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Transformation of cowpea (*Vigna unguiculata* L. Walp.) by *Agrobacterium* infiltration

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ABSTRACT
Objectives: Until recently, stable genetic transformation of cowpea through tissue culture technique could not be established. The aim of this work was to optimize inoculation and cocultivation medium factors for cowpea transformation and avoid in vitro regeneration procedures in obtaining transgenic cowpea through vacuum infiltration of embryos.
Methodology and results: Using plasmid pCAMBIA 1301, influence of inoculation and cocultivation media compositions on transient gene expression were determined. Embryos were inoculated on Murashige and Skoog (MS) or yeast extract broth (YEB) solutions supplemented with either acetosyringone, 0.05% silwet L-77, both or none and then cocultivated on MS solid medium for 24 hours. Gus assays showed that inoculating explants in MS containing acetosyringone and 0.05% silwet L-77 gave the highest transformation frequency (55.3%). When tested, the presence of acetosyringone in the cocultivation medium increased transformation frequency by 15.35%. When untransformed control seeds were screened on hygromycin and phosphinotricin 100% shoot and root growth inhibition were obtained at 50mg/l and 5mg/l, respectively. Secondly, cowpea embryos were transformed with Agrobacterium cells carrying two plasmids, ptjk 142 and pCAMBIA 1301, by inoculating them in media optimized as above and subjecting to two rounds of vacuum infiltration. Thereafter they were cocultivated for nine days on MS selection-free medium where they germinated and produced mature plantlets. T_1 seeds were selected on antibiotic concentrations determined as above. Plants surviving both phosphinotricin and hygromycin showed gus positive reactions when subjected to gus histochemical assay and amplified the gus primer during molecular analysis. Phosphinotricin resistant plants also amplified bar primers. Percentage transformation based on total number of T_1 seeds and number of plants with positive PCR reaction using both primers was 2.5 % for pCAMBIA 1301 and 3.9 % for ptjk 142 plants.
Conclusion and application of findings: The integration of transgenes into cowpea by Agrobacterium infiltration of embryos germinating on selection free medium has been demonstrated. This technique has the potential of rapidly producing stably transformed cowpea and avoiding all the limitations imposed by de novo regeneration in tissue culture.
Key words: cowpea, genetic engineering, Agrobacterium infiltration, acetosyringone, transient gene expression.

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INTRODUCTION

Cowpea (*Vigna unguiculata* L.Walp.) is a large-seeded food legume cultivated for its protein-rich seeds by poor farmers in Sub-Saharan Africa and Asia (Sahoo *et al*., 2003). According to FAOSTAT (2005), its total world production is estimated to be about 3.8 million tons. Sixty-six percent of the 12.5 million hectares cultivated world-wide are in West and Central Africa (Singh *et al*., 2000). Cowpea contains about 25% protein and so it is a cheap source of protein in the daily diet of urban and rural populations. Being a fast-growing crop it curbs erosion by covering the ground, fixes atmospheric nitrogen, and its decaying residues contribute to soil fertility.

However the yield of traditional cultivars on farmers' fields is far from optimal due to several abiotic and biotic constraints. Prominent among the biotic stresses are insects which attack the crop from the vegetative stage to flowering, podding and storage. These include aphids, pod borers (*Maruca vitrata*), pod-sucking bugs (*Clavigralla tomentosicollis*) and flower thrips (Jackai & Adalla, 1997).

Attempts at crossing wild *Vigna* species with cowpea in order to breed for insect resistance have been futile (Fatokun, 2000; Singh *et al*., 2000). The use of chemical pesticides for the control of insects is associated with several risks and damage to human health and environment. Genetic transformation offers new possibilities of transferring useful genes from other sources into cowpea to address these yield constraints. However the use of gene technology in cowpea improvement has been hindered by the lack of an efficient transformation technique (Popelka *et al*., 2004). *Agrobacterium*-mediated gene transfer is the most popular transformation technique as many plant species, including cowpea, have been found susceptible to several *Agrobacterium* strains (Garcia *et al*., 1986; Fillippone, 1990; Walden & Wingender, 1995; Chaudhury *et al*., 2007). However, lack of reliable and reproducible regeneration protocols and recalcitrance to in vitro manipulations common with most large-seeded legumes has been a barrier to the effective use of this method (Christou, 1992; Monti *et al*.,1997). Although direct shoot organogenesis have been obtained from primary leaves (Muthukumar *et al*.,1995) mature cotyledons (Pellegrineschi, 1997) and cotyledonary nodes (Machuka *et al*., 2000) in a few varieties, the frequency has been very low.

Reports available on stable *Agrobacterium*-mediated transformation of cowpea with transmission of transgenes to sexual progeny involved the inoculation of mature cotyledons (Muthukumar *et al*., 1996) embryonic axes (Popelka *et al*., 2006) and cotyledonary nodes (Chaudhury *et al*., 2007; Solleti *et al*., 2008). These efforts attained progressive increases in transformation efficiency from 0.003 to 0.76 and then to 1.64 %. Solleti *et al*. (2008) noted that inefficient TDNA delivery to regenerating cells of embryo-derived explants had been responsible for the low transformation efficiency and suggested that to enhance transformation rate early TDNA transfer steps must be optimized.

In the model legume, *Medicago truncatula*, gene transfer was significantly increased by vacuum infiltration of *Agrobacterium* cells into entire plants with subsequent selection of transformants from T_1 progenies (Trieu *et al*., 2000). This technique by-passes the need for *de novo* regeneration. Further, vacuum-infiltration has been successfully used for the transformation of recalcitrant plants such as *Triticum aestivum* and *Pinus radiata* (Amoah *et al*., 2000; Charity *et al*., 2002). However, transformation by vacuum-infiltration has not been previously carried out on cowpea.

The inclusion of acetosyringone, a virulence gene inducer, into inoculation and cocultivation media during Agrobacterium transformation of several species is now common. Others pretreat explants with surfactants like Silwet to increase bacterial cell penetration and hence TDNA transfer (Clough & Bent, 1998; Wu *et al*., 2003). The aim of this work was to evaluate the effect of various additives in inoculation and cocultivation media on transient gene expression and apply the optimized conditions for stable transformation of cowpea by *Agrobacterium* infiltration of seed embryos.

MATERIALS AND METHODS

Plant material and bacterial strain: Cowpea variety IT86D-1010 and the disarmed *Agrobacterium tumefaciens* strain pGV3850 containing the binary vector pCAMBIA 1301 were used for the transient gene expression experiment while the same strain containing the vector ptjk 142 was included in the vacuum infiltration experiment. Two related experiments were conducted. One was set up to determine the effect of bacteria inoculation medium on transient Gus gene expression in cowpea embryos. The second was to determine the effect of embryo cocultivation medium on gene expression in cowpea embryos.

Effect of inoculation medium was studied in a 2X4 factorial experiment laid out in a completely randomized design with three replications. The first factor was medium type, i.e. M and Y. In medium M bacterial cells were suspended in MS solution (James *et. al.*, 1993) while in medium Y, bacterial cells were suspended in yeast extract broth (YEB) solution. The second factor was the additive solutions in the medium. Two additive solutions, silwet (0.05%) and acetosyringone (100 μM), were used either singly or in combination to make four additive treatments denoted as 1, 2, 3 and 4. 1= no additive added (controls); 2= only acetosyringone added; 3= only silwet added; 4= both acetosyringone and silwet added. Eight embryos per replicate were immersed in each of the eight inoculation media combinations prepared.

