**C**).

Table 3.1: Combinations of various media used for the induction of callus, roots and shoots from the internodal explants

Series	Media	Media composition
1	CIM	Gamborg's B5 3.2 g/l, sucrose 30 g/l, Agar 0.8%, NAA 0.4 mg/l, pH 5.6- 5.8
2	SIM	Gamborg's B5 3.2 g/l, sucrose 30 g/l, Agar 0.8%, NAA 0.2 mg/l, pH 5.6- 5.8
3	MM	MS 4.4 g/l, sucrose 30 g/l, Agar 0.8%, IAA 0.1 mg/l, cefotaxime 250 mg/L, kanamycin 100 mg/l, pH 5.6- 5.8

genotype of the transformed explant was a deciding factor in its growth and development. Some hormones and media combinations showed only callus formation, while some showed rooting and shooting too. During the first week of inoculation, little morphological differentiation was observed. The cut ends began to swell and slight increase in tissue volume was observed. Callus formation took place from both sides of the explants which began to increase in size in the following weeks. Root formation was evident from the third week depending on the plant genotypes and the concentrations of the hormone(s) used. Use of MS media with 2,4-D and TDZ yielded significantly good percentage of calli, but showed no shoot or root development. Similar results were observed when cytokinin (BAP and/or Zeatin) was used alone or in combination with auxin and gibberellin (NAA and GA_3) (**Table 3.2-3.5**). This result further corroborated with earlier result observed by **Alam et al. (2010)** in which they observed that the use of TDZ or BAP produced callus instead of shoot development [327]. In case of leaf and petiole explants, a significant amount of callusing up to 94% was observed in all the cultivars used. However, rooting was observed only with the use of NAA alone irrespective of the media used (**Fig. 3.3, Table 3.2 and Fig. 3.4, Table 3.3**). Nonetheless, in spite of reasonable callusing no rooting was observed even the solitary use of NAA in case of root explants (**Fig. 3.5 and Table 3.4**). Contrastingly, internodal explants were proved to be one of the most *bona fide* sources for transformation as evident from its higher percentage of rooting in all the cultivars tested (**Fig. 3.6 and Table 3.5**). Considering all the combinations of media and hormones used, NAA was proved to be the most effective phytohormone suggesting that media containing NAA alone might be capable for developing a transformation system in sweet potato. However, when different concentrations of NAA were used, even though a fair amount of callusing was observed in the explants in four weeks duration, the percentage of root development remained low in all of the genotypes. To counter this, cv. SP-9, SP-11 and SP-12 including SP-17 were tested for transformation using the internodal explants with Gamborg's B-5 basal medium supplemented with NAA (0.2 mg/l). Interestingly, we obtained a reasonably higher percentage of callusing as well as rooting in all the cultivars except cv. SP-12 in Gamborg's B-5 supplemented with NAA (**Table 3.6**). Albeit in cv. SP-12, callusing was considerably higher but no rooting was observed. However, out of the six genotypes tested for their capability for genetic transformation cv. SP-6 and SP-17 performed better. Thereafter, only internodal explants of cv. SP-6 and SP-17 were used for further transformation and a different regeneration procedure was then used with single

hormone utilization. The internodal explants were first kept on the callus inducing media (CIM) [3.2 g/l Gamborg's B-5 with minimal organics, 30 g/l sucrose, 0.8% agar, 0.4 mg/l NAA, adjusted to pH 5.6-5.8] with increasing concentrations of NAA. Within 3 weeks, callusing was observed from both the corners of the internodal explants followed by initiation of the roots. Once the complete rooting took place, the infected explants were transferred to low auxin shoot induction media (SIM) [3.2 g/l Gamborg's B-5 with minimal organics, 30 g/l sucrose, 0.8% agar, NAA (0.1-0.4 mg/l), adjusted to pH 5.6-5.8]. After 7-8 weeks or even sometimes after 10 weeks, 1-2 microshoots were produced from the same callus in SIM. (**Fig. 3.7 and Table 3.7**). The regeneration efficiency was calculated as the number of regenerations obtained over the total number of explants responding to rooting and callusing. The overall regeneration efficiency ranged between 40-70% which appears to be the best at least for any endogenous sweet potato genotype. Interestingly overall performance of cv. SP-6 was found to be better when compared with SP-17, suggesting its better adaptability and efficacy since regeneration efficiency of cv. SP-6 was still better than that of SP-17. Therefore, for further transformation of *AmA1* this method was extended in cv. SP-6.

3.3.3 Transformation of *AmA1* in cv. SP-6

To introduce the pSB8 and pSB8ß constructs into sweet potato genotypes, a simple *Agrobacterium* mediated genotype independent regeneration and transformation protocol was developed by the transformation of *GUS* as a reporter gene. The cv. SP-1, SP-6, SP-9, SP-11, SP-12 and SP-17 were tested with different media and hormones, but out of 6 genotypes tested, SP-6 and SP-17 showed better results in regeneration as well as transformation with Gamborg's B-5 basal medium supplemented with phytohormone NAA. For the transformation of *AmA1* constructs, we selected cv. SP-6 due to its better efficacy for genetic transformations. 18 putative transformants were obtained in case of pSB8 construct whereas 15 putative transformants were obtained in case of pSB8ß construct (**Fig. 3.8 and Table 3.8**).

