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Full Length Research Paper

Factors Affecting *Agrobacterium*-mediated Transformation and Callus Induction of Sudan's Cotton Genotypes

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The objective of this study was to optimize conditions affecting transformation of insect resistance gene (*cry 1Ac*) into locally grown cotton cultivars (Hamid, Barakat- 90) using a binary *Agrobacterium tumefaciens* vector. The highest percentages (99.33 and 85.67%) of callus induction from hypocotyls of Hamid and Barakat- 90, respectively were observed in MS medium supplemented with 2 mg/L 2-isopentynyl adenine (2iP) + 2 mg/ L IAA. Transformation/callus induction protocol for cotton cultivars were developed using *A. tumefaciens* EHA105 strain harboring the pCAMBIA 2301 vector which contains a *cry 1Ac* gene coding for insecticide resistance under the transcriptional control of cauliflower mosaic virus 35S promoter. Results showed that 16.1—22.3% of the co-cultivated explants were Hygromycin resistant and were considered as putative transformants. Transformation was further confirmed by GUS histochemical assay and PCR amplification of the *35S* promoter, *Nos* terminator, Hygromycin phosphotransferase (*hpt)* and Kanamycin resistance (*nptII*) genes which were found on the inserted construct. *GUS* expression was observed in transformed cotton calli but never in the non-transformants (control). Also, transformed calli gave the target PCR amplicons for all of the examined genes while the untransformed tissues didn't. Transformation percentages of 16.7 for Hamid and 11.9 for Barakat- 90 were reported.

Keywords: Transformation, callus, cotton, *Agrobacterium tumefaciens*.

INTRODUCTION

Cotton is an economically important crop that is grown throughout the world. It belongs to the genus *Gossypium* in the family Malvaceae. The genus comprises 50 species four of which are cultivated *G. arboretum* L. and *G. herbaceaum* L. are diploid (2n=26), while *G. barbadense* and the most widely grown species *G. hirsutum* L. are tetraploid with 2n=52 (Kumeria et al. 2003). Cotton is considered as a source of fiber, food and feed. It has been estimated that 180 million people depend on cotton fiber production (Ganesan et al. 2009). More than three hundred thousand families in the Sudan depend on cotton for earning their livelihood. Several other thousands are engaged in cotton related activities (Abdellatef and Khalafalla 2008). Sudan produces two types of cotton: the Egyptian and the American cotton (Abdelrahman and Abdalla 2006). Cotton in Sudan is attacked by a complex of

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insect pests. The normal practice to combat these insects is insecticide spraying. Insecticides from different chemical classes are generally used. However, the number of sprays per season kept increasing, reaching a maximum of 9.25 sprays (Abdelgader 2012). This might be attributed to a reduced efficacy of the used insecticides as a result of resistance development by the major cotton pests and/or the rapid recovery of treated pests as a result of destruction of their natural enemies (Edge et al. 2001; Wu and Guo 2003). Decreasing the use of pesticide through the development of insect resistant cotton varieties remains a major aim of plant breeding and biotechnology (Pannetier et al. 1997). The use of conventional plant breeding to introduce insect resistance genes into cotton has been successful. However, the process is limited by the long time it takes to develop improved varieties through back crossing and phenotypic selection (Ouma et al. 2004). The availability of genetic engineering technique to bring changes at the molecular level is an additional tool in the hand of breeders who have been limited by the viable genetic material with which they could work (Hussain et al. 2007). Although plant transformation is an attractive mean for developing insect resistant cotton varieties but its use requires an effective regeneration system from a somatic tissue. Cotton, like many plants, is recalcitrant to regeneration from protoplast, leaf or callus tissue and only few cultivars such as Cocker and Siokra are regenerable (Feng et al. 1998, Kumeria et al. 2003). Although regeneration efficiency via somatic embryogenesis has been improved in cotton in the past years (Aydin et al. 2004; Sun et al. 2006; Khan et al. 2006; Kouakou et al. 2007; Divya et al. 2008; Michel et al. 2008; Wang et al. 2008, Han et al. 2009; Hussain et al. 2009), genotype-dependent responses and prolonged culture periods still remain two of the major problems associated with cotton regeneration (Ozyigit and Gozukirmizi 2008). The objective of this study is to develop transformation protocol for Sudanese cotton cultivars using Cambia construct harboring *Bt* gene.