Effect of cocultivation medium was studied in a 2X2 factorial experiment laid out in a completely randomized design with three replications. The first factor was the bacterial inoculation medium, M and Y, both containing acetosyringone (100 μM). The second factor was the cocultivation medium composition. Embryos were inoculated for 30 minutes and subsequently cocultivated with the bacteria cells on solid MS medium having either 1.0 mg/l BAP only or 1.0 mg/l BAP plus 100 μM acetosyringone for 24 hours. After cocultivation, assessment and analysis of Gus assay was done. Treatment effect on transformation rate was estimated as percentage of treated embryos showing Gus stained sectors. Data were subjected to analysis of variance (ANOVA) using the GLM procedure and means separated by the Duncan's multiple range test (SAS institute, 1989). Standard error and LSD values were determined.

Gus histochemical assay: Explants inoculated with Agrobacterium and cultured on cocultivation media described above were stained with a solution of X-Gluc (Clonetech), a chromogenic substrate (Jefferson *et. al.*, 1987;1989) and incubated in the dark at 37° C. Samples were cleared with 100% ethanol and evaluated for gus reaction.

Vacuum infiltration of *Agrobacterium* into embryo explants: This experiment was carried out using the cowpea variety IT96D-734 obtained from IITA gene bank and two *Agrobacterium* strains-pGV2260 containing plasmid ptjk 142 from University of Gent, Laboratory of Genetics, Belgium and pGV3850 containing plasmid pCAMBIA 1301 from CAMBIA (Centre for Application of Molecular Biology in Agriculture), Australia.

Seeds were sterilized in 0.75% (w/v) calcium hypochlorite overnight in 500 ml conical flasks. These were then rinsed in three changes of sterile distilled water. Hundred embryos each were excised asceptically from the seeds and inoculated separately with either *Agrobacterium* culture containing plasmids ptjk 142 (having the phosphinotricin resistance gene) or plasmid pCAMBIA 1301 (having the hygromycin resistance gene). *Agrobacterium* containing the ptjk 142 plasmid were cultured for about 48 hours in Luria Bertani (LB) bacterial growth medium supplemented with 50mg/l streptomycin and 150mg/l spectinomycin while *Agrobacterium* containing the pCAMBIA1301 plasmid vector were grown on YEB supplemented with 100mg/l rifampicin and 50mg/l kanamycin. The cells were harvested by centrifuging at 4000xg for 10 min and re-suspended in a liquid MS medium that is about one-quarter the volume of the original bacterial growth medium. One hundred micro moles of Acetosyringone solution was then added into the suspension, which was allowed to grow on a shaker (200 revolutions per minute), for about 5 hours. Silwet solution (0.05%) was also added to the *Agrobacterium* suspensions. Sterile embryos obtained earlier were decapitated and vacuum infiltrated inside a 5ml sterilin tube containing the bacterial suspensions at 28 inch Hg for 2-5 min using the vacuum chamber of the PDS helium particle delivery system. Thereafter, the embryos were blot dried on sterile paper towel for five minutes and cultured for five days on MS cocultivation medium containing 3% sucrose, 8.0 g/l agar, 1 mg/l BAP, 160 mg/l thymine, 160 mg/l putrescine and 0.5 mg /l casein hydrolysate. The embryos were re-infiltrated and cocultivated for between 2-4 days. The embryos were then washed in MS liquid solution containing 1 mg/l BAP and 1,250 mg/l Cefotaxime (a bactericidal agent)

overnight to kill the *Agrobacterium* cells and prevent them from overgrowing on the infiltrated explants.

Explant shoot elongation and rooting: Surviving plantlets were placed on MS basal medium containing 3% sucrose and 0.05mg/l naphthalene acetic acid (NAA) in 100ml magenta bottles where the shoots elongated and roots were produced. After about two weeks plantlets were transferred to peat in a containment room where they were conditioned before transplanting to soil in pots after about one week. These were transferred to a screenhouse where they were nurtured till T_1 seeds were obtained.

Screening of progenies on bialaphos (commercial brand of phosphinotricin) and hygromycin: Whole seeds of T_1 progenies were sterilised by gas produced by a mixture of 12N HCl and bleach solution (La Croix) and screened on 40-50 mg/l hygromycin (for plants infiltrated with pCAMBIA1301 bacteria), and 5mg/l bialaphos (for plants infiltrated with ptjk 142 bacteria). The concentrations of hygromycin and bialaphos used here were determined from the selection system established above that is most effective for eliminating non-transformed cowpea explants to enable selection of transformed cowpea. The number of surviving explants was determined after 9-12 days on bialaphos and hygromycin selection. After selection of plantlets on antibiotics, leaf samples were collected and stained with X-Gluc solution for Gus histochemical assay as described previously.

PCR analysis of progenies: The T_1 and T_2 generations obtained from antibiotic selection were screened for the Gus and PAT (or BAR) genes by PCR and Gus histochemical assay on their leaves. The co-transformation frequencies were evaluated by PCR analysis based on the presence of Gus and BAR genes in plants transformed with the ptjk 142 plasmid and based on the Gus and hpt II genes in plants transformed with the pCAMBIA 1301 plasmid. Hygromycin resistance gene, hpt II was scored in T_0 and T_1 plants based on survival of their progeny seeds when screened on 50 mg/l hygromycin. The expected amplification products for gus and bar genes are 480 and 372 base pairs, respectively. The primer sequences for bar obtained from Integrated DNA Technologies, U.K. are as follows;

PATJV1 [P1] 5'- CAG GAA CCG CAG GAG TGG A - 3'

PATJV2 [P2] 5'- CCA GAA ACC CAC GTC ATG CC -3'

Plants that survived on hygromycin and bialaphos selection medium, when controls had died, were assayed for Gus expression and analysed by PCR using Gus primers obtained from MWG-biotech-Gmbh, Germany, to detect specific Gus sequences introduced into the plant genome. Genomic DNA extracted from leaf samples of hygromycin resistant plantlets were used.

The primer sequences are as follows:

GUS down [P1] 5'- GGC AAT ACT CCA CAT CAC CAC G - 3'

GUS up [P2] 5'- GGC GAA CAG TTC CTG ATT AAC C - 3'

Amplification reactions were performed in 50-µl reaction volumes containing a cocktail of 5.0µl 10X thermophilic buffer, 4.0µl 25mM $MgCl_2$, 1.0µl 10mM dNTP, 3.0µl 5% Tween 20, 4.2 µl primer 1, 5.0 µl primer 2, 0.5µl 5U/µl Taq polymerase enzyme, 22.3µl sterile distilled water, and 5.0 µl genomic DNA.

Amplification was performed in a Perkin Elmer-Cetus DNA cycler 480. The reaction mixtures were pre-heated at 95° C for 3 min and then subjected to 30 cycles of 1 min at 94° C; 1 min at 55° C; 1 min at 72° C and finally, another cycle of 10 min at 72° C. The expected Gus fragment to be amplified is of about 480-basepair length. The fastest available transition between each temperature was used. Amplification products were analysed by electrophoresis in 1.4% agarose gels and detected by staining with ethidium bromide.

RESULTS

Optimization of culture conditions and development of transformation protocol: Preliminary experiments to develop transformation protocol involved optimization of inoculation medium composition and subsequent cocultivation medium composition to enhance transformation efficiency. After inoculating cowpea embryos for thirty minutes and cocultivation for 24 hours Gus assay was carried out in order to estimate transient gene expression. Gus activity was observed as deep blue sectors (Fig 1). Transformation frequency was expressed as percentage of treated embryos showing Gus stained sectors.