3.3.4 Selection of putative transformants

For the selection of the putative transformants, the suitable concentration of kanamycin was standardized to circumvent the untransformed escapes. Bacteriostatic agents such as cefotaxime (250 mg/l) and kanamycin (100 mg/l) were added to the media (SIM) for selection.

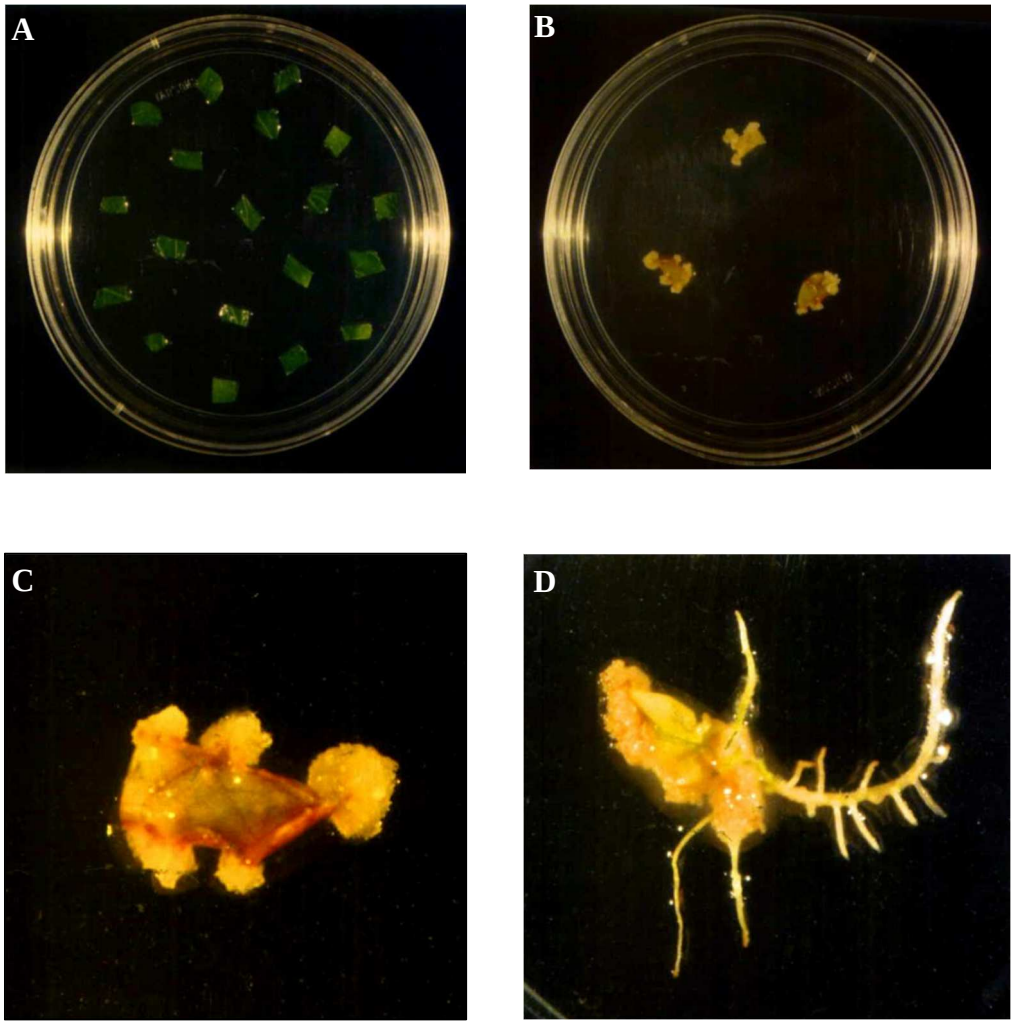

Fig. 3.3. Differential response of leaf explants in culture condition. Transformed leaf discs (**A**), Initiation of callusing (**B**), Callusing from leaf explant (**C**) and Leaf explant showing development of roots (**D**).