MATERIALS AND METHODS

Before surface sterilization, cottonseeds were kept under flowing tap water for 1 hour and they were surface sterilized by immersion in 70 % ethanol for 3 minutes, followed by stirring in 20 % commercial bleach for 20 minutes. The surface sterilized seeds were rinsed 3 times with sterile distilled water for 5 minutes and they were dried onto filter papers. Hypocotyl (1 cm long) explants were excised from 5-7 days old seedlings. Hypocotyls were isolated by cutting at the base of the cotyledons and were then placed horizontally on the surface of the regeneration medium.

Callus induction

Hypocotyl explants of cotton cultivars (Hamid and Barakat-90) were placed in MS medium supplemented with 30 g/L sucrose plus one of the following growth regulators combinations:

a. 0.1 mg/L 2, 4- D + 0.5 mg/L Kinetin.
b. 0.1 mg/L 2, 4- D + 1mg/L Kinetin.
c. 2.5 mg/L NAA + 2mg/L Kinetin + 0.25 mg/L BA.
d. 2mg/L 2iP + 2 mg/L IAA.

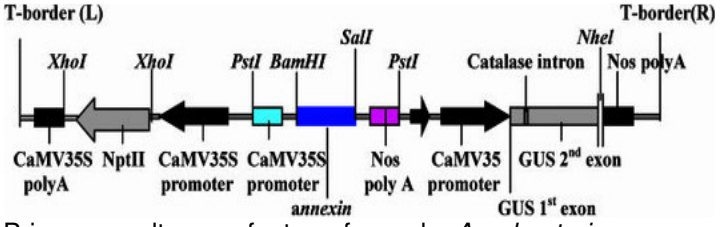
The percentages of callus induction were calculated and recorded. After about three weeks of incubation, developed calli were sub-cultured on fresh medium for another four weeks for proliferation.

Agrobacterium- mediated cotton transformation

Depending on the callus induction optimization results, the combination of 2mg/L 2iP + 2 mg/L IAA were used for transformation studies.

Agrobacterium strain

For transformation, a gene coding for an insecticidal protein (*cry 1Ac*), cloned in *A. tumefaciens* EHA105 strain was used. The construct containing the *cry1Ac* gene been inserted into a pCAMBIA 2301 vector which was then cloned to the mentioned *A. tumefaciens* strain. The *cry1Ac* gene was under the transcriptional control of cauliflower mosaic virus 35S promoter (CaMV-35S) and includes a nopaline synthase gene (*Nos*) as a terminator, Hygromycin phosphotransferase (*hpt)* and neomycin phosphotransferase II (*npt*II) genes; conferring Kanamycin and Hygromycin resistance, respectively; as selection markers for transformant plants and bacteria, respectively and GUS-intron (*uidA*-β-glucuronidase) as a reporter gene, a diagrammatic representation of the construct is as follows:



Primary culture of transformed *Agrobacterium* was prepared by inoculating 30 ml of LB medium with a single colony and 100mg/L of Kanamycin was added. Inoculated medium was then incubated at 28°C and 120 rpm for 48 h. The secondary culture was initiated by inoculating 1 ml of the primary culture in 50 ml of LB medium without antibiotic. The culture was then incubated at 28°C and 120 rpm for 48 h. The culture was then diluted to the target O.D by adding MS basal medium.

Minimum Inhibitory Concentration (MIC) of Cefotaxime for bacteria

MIC for Cefotaxime was determined by the plate dilution method (Andrews, 2001). 100, 200, 300, 400 and 500mg/L of Cefotaxime were studied. The minimum concentration of Cefotaxime that inhibited the growth of *Agrobacterium* was used in the selection medium.

Conditions' optimization for enhanced transformation process

The effect of eight different parameters on transformation and regeneration were studied in a series of experiments. The parameters studied were: Explants pre-culture period, *Agrobacterium* concentration and infection (immersion of explants in bacterial suspension) period, acetosyringone inclusion, co- cultivation period, Cefotaxime concentration and sensitivity of explants to Hygromycin.