Figure 1: Decapitated cowpea embryos showing Gus stains following *Agrobacterium tumefaciens* inoculation and cocultivation with plasmid pCAMBIA 1301.

Murashige and Skoog (MS) medium generally resulted in higher transformation rate than Yeast extract broth (YEB) (Fig. 2). For MS the least (20.8%) transformation rate was obtained with inoculation medium having no additive while the highest (55.3%) was recorded when both acetosyringone and silwet were added. For YEB the least (16.7%) transformation frequency was obtained with inoculation medium having no additive while medium with acetosyringone alone gave highest (27.8%) frequency, although this was not significantly different from medium having both acetosyringone and silwet combined (22.5%) ($p > 0.05$).

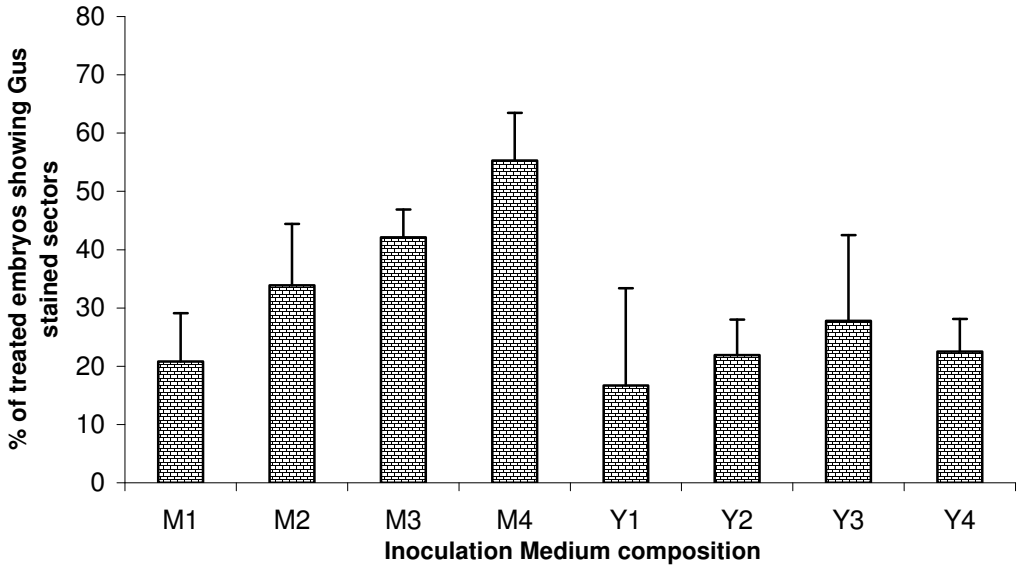


Figure 2: Effect of *Agrobacterium* inoculation medium on transient gus gene expression in cowpea embryos. Bars on columns indicate standard error. M = Murashige & Skoog medium; Y = Yeast extract broth bacterial growth medium 1 = No additive 2 = + Acetosyringone; 3 = + Silwet; 4 = +Acetosyringone + Silwet.

The effect of acetosyringone in the cocultivation medium on transformation was tested by inoculating embryos in MS and YEB both containing acetosyringone and subsequently cocultivating on solid MS+BAP medium with or without acetosyringone. The presence of acetosyringone in the cocultivation medium increased transformation percentage by 15.35% (Fig 3.). Cocultivation medium had a significant effect on percentage of treated embryos showing Gus stained sectors ($p<0.05$). Analysis of variance for the 2X2 experiment shows that interaction between inoculation medium type and cocultivation medium composition was significant ($p<0.05$) (Table1).

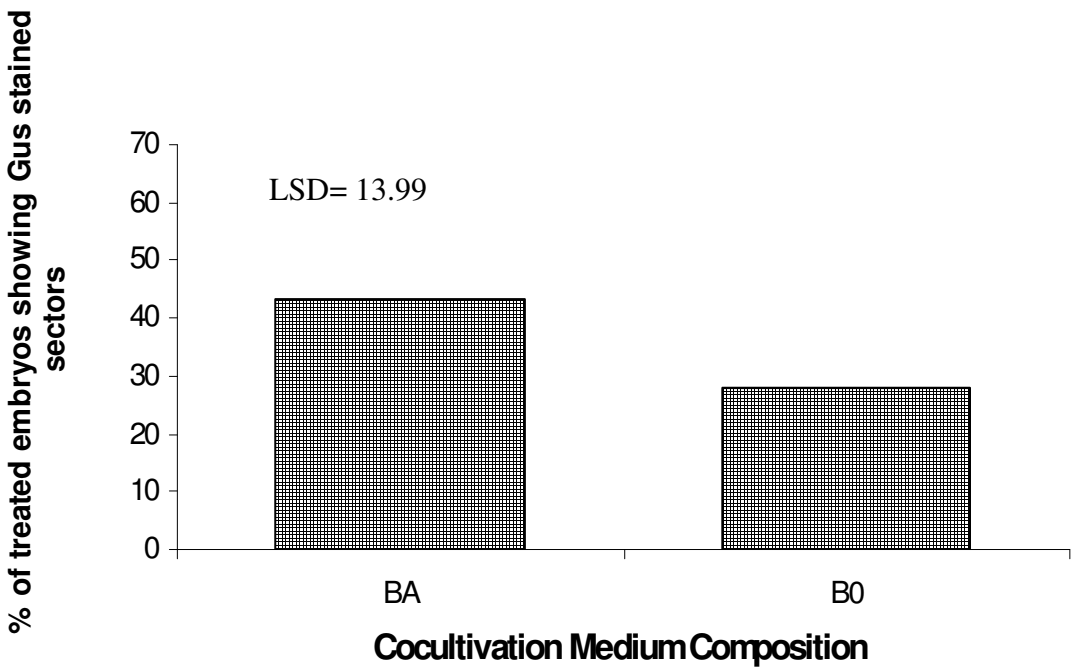


Figure 3: Effect of cocultivation medium composition on transient Gus gene expression in cowpea embryos (BA = MS + ACETOSYRINGONE; B0 = MS – ACETOSYRINGONE)

Table 1: Analysis of variance (ANOVA) of data on percentage of cowpea explants showing Gus stains on at least half the areas of the embryos after inoculation and cocultivation.

Source	DF	SS	F Value	Pr > F
Inoculation medium	1	524.04	1.94	0.2016
Cocultivation medium	1	706.87	0.26	0.6269
Inoculat* X Cocultivat*	1	1907.64	8.07	0.0218
Error	8	883.48		
Total	11	4022.03		

NB: Interactions between Inoculation and cocultivation media had significant effect on transient Gus expression in cowpea embryos

Interaction means are shown in Fig. 4 and reveal that the best transformation frequency (62.5%) was obtained when embryos were inoculated in YEB medium containing acetosyringone and later cocultivated on MS containing acetosyringone. The percentage of Gus expression was drastically reduced if acetosyringone was supplied in this inoculation medium and not in the cocultivation medium. The reverse seemed to be the case with MS inoculation medium. The addition of acetosyringone in subsequent cocultivation medium after passing embryos through MS inoculation medium did not improve but rather reduced the transformation frequency (Fig. 4).