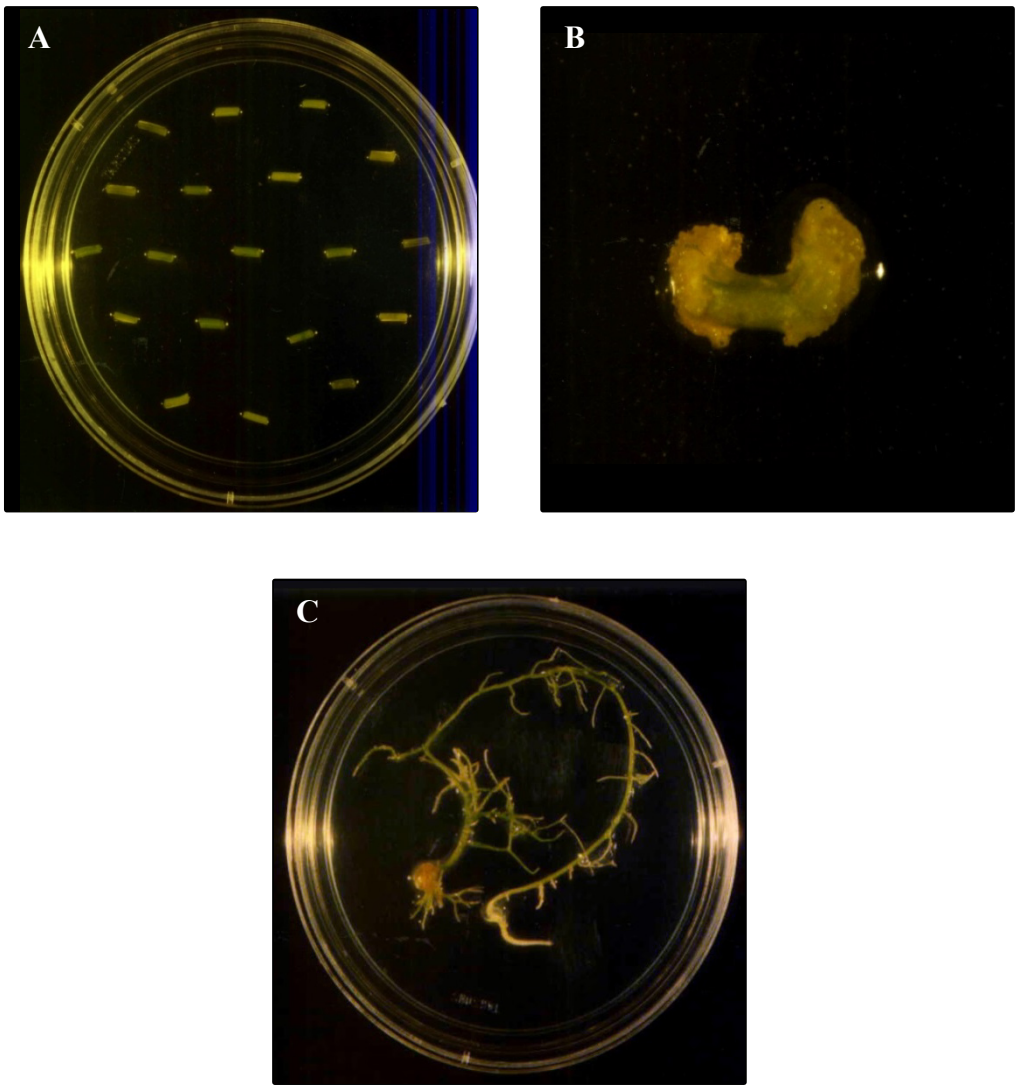

Fig. 3.4. Differential response of petiole explants in culture condition (**A**) Transformed petiole explants (**B**) Callusing from petiole explant and (**C**) Petiole explant showing vigorous development of roots