Effect of pre-culture period

Explants from 5-6 day-old seedlings were pre-cultured in a regeneration medium for 24, 48 and 72 hours to test for the best pre-culture period to be used in the transformation experiments.

Effects of bacteria concentration and infection period

Explants were dipped in different concentrations (OD 600= 0.74, 0.57, 0.48, 0.39) of *Agrobacterium* culture for 10, 20 or 30 min. The number of surviving explants was recorded after 13 - 15 days of incubation.

Effects of acetosyringone and co- cultivation period

Depending on the results obtained from the last experiment, explants were treated with a concentration of 0.4 (OD_{600}) *Agrobacterium* inoculum for 10 min. Cultured explants were blotted dry and co-cultivated on the co-cultivation medium (callus induction medium) or in co-cultivation medium supplemented with 40mg/L acetosyringone and incubated at 28 ºC under dark conditions for 48 and 72 hours. Numbers and % of survived explants were recorded.

Effect of Cefotaxime on the frequency of callus induction

Different concentrations (100, 200, 300,400, 500 mg/ L) of Cefotaxime were tested for their effect on callus induction. The cultured explants were incubated for 4 weeks at 16-hrs photoperiod at 50µmol/m^2/S, with day/night temperature of 20 - 25°C and the number of explants induced callus was recorded.

Determination of lethal dose of Kanamycin

The lethal dose (LD) for Kanamycin was determined by inoculating hypocotyl explants on MS medium supplemented with various concentrations of Kanamycin (0, 30, 50, and 75mg/ L). The percentages of survived explants were recorded after four weeks.

Sensitivity of cotton explants to Hygromycin:

To screen for an appropriate Hygromycin concentration for transformants' selection, 50 explants were transferred to the selection medium supplemented with different concentrations of Hygromycin (5, 15, 25, 35, 50 mg/ L). The explants were incubated at a 16/8 hrs (light/dark) photoperiod at 25ºC. The number of explants that turned brown was recorded after 3 weeks and % of browning was calculated.

Transformation and callus induction

After 48 hrs of pre-culturing, explants were carefully submerged in *Agrobacterium* inoculums (OD_{600}= 0.38-0.49) with gentle swinging for 10 min. The explants were dried on sterile filter papers and transferred to the co-cultivation medium and incubated, in the dark**,** at 28°C for 48 hrs. Explants were then washed by washing medium (liquid MS medium), dried on sterile filter papers and transferred to plates containing the selection medium1 (MS medium supplemented with 2 mg/L 2iP+ 2 mg/L IAA+ 500 mg/L Cefotaxime). The explants were left to regenerate at 25°C, with a 16/8 hrs (light/dark) photoperiod in the culture room. After 10 days explants were transferred to the selection medium2 (MS supplemented with 2.0 mg/L 2iP+ 2 mg/L IAA+25 mg/L Hygromycin+ 50 mg/L Kanamycin). Survivor explants were transferred weekly to a fresh selection medium2 for 4-6 weeks. The fresh calli were sub-cultured in a callus proliferation medium. A set of explants which were not co-cultivated with *Agrobacterium* were also regenerated, as described above, as a control. Transformation frequency was expressed as a percentage of the number of transformed explants recovered relative to the total number of those incubated. Any proliferated callus was considered as putative transformants.

All culture media used in transformation and callus induction experiments were solidified with 2mg/L gelrite (except for the washing medium) and its pH was adjusted to 5.7.

Authentication of putative transformants

To verify the presence of the introduced construct in the putative transformants (Hygromycin resistant explants), both histochemical and molecular techniques were utilized. The chromosomal DNA of plants obtained from cocultivated explants, control plants and plasmid DNA

Table 1: Percentages of callus induction from hypocotyl explants by different growth regulators

Growth regulators combination	* Explants developing into calli (%)	
	Hamid	Barakat-90
0.1mg/L 2, 4 D+ 0.5mg/L Kt	34	57.67
0.1mg/L 2,4- D + 1mg/L Kt	50.33	24.67
2.5mg/L NAA+ 2 mg/L Kt+ 0.25 BA	39.33	55.33
2mg/L 2iP+ 2 mg/L IAA	85.67	99.33

Plate 1: Callus induction of hypocotyl explants in 2iP- supplemented medium.

were used as template for PCR confirmation of the targeted *cry1Ac* specific gene. Four primers were used to amplify four regions: The cauliflower mosaic virus *35S* promoter (CaMV-35S), nopaline synthase *Nos* terminator, Hygromycin phosphotransferase (*hpt)* gene and neomycin phosphotransferase II (*nptII*).