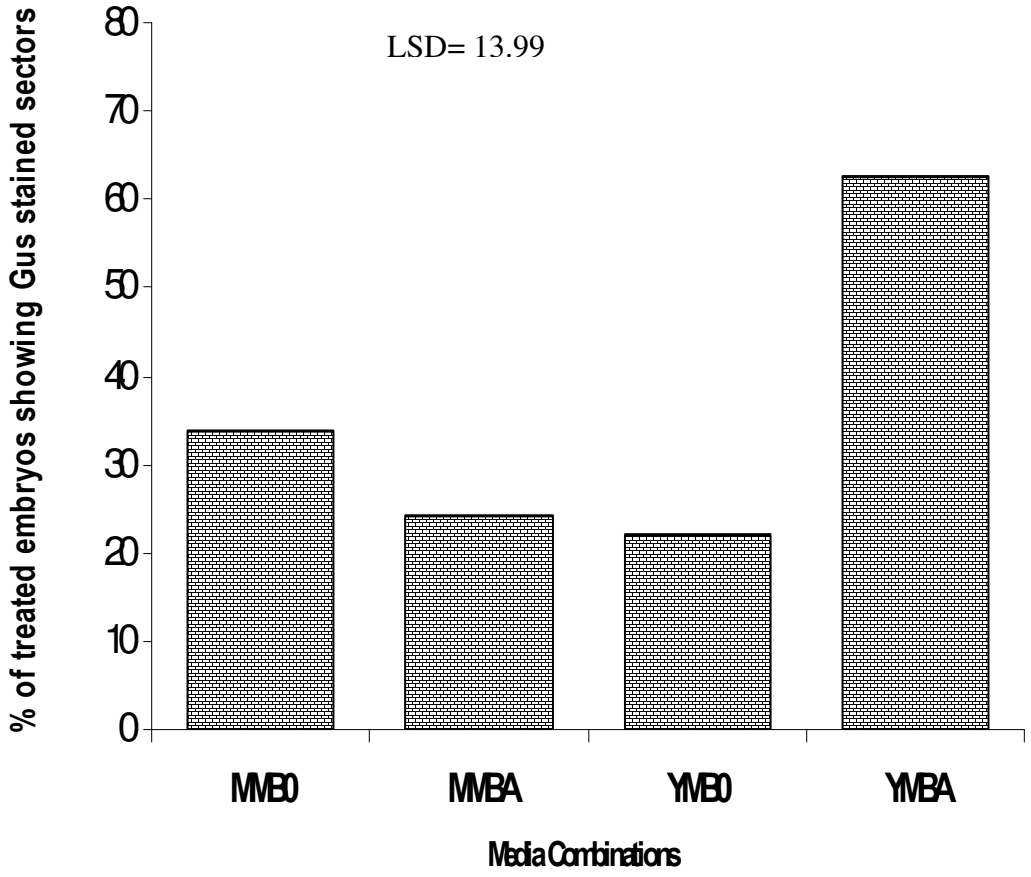


Figure 4: Effect of Agrobacterium inoculation medium and cocultivation medium interaction on transient gus gene expression in cowpea embryos. MMB0 = MS (Inoculation) and MS – acetosyringone (Cocultivation); [MMBA = MS (Inoculation) and MS + acetosyringone (Cocultivation); YMBO = YEB (Inoculation) and MS – acetosyringone (Cocultivation); YMBA = YEB (Inoculation) and MS + acetosyringone (Cocultivation)]

Following the optimization of media factors, embryos in subsequent transformation e periments were inoculated on medium containing both acetosyringone and silw*et al*ong with two rounds of vacuum infiltration before cocultivation.

In experiments performed initially to determine the tolerance of untransformed (control) cowpea embryos to hygromycin and phosphinotricin, the proportion of explants with growing shoots at 20-50 mg/l hygromycin were significantly ($p<0.05$) higher than those on selection free medium (Fig. 5a). Plants on 10 mg/l and selection-free media were not significantly different in their response to hygromycin. A drastic reduction (between 65 to 80%) occurred in percentage of explants with growing shoots when hygromycin concentration was above 20mg/l . Generally, hygromycin led to explant death by wilting of the developing plantlet and necrotic spots appeared all over the plant. At 50 mg/l, no embryo germinated or produced roots and all the explants died within 24 days of the experiment. The proportion of embryos with growing shoots was significantly higher at 10 mg/l hygromycin than at all other concentrations tested ($p<0.05$), but there was no significant difference in the effect of 40 and 50 mg/l hygromycin on shoot growth. The same trend was observed with regard to the effect of different hygromycin concentrations on root growth.

x

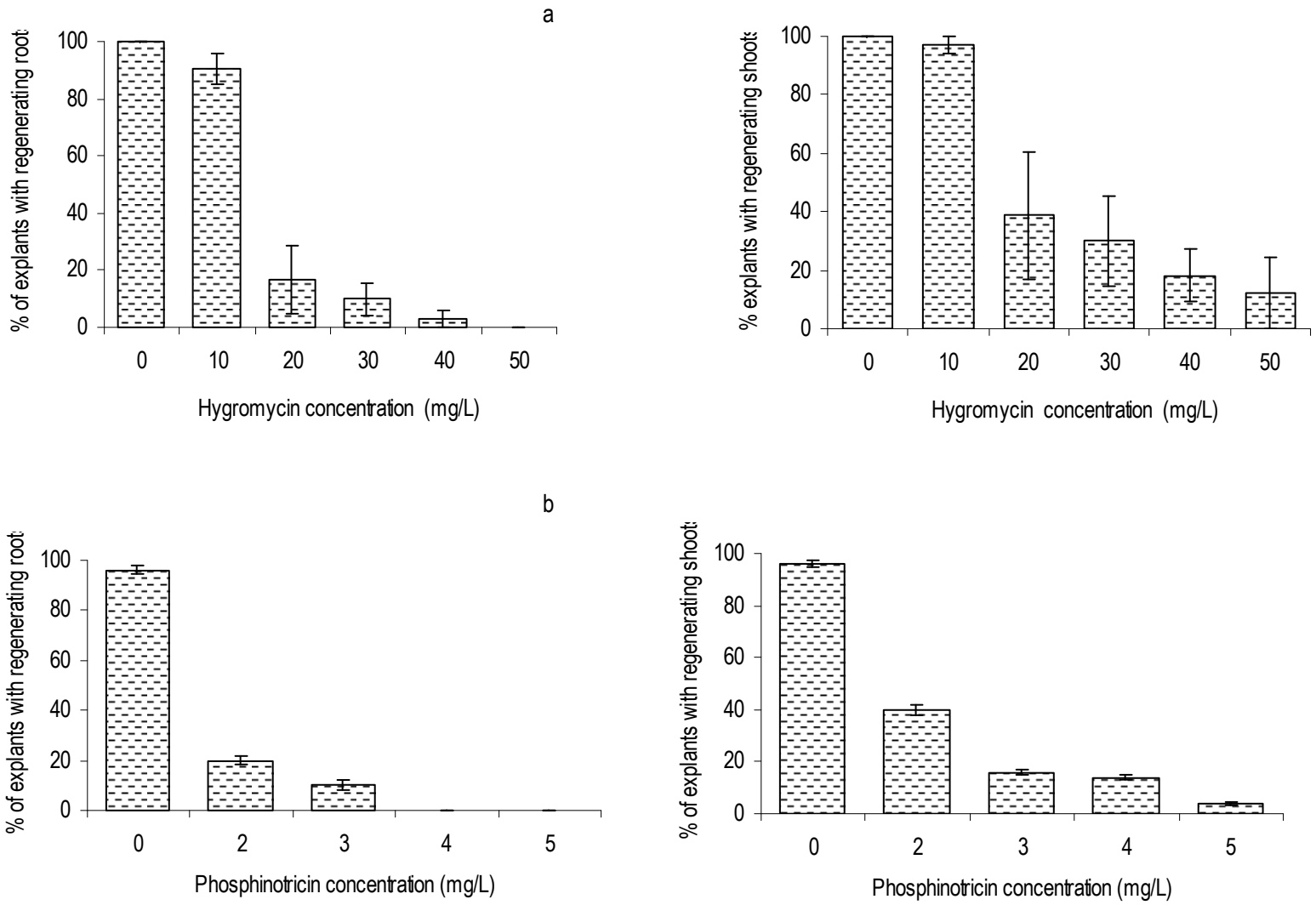


Figure 5: Response of non transformed cowpea to varying concentrations of selective antibiotics (a) hygromycin, and (b) phosphinotricin,. I = Standard error