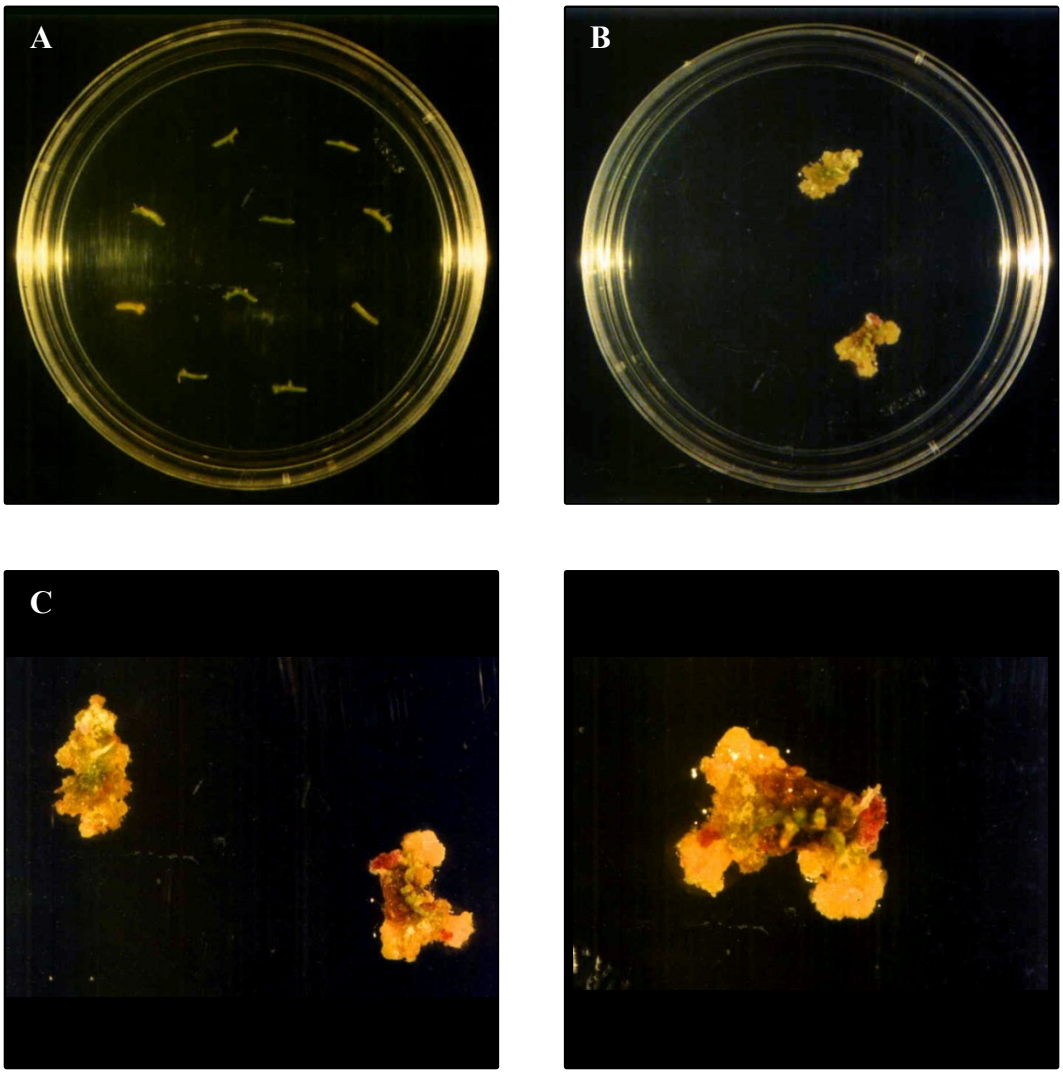

Fig. 3.5. Response of root explants in culture condition. Transformed root explants (**A**) showed the development of callus at different stages of callusing (**B**), (**C**) and (**D**). Though, no rooting was observed using different media and hormone combinations.

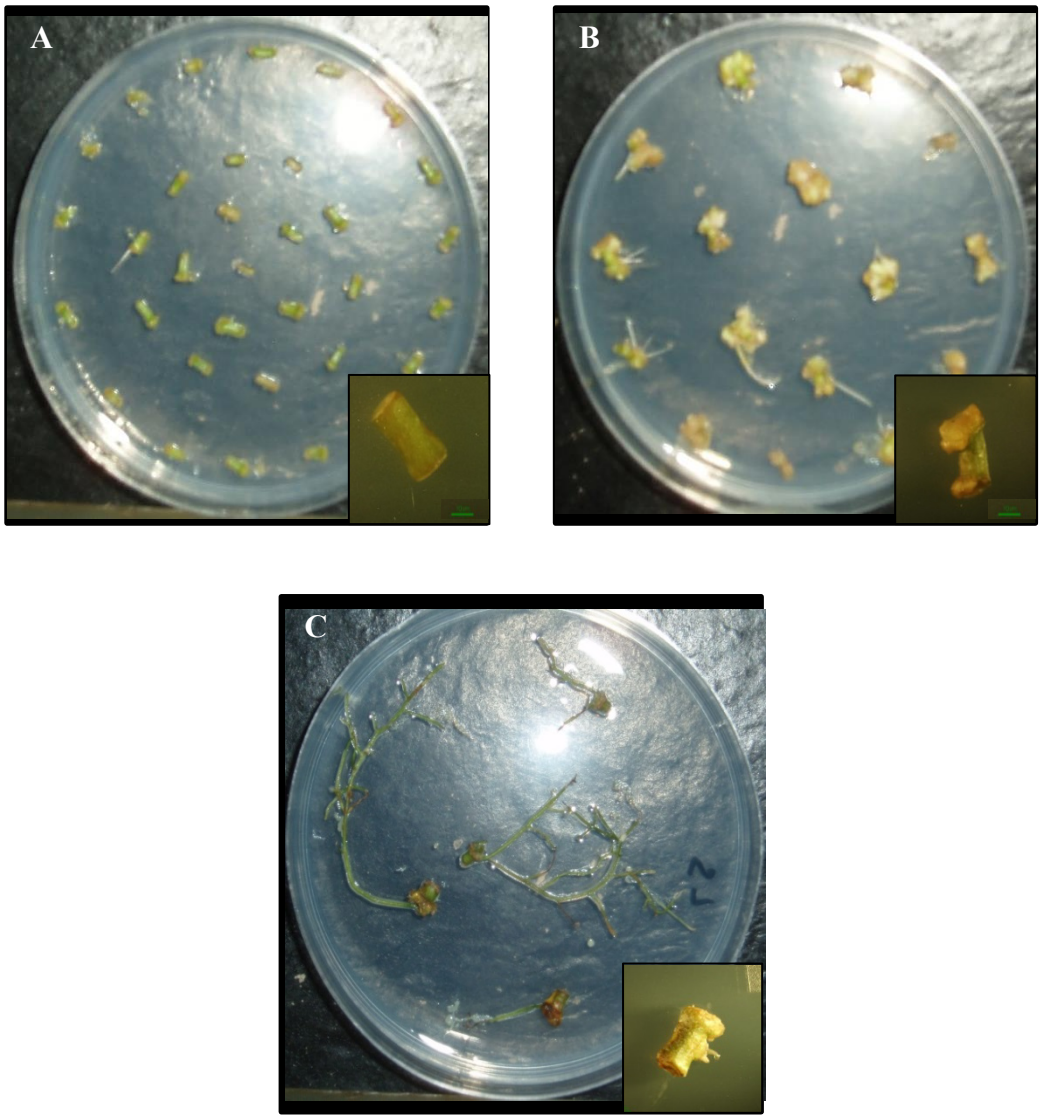

Fig. 3.6 Response of internodal explants infected by *A. tumefaciens*. *GUS* transformed internodal explants (**A**), Initiation of callusing and rooting (**B**) and (**C**) Fully developed root from internodal explants. A representative explant at different stages is shown in inset.