RESULTS AND DISCUSSION

Callus induction from hypocotyls

Hypocotyl explants from each cultivar were compared for their abilities to develop calli using four different growth regulators combinations, results are shown in Table 1. Highest callus formation for both cultivars was recorded in 2iP- medium (99.33 and 85.67% for Barakat-90 and Hamid cultivar, respectively). Callus formation was more vigorous in 2 mg/L 2iP +2 mg/L IAA (Plate 1). 2iP was previously reported to be effective for callus induction **(**Trolinder and Goodin, 1987; Nobre et al. 2001; Khan et al. 2010). Callus developed well in the presence of 2.5 mg/L NAA + 2 mg/L Kinetin+ 0.25 mg/L BA. Tripathy and Reddy (2002) reported 98% callus initiation in a hormonal combination of 0.25 mg/L NAA, 2mg/L Kinetin and 0.2 mg/L BA.

It should be emphasized that root formation was observed before callus induction in 2,4- D, Kinetin-supplemented medium (Plate 2). Trolinder and Goodin (1987) reported that root formation decreased the amount of available embryogenic calli. Different hormonal combinations of auxin (2,4-D) and cytokinin (Kinetin) at varying concentrations have been successfully used for callus induction in basal MS medium (Trolinder and Goodin 1987; Kumeria et al. 2003; Rajasekaran et al. 2000;

Plate 2: Rooting of hypocotyl explants in Kinetin- suplemnted medium before callus induction

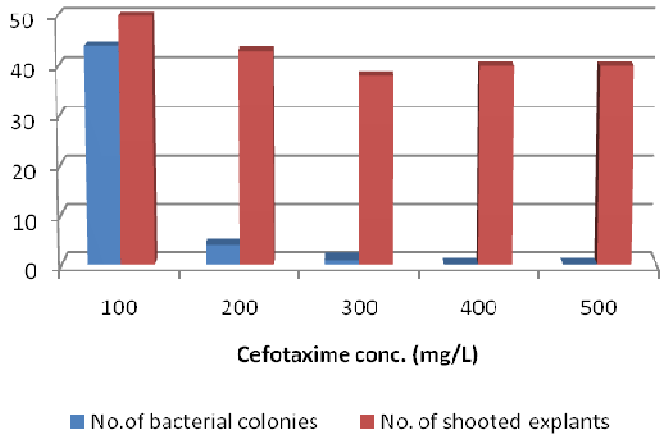


Figure 1: Effect of Cefotaxime on bacterial growth and shooting of explants

Leelavathi et al. 2004; Ikram-ul-Haq and Zafar 2004). Han et al. (2009) obtained the highest callus induction for five recalcitrant cotton cultivars in MS medium supplemented with a combination of 0.1 mg/L Kinetin, 0.1 mg/L 2, 4- D and 0.1 mg/L IBA.

Determination of suitable Cefotaxime concentration for both bacteria and shooting frequency

The effect of various concentrations of Cefotaxime on callus induction and bacterial growth was studied; results are shown in Figure 1. Cefotaxime at a concentration of 500 mg/L eliminated *Agrobacterium* growth. The highest shooting frequency of 50% was observed in Cefotaxime concentration of 100mg/L. However, Cefotaxime at 500 mg/L has resulted in 40% shooting frequency. Hence, Cefotaxime was used in the selection medium in a concentration of 500 mg/L to insure both bacterial elimination and higher shooting percentages. Guo et al. (2007); Li et al. (2009) and Khan et al. (2010) have also used 500 mg/L Cefotaxime in their selection media while Sumithra et al. (2010) used 400mg/L.