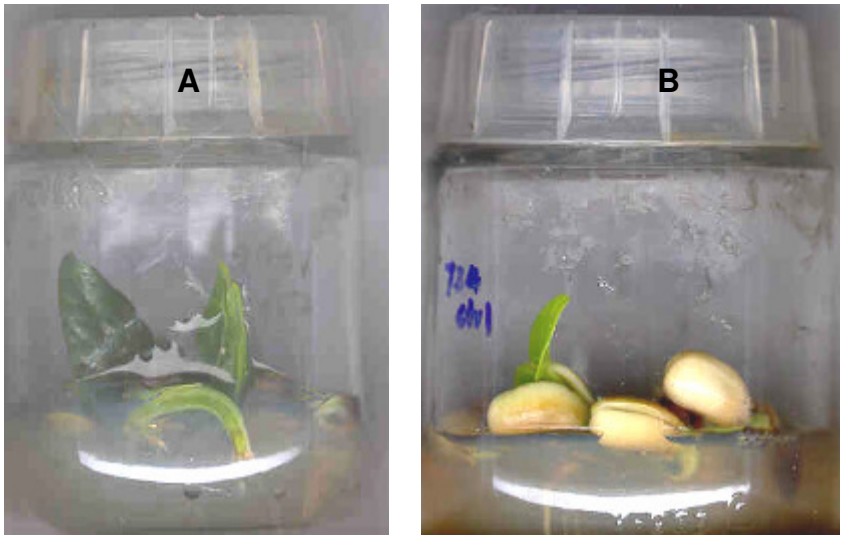
An effect of varying concentrations of phosphinotricin was observed on percentage of cowpea embryos producing roots and shoots over 22 days. The least concentration tested (2 mg/l) reduced shoot growth by more than 60% while root growth was reduced by about 80% (Fig. 5b). The antibiotic appeared to have more drastic effect on roots than on shoots. No embryo shoots survived 4 and 5 mg/l phosphinotricin, as roots were completely dead. At 5mg/l concentration, embryos did not germinate at all.

Effect of *Agrobacterium* infiltration on survival of cowpea plantlets and expression of selectable marker genes in T_1 progenies: Following the optimization of medium composition factors for enhanced transformation rates in inoculated cowpea embryos, 100 embryos each were inoculated and infiltrated with *Agrobacterium tumefaciens* strain PGV3850 containing the plasmid pCAMBIA 1301 carrying gus and hygromycin resistance genes used in transient transformation experiments above and strain PGV 2260 containing plasmid ptjk 142 carrying gus and phosphinotricin resistance genes.

A total of thirty two (i.e. 16%) embryos survived infection, two infiltrations, and germinated into healthy plantlets after nine days of cocultivation (Table 2). These seedlings grew when transferred to peat in a containment room before they were later moved into pots in the green house. The rate of growth of these infected seedlings was however slow when compared to uninfected seedlings, which might be due to prolonged infection and cocultivation with Agrobacterium. These seedlings produced T_1 seeds even though the number of seeds collected was lower than expected probably due to the small pot size. Putative T_1 transformants were selected from the progeny seeds of the infiltrated plants on two different selective antibiotics corresponding to the introduced selectable marker genes (Fig. 6). Percentage transformation based on selection on 50 mg/l hygromycin was higher than on 5 mg/l phosphinotricin (Bialaphos) (Table 2).

Table 2: *Agrobacterium* infiltration of cowpea embryos with two selectable marker genes.

Plasmid and *Agrobacterium* strain used	Selectable marker gene	Selective Antibiotic	No. of embryos infiltrated	No. of embryos survived	No. of T1 seeds collected	No. of resistant seedlings	Transformation Efficiency
ptjk 142 PGV 2260	PAT	5mg/l PPT	~100	22	126	10	7.94
pCAMBIA 1301 PGV3850	*Hpt* II	50mg/l HYG	~100	10	39	5	12.82



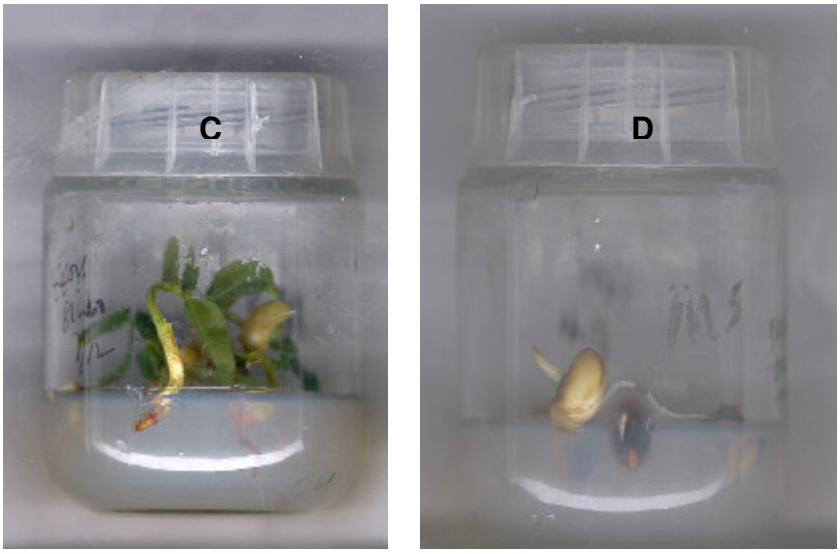


Figure 6: Evaluation of transformed and control seeds on phosphinotricin (5mg/l Bialaphos) (top) and 50mg/l Hygromycin (bottom). A and C - Transformed seeds . B and D - Untransformed control seeds .

Expression and inheritance of Gus gene: The susceptibility response of cowpea embryo to Agrobacterium infection that was determined initially by scoring the transient GUS activity 24 h after infection resulted in blue color development on the embryonal axes (Fig.1). The background GUS activity resulting from bacterial presence in the tissue was eliminated by a plant intron that eliminates Gus activity in *A. tumefaciens* in the plasmid vectors. Deep blue sectors were seen on some parts of the leaflet and GUS activity was expressed in various organs and tissues of the T_1 and T_2 plants including the stipules (Fig. 7). None of the control plants expressed GUS.