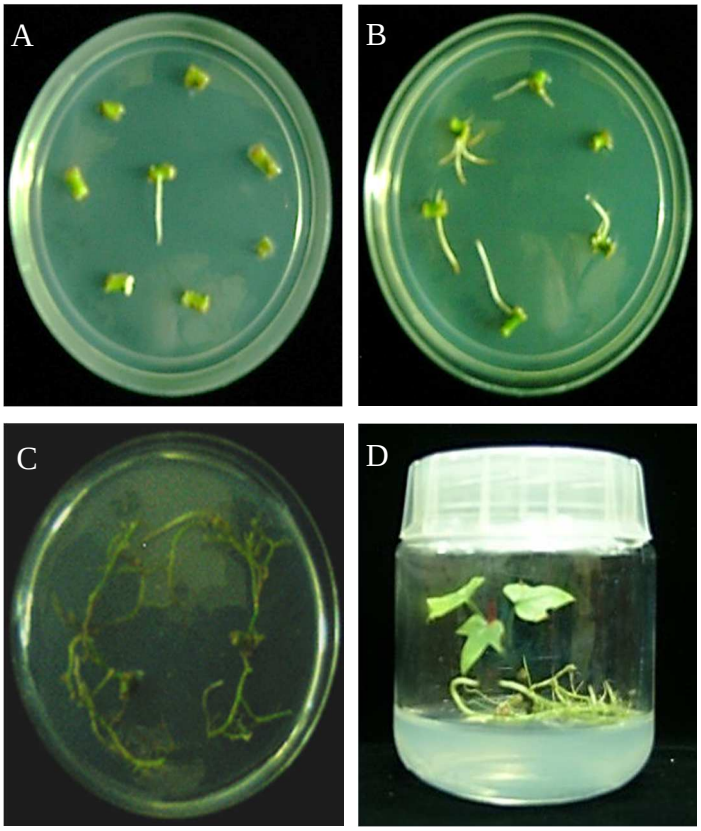

Fig. 3.7. Regeneration stages of putative transformants. The internodal explants infected by *A. tumefaciens* (strain EHA105) harbouring the expression plasmid pBI121 responded in a stage wise manner *viz.* callusing (**A**) followed by rooting (**B & C**) and shooting (**D**) in CIM and SIM, respectively.`

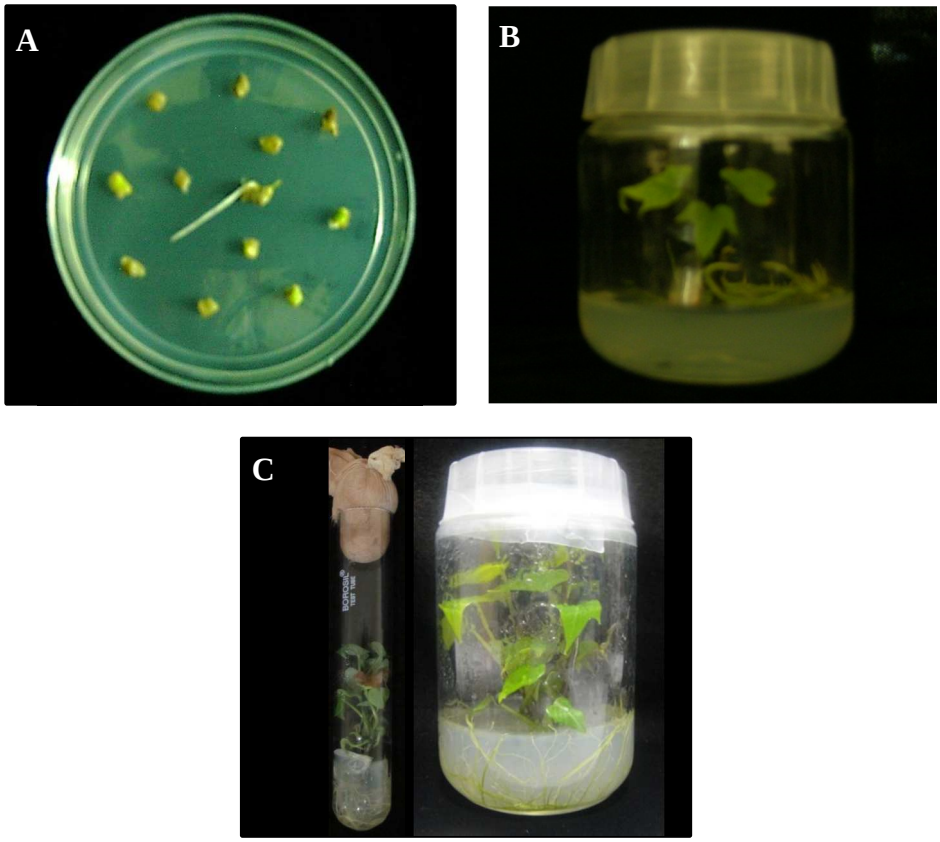

Fig. 3.8. Transformation of AmA1 expression plasmid using internodal explants. Different stages of regeneration to fully developed plants are shown. Transformed internodal explants showing initiation of rooting (**A**), Shooting (**B**) and a fully developed plant (**C**).