Determination of suitable Kanamycin and Hygromycin concentrations in selection medium:

To determine the least concentration of Kanamycin that kill the plant cells, hypocotyls were transferred into a medium containing Kanamycin at 0, 30, 50, and 75 mg/L and the number of elongated shoot apices was counted and recorded each week, results are presented in Figure 2. It is obvious that the number of calli has significantly decreased on MS medium containing kanamycin. Thirty seven percent of shoot apices survived after three weeks in MS medium containing 30mg/L Kanamycin. The minimum lethal concentration that kills all the explants within two weeks was 50 mg/L. Hence, Kanamycin at 50 mg/L was taken as the selection pressure in all subsequent transformation experiments. Fifty mg/ L Kanamycin was previously used for selection of transformed cotton explants by many authors (Tohidfar et al. 2005; Ganesan et al. 2009).

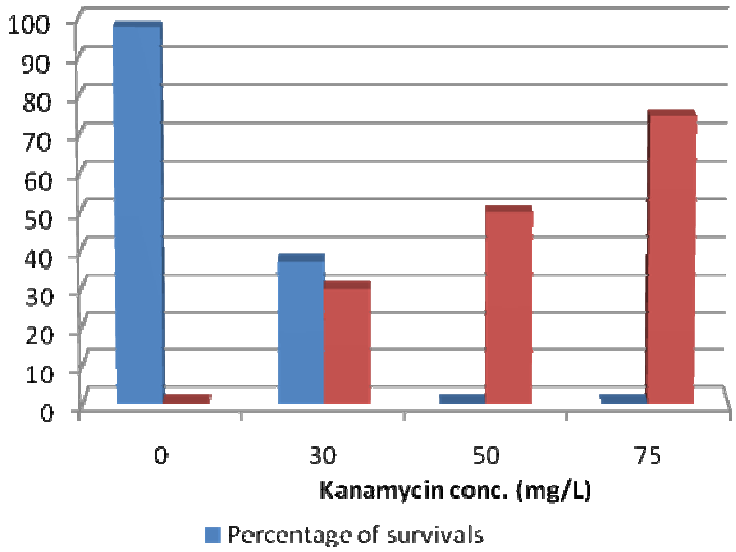


Figure 2: Effect of Kanamycin on explants' survival

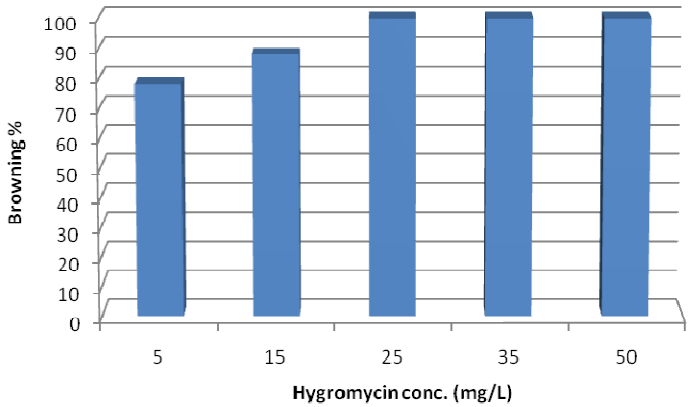


Figure 3: Effect of Hygromycin on explants' survival

Results presented in Figure 3 indicated that at a concentration of 25 mg/ L, Hygromycin killed all of the tested explants. Thus, this concentration was used for selection of transformants. A number of researchers have reported 25mg/L as the effective concentration for selection in many plants including cotton (Kumria et al. 2001; Sawahel 2001, Chaudhry and Rashid 2010). However, Bibi et al. (2013) reported 75 mg/L as a suitable concentration for selection.

Effect of pre- culture period

Pre-culturing of explants for two days in the regeneration medium has resulted in the highest % survivals (76% for hypocotyls explants) after co- cultivation. It seems that pre-culturing renders explants more tolerant to bacterial infection. Paramesh et al. (2010) reported that pre-culturing of explants helped not only in easy handling of explants during transformation process, but also reduces *Agrobacterium* contamination and death before callus formation due to insufficient strength of explants to bear the antibiotic treatments.