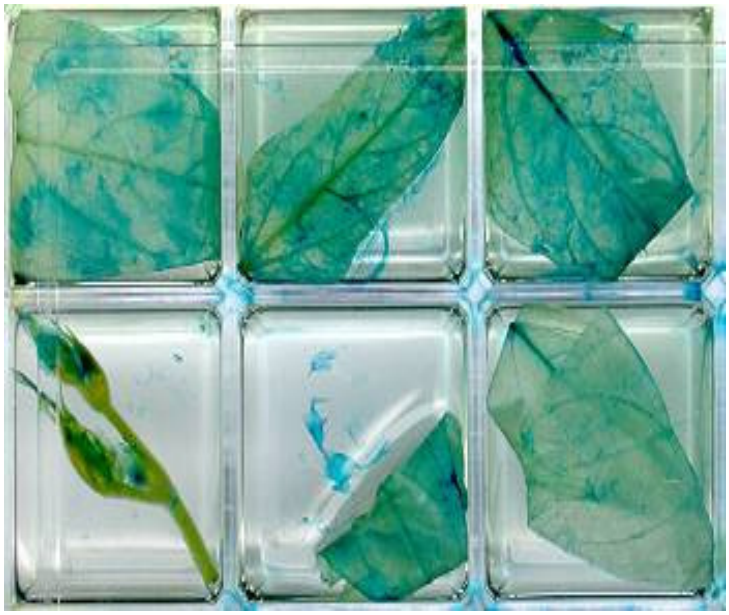


Figure 7: Histochemical Gus assay of leaves from T2 progeny obtained by Agrobacterium infiltration with plasmid ptjk 142. Gus stains observed mostly on the mid-veins of the leaves.

Molecular analysis of T_1 plants: Table 3 shows the PCR analysis of 8 T_1 progenies transformed with the ptjk 142 plasmid and analysed with PAT and Gus primers. Gus primers amplification product was 480 base pairs length (Fig. 8) while PAT primers amplification product was 372 base pairs length (Fig. 9). The frequency of co-transformation of the linked genes was evaluated using the Gus and PAT gene sequences in the plasmid. Six plants in all showed amplification for either of the two primers. Both primers amplified five out of these. On the bases of this T_1 progeny analysis, the co-transformation frequency was 83.33% (i.e. 5/6 x 100%). All the plants analysed showed the presence of the PAT gene sequence except plants G7 and G19 while only G15 lacked the Gus gene (Figs. 8 and 9). The last four plants on Table 3 (G6, G10,G11, and G14) were transformed with plasmid pCAMBIA 1301 and analysed for the presence of Gus gene. Three of them were analysed by screening their T_2 progenies for resistance to hygromycin (Table 3) and they all had resistant progenies. This was used to score for the presence of hygromycin resistance gene in T_1 plants (Fig. 6)) due to the non-availability of hygromycin resistance gene primers. Only the plant G6 showed presence of the Gus gene sequence. The other three did not show presence of the Gus gene but however tested positive for hygromycin resistance. Percentage transformation based on total number of T_1 seeds and number of plants with positive PCR reaction using both primers was 2.5 % for pCAMBIA 1301 and 3.9 % for ptjk 142 plants.

Table 3: Summary of the PCR analysis of T_1 phosphinotricin and hygromycin resistant plants transformed with plasmids ptjk 142 and pCAMBIA 1301, respectively.

Plasmid used	**T_1 plant**	**Gus PCR**	**Bar PCR**
ptjk 142	G5	+	+
	G7	-	-
	G13	+	+
	G15	-	+
	G16	+	+
	G17	+	+
	G18	+	+
	G19	-	-
pCAMBIA 1301	T_1 plant	Gus PCR	Hygromycin resistant progeny
	G6	+	ND
	G10	-	+
	G11	-	+
	G14	-	+

ND = Not determined

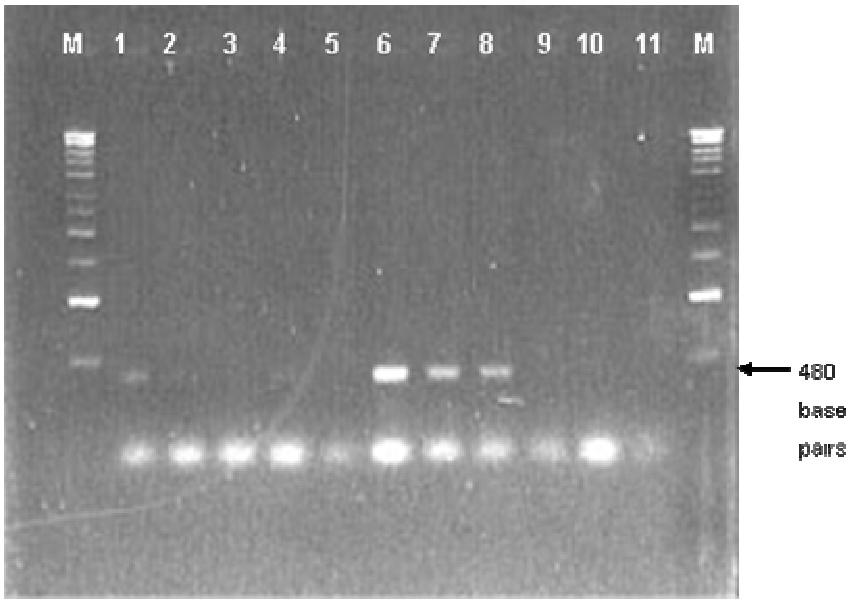


Figure 8: Gel electrophoresis of PCR amplified DNA fragments of Gus gene from T1 progenies of plants infiltrated with *Agrobacterium* containing the ptjk 142 plasmid.

Lane 1 = Positive plasmid control , Lanes 2-9 = DNA from T1 plants, Lanes 10-11 = Untransformed cowpea DNA negative and water controls, M = 1Kb Ladder DNA marker

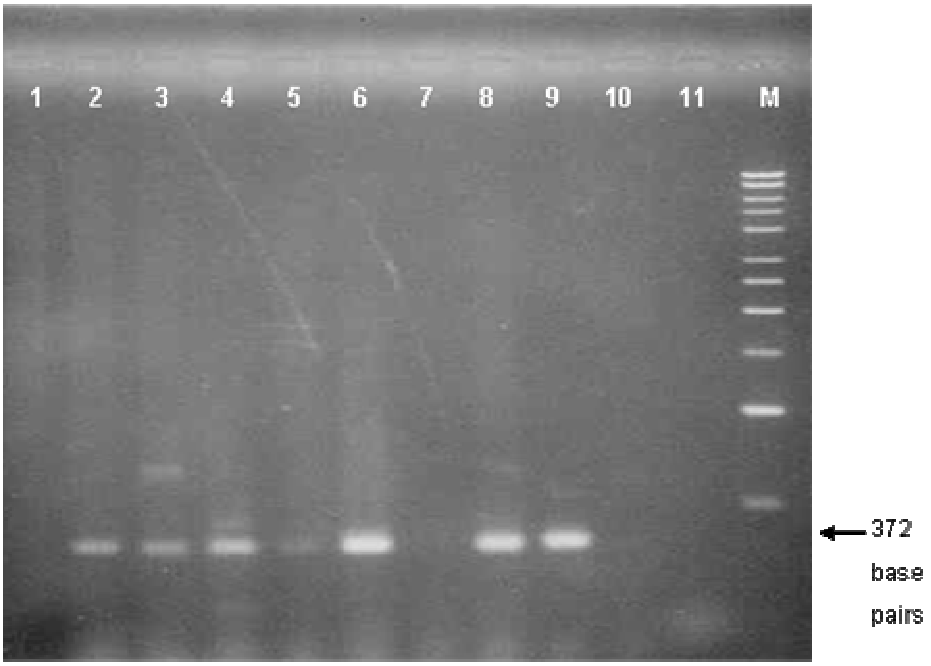


Figure 9: Gel electrophoresis of PCR amplified DNA fragments of Bar gene from T1 progeny of plants infiltrated with *Agrobacterium* containing the ptjk 142 plasmid.