Table 3.2: Differential response of leaf explants in various culture conditions

Cultivar	Callusing media and hormone(s)	No. of explants	Callus formed	Percent Callus formed	Subculturing media	Roots formed	Percent Root formed
SP-1	MS, BNG	46	9	19.5	MS, BNG, BAP , Kinetin		
	MS, BNGZ	19	18	94.7	MS, NAA		
	MS, 2,4D-BAP	45	23	51.1	MS, 2,4D-BAP		
	MS, Different conc. of NAA(0.1-0.8mg/l)	115	107	93.04	MS, Different conc. of NAA(0.1-0.8mg/l)	16	14.9
	LS, Different conc. of NAA(0.1-0.8mg/l)	120	98		LS, Different conc. of NAA(0.1-0.8mg/l)	18	18.3
SP-6	MS, BNG	162	109	67.2	MS, BNG (combination of different conc. of NAA & BAP)		
	MS , 2,4D-BAP	32	10	3.1	MS, 2,4D-BAP		
	MS, 2,4D-TDZ	80	69	86.25	MS, 2,4D-TDZ		
	MS, Different conc. of NAA(0.1-0.8mg/l)	85	79	92.9	MS, NAA (in different conc.)	13	16.4
	LS, BNG	82	13	15.8	LS, BNG		
	LS, Different conc. of NAA(0.1-0.8mg/l)	69	28	40.5	LS, NAA (in different conc.)	6	21.4
SP-17	MS, BNG	46	5	10.8	MS, BNG (diff. conc. of BAP)		
	MS, 2,4D-BAP	54	24	44.4	MS, BNG		
	MS, 2,4D-TDZ	60	30	50	MS, BNG (diff. conc. of BAP)		
	MS, Different conc. of NAA	60	46	76.6	MS, NAA in different conc.	8	17.7
	LS, BNG	58	12	20.6	LS, BNG		
	LS, Different conc. of NAA(0.1-0.8mg/l), LS	71	25	35.2	LS, NAA (in different conc.)	4	16

Table 3.3: Differential response of petiole explants in various culture conditions

Cultivar	Callusing media and hormone(s)	No. of explants	Callus formed	Percent Callus formed	Subculturing media	Roots formed	Percent Root formed
SP-1	MS, BNG	54	20	37	MS, BNG, BAP , Kinetin		
	MS, BNGZ	39	10	25.6	MS, NAA		
	MS, 2,4D-BAP	50	15	30	MS, 2,4D-BAP		
	MS, Different conc. of NAA(0.1-0.8mg/l)	77	58	75.3	MS, Different conc. of NAA(0.1-0.8mg/l)	10	17.2
	LS, Different conc. of NAA(0.1-0.8mg/l)	90	66	73.3	LS, Different conc. of NAA(0.1-0.8mg/l)	8	12.1
SP-6	MS, BNG	116	78	67.2	MS, BNG (combination of different conc. of NAA & BAP)		
	MS , 2,4D-BAP	62	23	37	MS, 2,4D-BAP		
	MS, 2,4D-TDZ	87	65	74.7	MS, 2,4D-TDZ		
	MS, Different conc. of NAA(0.1-0.8mg/l)	85	79	92.9	MS, NAA (in different conc.)	23	29.1
	LS, BNG	82	13	15.8	LS, BNG		
	LS, Different conc. of NAA(0.1-0.8mg/l)	72	33	45.8	LS, NAA (in different conc.)	6	18.1
SP-17	MS, BNG	76	12	15.7	MS, BNG (diff. conc. of BAP)		
	MS, 2,4D-BAP	58	14	24.1	MS, BNG		
	MS, 2,4D-TDZ	64	31	48.4	MS, BNG (diff. conc. of BAP)		
	MS, Different conc. of NAA	70	55	78.5	MS, NAA in different conc.	9	16.3
	LS, BNG	60	12	20	LS, BNG		
	LS, Different conc. of NAA(0.1-0.8mg/l), LS	65	25	38.4	LS, NAA (in different conc.)	4	16

Table 3.4: Differential response of root explants in various culture conditions

Cultivar	Callusing media	No. of explants	Callus formed	Percent Callus formed	Subculturing media	Roots formed	Percent Root formed
Sp-1	MS, 2,4D-BAP	35	23	65.71	MS, 2,4D-BAP		
	MS, Different conc. of NAA (0.1-0.8mg/l)	62	57	91.94	MS, Different conc. of NAA		
Sp-6	MS, 2,4D-BAP	12	10	83.3	MS, 2,4D-BAP		
	MS, Different conc. of NAA (0.1-0.8mg/l)	26	24	92.3	MS, Different conc. of NAA		

Table 3.5: Differential response of internodal explants in various culture conditions