Effects of *Agrobacterium* concentration and infection period

Results presented in Figure. 4 and Table 2, showed that both *Agrobacterium* concentration and infection period have a significant effect on the number of surviving explants. The most suitable *Agrobacterium* inoculum density for transformation enhancement and contamination avoidance was found to be in the range of 0.39 - 0.48 OD_{600}. Similar to our results, Jin et al. (2005) reported that increasing *Agrobacterium* concentration did not always increase the transformation rate. This may be due to the fact that too high *Agrobacterium* concentration may lead to *Agrobacterium* overgrowth problems (Plate 3). Also, they reported that *Agrobacterium* at a concentration of 0.5 (OD=600) improved the efficiency of transformation. Contrary, higher concentration (OD600= 1.0–1.5) of

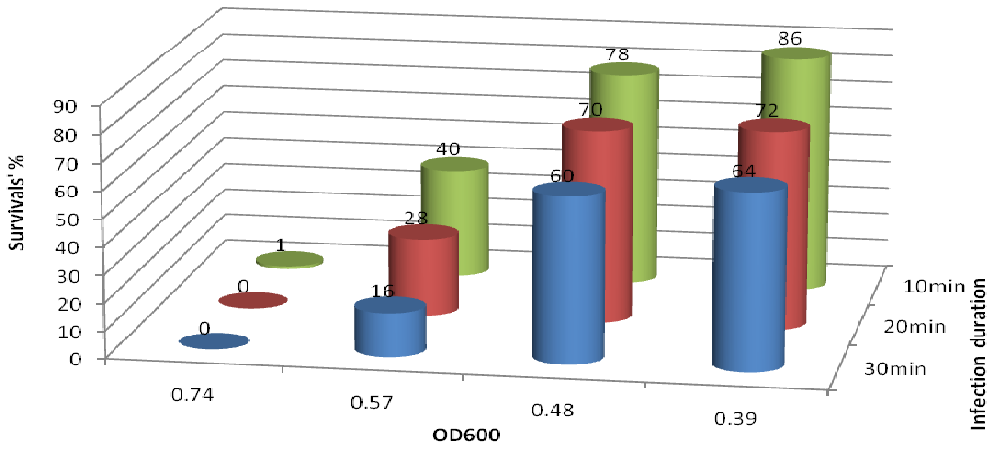


Figure 4: Effects of *Agrobacterium* concentration and infection period on the number of surviving explants

Table 2: ANOVA for the effect of *Agrobacterium* concentration and infection period

Source	df	SS	MS	F value
Agrobacterium concentration	3	2515.333	838.444	65.192**
Infection period	8	158.167	79.083	6.149*
Intercept	1	5125.333	5125.333	398.514**
Error	96	77.167	12.861	
Total	108	7876.000		

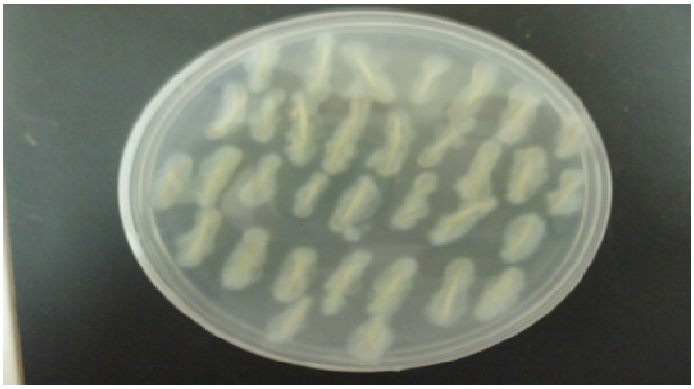


Plate 3: Effect of OD_{600}>0.48 *Agrobacterium* density on explants' survival (note the excessive overgrowth associated with *Agrobacterium*)

Agrobacterium cells improved the efficiency of transformation, as reported by Hamilton et al. (1996) and Amoach et al. (2001).