Lanes 1-8 = DNA from T1 plants, Lane 9 = Positive plasmid control, Lanes 10-11 = Untransformed cowpea DNA negative and water controls, M = 1Kb Ladder DNA marker

DISCUSSION

Until recently cowpea transformation with successful in vitro regeneration of transgenic progeny was extremely difficult. Infiltration with Agrobacterium is a method that can achieve stable gene transfer without tissue culture regeneration and also reduce the time interval for obtaining transgenics (Grabowska & Filipeki, 2004). In line with the aim of this work total time spent in culture was reduced to twenty one to twenty three days compared to about 5 months (Popelka et al., 2006; Chaudhury et al., 2007) and plantlets were produced without de novo regeneration.

Cocultivation of explants on selection-free media was done according to Trieu *et al*. (2000). Antibiotic screening of T1 seeds made identification of transformed progeny easy but the ratio of antibiotic resistant to susceptible did not obey Mendelian segregation pattern, which suggests that germline transformation was very low. The results of PCR analyses indicate that the Gus and bar genes were integrated in cowpea progenies.

According to Grabowska and Filipecki (2004), the number of infiltrations, Agrobacterium inoculation medium composition, the application of vacuum during transformation, and developmental stage of the transformed plants are among the important factors which influence the efficiency of transformation during *Agrobacterium* infiltration of plants.

Bechtold *et al.*, (1993) and Clough and Bent (1998) made use of MS medium containing BAP, sucrose and surfactant Silwet L-77 which reduces the surface tension better than most surfactants and keeps bacterial suspension longer on plant thus improving penetration of the bacteria into the intercellular spaces.

In addition to sucrose and silwet, another important component of inoculation media which significantly increases transgene expression is acetosyringone. The level of *Agrobacterium* infection in plants has been linked to the activities of virulence (*vir*) genes in the Ti plasmid of *Agrobacterium tumefaciens* which are induced by plant phenolic compounds like acetosyringone and sinapyl alcohol (Zambryski, 1992; Nan *et. al*., 1997) . In this study it was observed that the presence of silwet in the medium alone resulted in higher transient gene expression than acetosyringone. However, the presence of the two resulted in a greater transformation rate than either of them singly. This observation might suggest that the enhanced penetration of *Agrobacterium* into the cell walls and intercellular spaces by silwet is a more important determinant of transformation in cowpea embryos than induction of virulence genes through acetosyringone. These observations are supported by results obtained with *Arabidopsis* transformation (Clough & Bent, 1998) as the presence of this surfactant doubled the transformation efficiency

Agrobacterium-infiltration has been successfully used for the stable transformation of recalcitrant plants such as *Triticum aestivum, Pinus radiate*, banana, Lentil, radish and *Medicago truncatula* (Amoah *et al*., 2000; Trieu *et.al.*, 2000; Curtis & Nam, 2001;Charity *et al*., 2002; Mahmoudian *et al*., 2002; Acereto-Escoffie *et al*.,2005).

The present study showed that the presence of acetosyringone in both inoculation and cocultivation media is better than when in inoculation medium alone. This implies that continuous exposure of cowpea explants after inoculation to acetosyringone is likely to produce much higher transformation frequencies.

Dosage-response experiments utilising cowpea embryonic axes have not been well documented. Dekeyser *et.al.* (1989) evaluated a number of selectable markers for rice transformation. Their results indicated that hygromycin and phosphinotricin were highly effective as selective agents. However Christou *et al.* (1991) has cautioned that the number of escapes (i.e non-transformed plants that survive selective pressure) should be limited. This study, by evaluating a range of concentrations, has shown that cowpea progeny embryos can be selected at 5 mg/l Bialaphos (Phosphinotricin) and 50 mg/l hygromycin within two weeks during which all non-transformed controls had died. Earlier on, Kononowicz *et.al.* (1997) while carrying out a dose-response experiment with cowpea embryos and cotyledons concluded that 1 mg/l bialaphos was lethal for cowpea when used for continuous selection and regeneration of explants bombarded with the bar gene. Putative transformants from their experiment were claimed to be southern positive when hybridised with a bar probe although the evidence did not seem convincing (Machuka *et. al*., 2002). However, sensitivity of plants to selective agents and effectiveness of the selection process does not depend on the amount of phytotoxin used only but also on the type of explant used and the duration of the selection period (Galum & Breiman, 1997).

CONCLUSION

The methodologies of Trieu *et.al.* (2000) and Clough and Bent (1998) who infiltrated *Medicago truncatula* and *Arabidopsis thaliana* respectively *in planta* and allowed them to set large numbers of seeds that were later subjected to selection on predetermined antibiotic dosages was adopted. Molecular analyses confirm that progenies obtained were transformed. Cowpea embryos infiltrated with *Agrobacterium tumefaciens* carrying the hygromycin resistance and bar (PPT) genes gave rise to T_1 progenies that survived 50 mg/l hygromycin and 5 mg/l bialaphos (phosphinotricin). These were confirmed by polymerase chain reaction analysis to contain the inserted gene sequences. The presence of a plant intron (Vancanneyt *et.al.*,1990) that eliminates Gus activity in *A. tumefaciens* in these plasmids makes it unlikely that the PCR amplification of DNA from transformed and antibiotic resistant progeny are a result of bacterial contamination. In summary, this work demonstrates the inheritance of foreign genes in cowpea progeny through *Agrobacterium* infiltration and the beneficial effect of acetosyringone and silwet inclusion in the inoculation and cocultivation media on transgene expression. It also shows the efficiency of hygromycin and phosphinotricin as selectable markers for cowpea transformation.

REFERENCES

Acereto-Escoffie POM, Chi-Manzanero BH, Echeverria-Echeverria S, Grijalva R, James KA, Gonzalez-Estrada T, Castano E, Rodriguez-Zapata LC, 2005. *Agrobacterium*-mediated transformation of *Musa acuminate* cv. "Grand Nain" scalps by vacuum infiltration. Scientia Horticulturae 105: 359-371.

Amoah BK, Wu H, Spark C, Jones HD, 2000. Factors influencing Agrobacterium-mediated transient expression of uid A gene in wheat inflorescence tissue. Journal of Experimental Botany 52: 1135-1141.

Cangelosi GA, Ankenbauer RG, Nester EW, 1990. Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proceedings Natational Academy Science. 87: 6708-6712.

Charity JA, Holland L, Donaldson SS, Grace L, Walter C, 2002 Agrobacterium-mediated transformation of Pinus radiate organogenic tissue using vacuum infiltration. Plant Cell Tissue Organ Culture 70: 51-60.

Chaudhury D, Madanpotra S, Jaiwal R, Saini R, Kumar PA, Jaiwal PK, 2007 *Agrobacterium tumefaciens*-mediated high frequency genetic transformation of Indian cowpea (*Vigna unguiculata* L. Walp.) cultivar and transmission of transgenes into progeny. Plant Science 172: 692-700.

Christou P, 1992 Genetic engineering and *in vitro* culture of crop legumes. Technomic Publishing, Pennsylvania. 307 pp.

Christou P, Ford TL, Kofron M, 1991 Production of transgenic rice (Oryza sativa L.) plants from agronomically important indica and japonica varieties via electric dischage paricle acceleration of exogenous DNA into immature zygotic embryos. Biotechnology 9: 957-962.