Cultivar	Callusing media	No. of explants	Callus formed	Percent Callus formed	Subculturing media	Roots formed	Percent Root formed
SP-1	MS, BNG	100	77	77	MS, BNG (combination of different conc. of NAA & BAP)		
	MS, 2,4D-BAP	62	31	50	MS, 2,4D-BAP		
	MS, 2,4D-TDZ	90	49	54.4	MS, 2,4D-TDZ		
	MS, Different conc. of NAA(0.1-0.8mg/l)	78	58	74.3	MS, NAA (in different conc.)	8	13.7
	LS, BNG	68	10	14.7	LS, BNG		
	LS, Different conc. of NAA(0.1-0.8mg/l),	80	48	60	LS, NAA (in different conc.)	6	12.5
SP-6	MS, BNG	170	119	70	MS, BNG (combination of different conc. of NAA & BAP)		
	MS, 2,4D-BAP	42	35	83.3	MS, 2,4D-BAP		
	MS, 2,4D-TDZ	80	69	86.25	MS, 2,4D-TDZ		
	MS, Different conc. of NAA(0.1-0.8mg/l)	70	65	92.8	MS, NAA (in different conc.)	15	21.42
	LS, BNG	65	11	16.9	LS, BNG		
	LS, Different conc. of NAA(0.1-0.8mg/l),	60	27	45	LS, NAA (in different conc.)	12	20
SP-17	MS, BNG	42	5	11.9	MS, BNG , diff. conc. of BAP		
	MS, 2,4D-BAP	40	24	60	MS, BNG		
	MS, 2,4D-TDZ	60	30	50	MS, BNG (diff. conc. of BAP)		
	MS, Different conc. of NAA	45	36	80.0	MS, NAA in different conc.	8	17.7
	LS, BNG	50	9	18	LS, BNG		
	LS, Different conc. of NAA(0.1-0.8mg/l)	62	21	35	LS, NAA (in different conc.)	10	16.12

MS: Murashige and Skoog; LS: Linsmaier and Skoog; NAA: Naphthaleneacetic acid

Table 3.6: Differential responses of transformed internodal explants of different cultivars in Gamborg's B-5 supplemented with NAA

Cultivars	Callusing medium	No. of explants	Callus formed	Percent Callus formed	Subculturing medium	Rooting	Percent Rooting
SP 9	Gamborg's B-5+ NAA (0.2 mg/l)	78	56	71.7	Gamborg's B-5+ NAA (0.2 mg/l)	7	12.5
SP 11	Gamborg's B-5+ NAA (0.2 mg/l)	62	46	74.1	Gamborg's B-5+ NAA (0.2 mg/l)	10	21.7
SP12	Gamborg's B-5+ NAA (0.2 mg/l)	67	51	76.1	Gamborg's B-5+ NAA (0.2 mg/l)	0	0
SP 17	Gamborg's B-5+ NAA (0.2 mg/l)	97	75	77.3	Gamborg's B-5+ NAA (0.2 mg/l)	39	52

BNG (0.1mg/l BAP, 0.1mg/l NAA, 0.3mg/l GA3); BNGZ (0.1mg/l BAP, 0.1mg/l NAA, 0.3mg/l GA3, 0.1 mg/l Zeatin); 2,4D/ BAP (10 µM/ l, 1.0 µM/l BAP); BAP (0.1- 0.4 mg/l BAP); Kinetin (0.5 mg/l), TDZ (0.05-1.5 mg/l).

Table 3.7: Transformation efficiency of the internodal explants of cv. SP-6 and SP-17

Cultivar	Set	No of explants	Rooting and callusing	Shooting	Percent Shooting
SP-6	1.	55	30	15	50
	2.	25	13	9	70
	3.	37	14	9	64
SP-17	1	42	26	11	42
	2	30	16	6	40
	3	35	13	6	47

Table 3.8: *AmA1* transformed internodal explants of cv. SP-6

Construct	Set	Medium	No of explants	Rooting and callusing	Shooting	Percent Shooting
pSB8	1.	Gamborg's B5+ NAA	52	20	6	30
	2.	Gamborg's B5+ NAA	50	25	5	20
	3.	Gamborg's B5+ NAA	30	12	4	33.3
	4	Gamborg's B5+ NAA	40	25	3	12
pSB8β	1.	Gamborg's B5+ NAA	66	40	5	12.5
	2.	Gamborg's B5+ NAA	76	36	7	19.4
	3.	Gamborg's B5+ NAA	32	12	3	25

25 and 15 regenerated lines survived the kanamycin selection from SP-6 and SP-17 respectively in the *GUS* transformed lines. However, in case of *AmA1* putative transformants all the regenerated lines from pSB8 and pSB8ß constructs had shown positive kanamycin selection.

3.4 Discussion

The suitable media and hormone combination as well as the source of explants are decisive factors in obtaining high regeneration efficiency in case of sweet potato regeneration and transformation. Sweet potato transformation is highly genotype and explants dependent [207]. To establish a simple, robust, efficient and genotype independent regeneration and transformation system in sweet potato, initially different sources of explants and genotypes were considered with varying media and hormone combinations. Based on the responses of different genotypes in various culture conditions internodal explants on Gamborg's B-5 basal medium supplemented with plant hormone NAA was proved to be a better choice. Next, two genotypes *viz.* SP-6 and SP-17 were selected for transformation of *GUS* with internodal explants and *in vitro* regeneration experiments were carried out. Hitherto, several approaches have been used to produce transgenic sweet potato especially the electroporation of protoplasts [226, 227] and particle bombardment [228, 229]. In particular, the *Agrobacterium tumefaciens* mediated transformation system has been widely used because of its efficiency, simplicity and stability of the introduced gene. The first successful such transformation protocol for sweet potato was based on the formation of hairy roots using leaf discs as explants by *Agrobacterium rhizogenes* [240, 241]. However, morphological abnormalities shown by regenerated transgenic plants were a big question mark for the method. *A. tumefaciens* mediated transformation in sweet potato was also well established by several workers [161, 242-244]. In general, these procedures have been very genotype-dependant with lower transformation efficiency [247-250], and often difficult to reproduce [159]. *Agrobacterium*-mediated transformation in sweet potato has also been well applied for regeneration via somatic embryogenesis using somatic embryos or organs as explants by several workers [176, 187, 250-252].