On the other hand, at all *Agrobacterium* concentrations an infection period of 10 minutes gave the highest percentage (86%) of surviving explants compared to 20 and 30 min. (Figure. 4). Seven minutes of infection was successfully used for efficient transformation in cotton by Longxu et al. (2005). Similar to our results, Zhao et al. (2006) reported that an infection period of 10 min increases the transformation rate compared to 20 and 30 min. This may be due to *Agrobacterium* overgrowth problems associated with long infection periods. However, Leelavathi et al. (2004) reported a simple and efficient cotton transformation protocol using an infection period of 20 min.

Table 3: Effect of acetosyringone in co- cultivation medium and co- cultivation duration on survival's percentages

Acetosyringone conc. (mg/L)	No. of shoot apices tested	No. and % of surviving explants after 48 hrs. of co-cultivation	No. and % of survivals after 72 hrs of co-cultivation
0	40	7 (17.5%)	0 (0%)
40mg/L	40	21 (52.5%)	4 (10%)

Table 4: ANOVA for the effect of acetosyringone and co- cultivation duration

Source	df	SS	MS	F value
Acetosyringone	1	18.062	18.062	65.192**
Co- cultivation duration	1	33.062	33.062	6.149*
Intercept	1	68.062	68.062	398.514**
Error	13	13.812	1.062	
Total	16	133.000		

Table 5: *Survived explants after 6weeks of incubation in selection media

Cultivar	Type of explants	No. of Total co-cultivated explants	No. of Kanamycin and Hygromycin- resistant explants (putative transformants)	% of putative transformants
Hamid	Hypocotyl	215	48	22.3
Barakat- 90	Hypocotyl	260	42	16.1

*Results based on pooled data of three replicates

Effects of acetosyringone and co-cultivation duration

Acetosyringone is one of the phenolic compounds secreted by wounded plant tissue and is known to be a potent inducer of *Agrobacterium vir* genes (Stachel et al. 1985). Results obtained in Table 3 and 4 indicate that, after 2 days of co- cultivation, the use of acetosyringone significantly increased survivals from 17.5% to 52.5% in acetosyringone- free and acetosyringone- supplemented MS media, respectively. This is similar to the findings of Jin et al. (2005) who reported that acetosyringone significantly improved transformation efficiency. Several reports suggested that acetosyringone inclusion in infection and/ or the co-cultivation media can significantly enhance *Agrobacterium* virulence and consequently transformation efficiency (Sunikumar et al. 1999; Jin et al. 2005; Mogali et al. 2011). However, acetosyringone- free co- cultivation medium was successfully used by Tohidfar *et al*. (2008).

Co- cultivation period also has a significant effect on the percentage of surviving explants in both free and acetosyringone- supplemented MS media. After 72 hours, the percentages of survivals have sharply declined from 52.5% to 10% in acetosyringone- supplemented medium and from 17.5 to 0 % in the acetosyringone- free medium. This sharp reduction may be attributed to the observed *Agrobacterium* overgrowth. The influence of co-cultivation period on *Agrobacterium* mediated transformation has also been reported in cotton and a number of other plant species (Mohan and Krishnamurthy, 2003; Jin et al. 2005; Ganesan et al. 2009). Shuangxia et al. (2005) and Sumithra et al. (2010) reported that co-cultivation duration with 48 h was optimum for transforming embryogenic callus of cotton.