Clough SJ, Bent AF, 1998 Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant Journal 16(6): 735-743.

Curtis IS, Nam HG, 2001 Transgenic radish (Raphanus sativus L. longipinnatus Bailey) by floral dip method plant development and surfactant are important in optimizing transformation efficiency. Molecular Breeding 8: 37-52.

Dekeyser R, Claes B, Marichal M, 1989 Evaluation of selectable markers for rice transformation. Plant Physiology 90: 217-223.

Egnin M, Mora A, Prakash C,1998 Factors enhancing *Agrobacterium tumefaciens*-mediated gene transfer in peanut (*Arachis hypogea* L.). In Vitro Plant Cellular and Developmental Biology 34: 310-318.

FAOSTAT(2005) http://Fao.stat.fao.org cited July, 2008.

Fatokun CA, 2000 Breeding cowpea for resistance to insect pests: attempted crosses between cowpea and Vigna vexillata.Pp52-61 In: C.A Fatokun, S.A. Tarawali, B.B. Singh, P.M. Kormawa and M. Tamo (eds.) Challenges and opportunities for enhancing sustainable cowpea production. Proceedings of the World Cowpea Conference III held at the International Institute of Tropical Agriculture, Ibadan, 4-7 September 2000.

Filippone E, 1990 Genetic transformation of pea and cowpea by co-cultivation of tissues with *Agrobacterium tumefaciens* carrying binary vectors. Pp.175-181, In: Ng, N.Q and Monti, L.M. (eds.) Cowpea Genetic Resources: Contributions in cowpea exploration, evaluation and research from Italy and IITA. IITA, Ibadan.

Galum E, Breiman A, 1997 Transgenic plants. Imperial college press. 376p.

Garcia JA, Hille J, Godbach R, 1986 Transformation of cowpea *Vigna unguiculata* cells with an antibiotic resistance gene using a Ti-plasmid-derived vector. Plant Science 44: 37-46.

Grabowska A, Filipecki M, 2004 Infiltration with agrobacterium- the method for stable transformation avoiding tissue culture. Acta Physiologia Plantarum 26: 451-458.

Horsch RB, Fry JE, Hoffman N, Eichlz D, Rogers SG, Fraley RT, 1985 A simple and general method for transferring genes into plants. Science 277: 1229-1231.

Jackai LEN, Adalla CB, 1997 Pest management practices in cowpea: a review. Pp. 240-258, In: B.B. Singh, D.R. Mohan Raj, K.E.Dashiell, L.E.N. Jackai (eds.) Advances in Cowpea Research. IITA and Japan International Research Centre for Agricultural Sciences (JIRCAS). I.I.T.A., Ibadan, Nigeria.

Kononowicz AK, Cheah KT, Narasimhan ML, Murdock LL, Shade RE, Chrispeels MJ, Filippone E, Monti LM, Bressan RA, Hasegawa PM, 1997 Developing a transformation system for cowpea (*Vigna unguiculata* L.Walp). Pp. 361-371, In: B.B. Singh, D.R. Mohan Raj, K.E.Dashiell, L.E.N. Jackai (eds.) Advances in Cowpea Research. IITA and Japan International Research Centre for Agricultural Sciences (JIRCAS). IITA, Ibadan, Nigeria.

Machuka J, Adesoye A, Obembe OO, 2000 Regeneration and Genetic transformation in cowpea. Pp 185-196, In: C.A Fatokun, S.A. Tarawali, B.B. Singh, P.M. Kormawa and M. Tamo (eds.) Challenges and opportunities for enhancing sustainable cowpea production. Proceedings of the World Cowpea Conference III held at the International Institute of Tropical Agriculture, Ibadan, 4-7 September 2000.

Mahmaudian M,Yucel M,Oktem HA, .2002 Transformation of lentil (Lens culinaris M.) cotyledonary nodes by vaccum infiltration of Agrobacterium tumefaciens. Plant Molecular Biology Report 20: 251–7.

Monti LM, Murdock LL, Thottappilly G, 1997. Opportunities for biotechnology in cowpea. Pp. 341-351, In: B.B. Singh, D.R. Mohan Raj, K.E.. Dashiell, and L.E.N. Jackai (eds.) Advances in cowpea research, IITA and Japan International Research Centre for Agricultural Sciences (JIRCAS). IITA, Ibadan, Nigeria.

Muthukumar B, Mariamma M, Gnanam A, 1995 Regeneration of plants from primary leaves of cowpea. Plant Cell Tissue and Organ Culture 42: 153-155.

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

Nan GL, Tang CS, Kuehnle AR, Kado CI, 1997 *Dendrobium* orchids contain an inducer of *Agrobacterium* virulence genes. Physiological and Molecular Plant Pathology 51 (6) : 391-399.

Pellegrineschi A, 1997 *In vitro* plant regeneration via organogenesis of cowpea (*Vigna unguiculata* (L.) Walp.). Plant Cell Reports 17: 89-95.

Pena L, Cervera M, Juarez J, Navarro A, Pina JA, Navarro L, 1997 Genetic transformation of lime (*Citrus aurantifolia* Swing.): factors affecting transformation and regeneration. Plant Cell Reports 16 :731-737.

Popelka JC, Gollasch S, Moore A, Molvig L, Higgins TJV, 2006 Genetic transformation of cowpea (Vigna unguiculata L.Walp)and stable transmission of transgenes to progeny. Plant Cell Reports 25:304-312.

Popelka JC, Terryn N, Higgins TJV, 2004 Gene technology for grain legumes: Can It contribute to food challenge in developing countries? Plant Science 167:196-206.

Sahoo L, Sugla T, Jaiwal PK, 2003 In vitro regeneration and transformation of cowpea, mungbean, urdbean, and azukibean. In:Biotechnology for the improvement of legumes Part-B. Kluwer Acad. Publishers, Netherlands, pp.89-120.

Singh BB, Ehlers JD, Sharma B, Freire-Filho FR, 2000 Recent progress in cowpea breeding. pp.22-40.In: Proceedings of World Cowpea Conference III, IITA, Ibadan, Nigeria, 4-7 September.

Solleti SK, Bakshi S, Sahoo L, 2008 Additional virulence genes in conjunction with efficient selection scheme, and compatible culture regime enhance recovery of stable transgenic plants in cowpea via Agrobacterium tumefaciens-mediated transformation. Journal of Biotechnology 135:97-104.

Trieu AT, Burleigh SH, Kardailsky IV, Maldonado-Mendoza IE, Versaw WK, Blaylock LA, Shin H, Chiou T, Katagi H, Dewbare GR, Weigel D, Harrison MJ, 2000 Transformation of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*. Plant Journal 22 (6): 531-541.

Vancanneyt G, Schmidt R, Connor-sanchez AO, Willmitzer L, Rocha-sosa M, 1990 Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Molecular General Genetics 220 :245-250.

Walden R, Wingender R, 1995 Gene transfer and plant regeneration techniques. Trends in Biotechnology. 13: 324-331.

Wu R, Sparks C, Amoah BK, Jones HD, 2003 Factors influencing successful Agrobacterium-mediated. Genetic transformation of wheat**.** Plant Cell Reports 21:259-268.

Zambryski P, 1992 Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annual Review Plant Physiology and Plant Molecular Biology 43: 465-490.

Zhang Z, Coyne DP, Mitra A, 1997 Factors affecting *Agrobacterium*-mediated transformation of common bean. Journal American Society Horticultural Science 122 (3): 300-305.