Over the years, *E. coli uidA* gene encoding GUS has been the most chosen reporter gene and widely used to assess the transient and stable transformation in plants. The GUS gene fusion system has found extensive application in plant gene expression studies due to enzyme stability and the high sensitivity [328]. One of the most critical factors to develop a high efficiency

transformation protocol is the use of a hypervirulent strain of *A. tumefaciens* and an appropriate medium for optimum infection of explants. Therefore to mobilize the binary expression plasmid pBI121 containing the *uidA* and *nptII* genes into *Agrobacterium tumefaciens* EHA105, triparental mating strategy was used. In plant cell culture, growth and morphogenesis are significantly affected by the type(s) of media and the concentrations of growth regulators. Plant growth regulators are conceivably the most important components affecting shoot regeneration capacity of explants. Thus, optimization of the correct combinations of auxins and cytokinins is indispensable for high frequency shoot regeneration. Also, the genotype of the transformed explants is a deciding factor for their growth and development. In present study, an oppressive impact of cytokinins was observed when used in combination with auxins in case of MS as well as LS media. Addition of either of these cytokinins inhibited root and shoot development in such a way that higher the cytokinin concentration, greater was the inhibitory effect; however, a corresponding increase in callus growth was also observed. Roots are the primary organ for the synthesis of cytokinins and thus rooted explants should conceivably be able to synthesize a little amount of cytokinins [176]. In addition, several tissues are considered to be cytokinin independent, and the findings of the present study also suggest that it is not necessary to supplement the regeneration medium with cytokinin for the regeneration of sweet potato.

In *Agrobacterium*-mediated transformation system, activation of the virulence genes in the Ti plasmid is modulated by molecular signals provided by wounded tissue that is mimicked by phenolic compounds such as acetosyringone or hydroxyacetosyringone. The use of acetosyringone has been reported earlier in various sweet potato transformation systems [250, 254]. However, a stable transformation system was developed without the addition of exogenous signaling compound, considering the predominant presence of phenolic compounds in sweet potato. In this study, the induction of root as well as the shoot was found to be from a single regenerative act. This differentiates somatic embryogenesis from organogenesis, wherein *de-novo* produced 'unipolar' regenerations, mainly shoots; require to be triggered with growth regulators for the stimulation of adventitious roots, thus producing a complete plant in two steps. Induction of somatic embryos can be directly from the cells of the explant cultured *in vitro* ('direct' or 'adventitious' somatic embryogenesis), or more frequently, from the dedifferentiated cells of a proliferative callus (indirect or induced somatic embryogenesis) after the explant tissue is artificially stimulated (*i.e.*, with growth regulators) to expand its

embryogenic proficiency [329]. In this study, rooting as well as the shooting was obtained from the explants in a single regenerative act that might possibly be the case of somatic embryogenesis. Intriguingly, the induction of somatic embryogenic tissues at high frequencies has been found to be limited to a few genotypes and when attempts were made to extend this into a wide range of genotypes, the majority was found to be recalcitrant or to respond at low frequencies. **Al-Mazrooei et al. (1997)** optimized the culture condition for somatic embryogenesis in 14 genotypes and obtained the regeneration frequency up to 68% [169]. It is evident that there has been a gap between the regeneration frequency and the transformation efficiencies. However, transformation efficiencies of up to 20% reported by **Luo et al. (2006)** from leaf petioles were higher than those of several other reports [187, 250, 252, 260]. A relatively rapid regeneration system (12-16 weeks) with a higher transformation frequency of 30.8% was also reported from stem explants [248]. In comparison with the prolonged regeneration steps required in the somatic embryogenesis [187, 250, 330], the rapid *de novo* organogenesis is likely to lower somaclonal variations [250]. We observed the regeneration in time span of 8-12 weeks, starting from the day of infection with *Agrobacterium* which also strengthens the claim for the development of a relatively more rapid regeneration system for sweet potato.

Selectable marker genes have a pivotal role in validating the plant transformation technologies as the marker genes allow to distinguish between the transformed and untransformed plant(s). While untransformed escapes are the common phenomenon in transgenic technology of plants, a suitable concentration of test antibiotic can reduce such risk [331]. To screen the true transformation events, high concentration of kanamycin at 100 mg/l was used and the kanamycin-resistant plants were considered for downstream analysis. As stated earlier, morphological abnormality is one of the major constrains in the development of a successful regeneration and transformation system. In the present study no detectable morphological difference between the wild-type and the transgenic plants was observed at this stage. The genetic transformation protocol reported here may be used to incorporate agriculturally and/or industrially important candidate gene(s) in sweet potato. It is likely that, by manipulating the regeneration process or better susceptibility to *Agrobacterium*, it will be possible to address issues of genotype-dependence and to improve transformation efficiencies in sweet potato

further. Indeed, a comprehensive analysis of successful transformation to appraise the transformation efficiency and the impact of transgene introduction as well is needed.