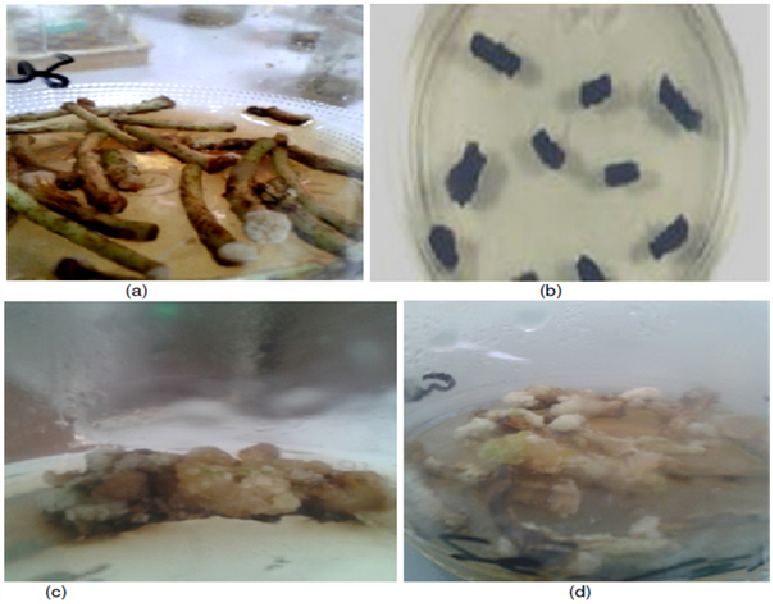


Plate 4: Transformation and callus induction from hypocotyl explants derived from cotton *cv.* Barakat – 90 cultivar:
(a) Induction of calli (*arrow indicated*) on selection medium (b) Control (non- treated explants)
(c) Subculture on selection medium (d) Proliferation on regeneration medium

Development of putative transformants

Protocols for transformation and subsequent callus induction of cotton cultivars have been developed on the basis of the results of the optimization experiments. The efficiency of the developed protocols was evaluated by determination of Hygromycin resistant explants and histochemical analysis of transient and stable GUS-gene expression. Hypocotyl explants of Hamid and Barakat-90 were treated with *Agrobacterium* and cultured on selective MS medium supplemented with 2mg/L 2iP + 2 mg/L IAA. The development of pale white calli at cut ends was observed after 4-5 weeks of incubation (Plate 4 a). The percentages of surviving explants, on Kanamycin-Hygromycin selection medium, of Hamid and Barakat-90 were 22.3 and 16.1%, respectively (Table 5). No callus was obtained in *Agrobacterium* untreated explants (control) where all of the hypocotyls turned black after 1-2 weeks of incubation (Plate 4 b). Proliferation of callus was achieved when it was transferred to a fresh medium for another four weeks (Plate 4 d).

Verification of transformation

Verification based on Gus assay

Survived explants in a medium containing 25mg/L Hygromycin + 50 mg/L Kanamycin showed GUS positive result suggesting that an integrated *GUS* gene was expressed at high levels under the control of the 35S promoter of cauliflower mosaic virus. Control explants which were not co- cultivated with *Agrobacterium* did not show any endogenous GUS activity.

PCR- based transformants' verification

The putative transgenic plants, when screened by PCR using specific primers targeting *35S* promoter, nopaline synthase (*Nos*) terminator, hygromycin phosphotransferase (*hpt)* and neomycin phosphotransferase II (*npt*II) (which confers Kanamycin resistance), gave target (as indicated by primers manufacturers) PCR amplicons; of 200bp , 180bp, 550bp and 500bp, respectively (Plates 5, 6, 7 and 8). The same fragments were detected in the amplified plasmid DNA (positive control) while no such amplicons were observed in untransformed (negative control) plants. This result confirms the successful insertion of the construct harboring the target insecticide resistance (*cry 1Ac*) gene. Based on these results, the percentages of transformation efficiency were calculated and recorded in Table 6. In this table, it is clear that not all of the survived explants proved to be transformants. A few untransformed tissues which were highly chimeric proliferated rapidly due to the lack of complete contact between explants and kanamycin/Hygromycin in the medium (Tohidfar et al. 2005). Transformation was verified by PCR in 75% and 73.8% of the putative transgenic calli of Hamid and Barakat- 90, respectively, that survived Kanamycin and Hygromycin pressure. Thus, the re- calculated transformation frequencies, based on the total co-cultivated explants, of Hamid and Barakat - 90 were

16.7 and 11.9, respectively which were within the range (8.8- 28%) previously reported by Leelavathi et al. (2004). The transformation percentage (16.7) reported for Barakat- 90-derived calli is comparable to the percentage (17.8) reported by Leelavathi et al. (2004) for green-colored cotton using hypocotyl explants. This percentage is less than those (20%-22.2%) reported by Tohidfar et al. (2005) but greater than what has been obtained (8%) by Wu et al. (2008). The variation in transformation % may be attributed to the effect of genotype (Park et al. 2003; Raj et al. 2005), bacterial strain (Islam et al. 2007; Guo et al. 2012) or explants type (Wu et al. 2006).

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