4.0 Result and Discussion

4.1 The effects of 6-BA on *Cryptocoryne willisii* shoots induction

The effects of different concentrations of 6-BA on shoot tip explants of *C. willisii* multiple shoots induction was summarised in **Table 4.1**. The number of normal shoots formed from single-node explants were recorded and analysed after 4 weeks of culture. Data (shown in **Appendix 8**) was analysed statistically and the results were found significantly contrasted using Duncan multiple comparison test (SPSS Inc., USA) at 95% confidence level as detailed in **Appendix 9**. This experiment is crucial for bulking up the target tissues required for transformation experiment and also important to ensure that the transformed explants could be regenerated and propagated into a whole individual plant at high frequency. Successful plant regeneration is a prerequisite for plant transformation (Rup and Sukanya, 1993).

As shown in **Figure 4.1**, the highest number of shoots (5.0 ± 2.0) was formed when shoot tip explants of *C. willisii* were cultured on MS media supplemented with 6 mg/ L of 6-BA (27 μM). The number of shoots formed decreased in MS media supplemented with lower or higher concentrations of 6-BA than 6 mg/ L. Besides, axillary shoots grown on these media were smaller in size as seen in **Table 4.1** (as indicated with dark red arrows). In

contrast, the same age cultures of axiliary shoots induced in media supplemented with 6 mg/ L of 6-BA exhibited better growth (**Figure 4.1**, as indicated with dark indigo arrows).

All of the explants used in the experiments produced varying numbers and sizes of normal shoots. This finding is in accordance with the work done by Sahidin, *et al*. (2007) though different combinations of hormones used in the experiments. However, no roots were formed for all of the *C. willisii* explants cultured in media containing 6-BA alone. Therefore, induced shoots with at least three leaves were then cut and subcultured onto fresh MS basal media without any plant growth regulators (MSO) for roots induction. **Figure 4.2** showed 8 weeks cultures of healthy rooted plantlets of *C. willisii* with approximately 5 cm height. Similiarly, subculturing on MSO media was found to increase the survival rate of adventitious shoots regenerated from the leaf explants of *Echinacea purpurea* (Koroch *et al.*, 2001).

Plant growth regulator (PGR) is a critical component in the media as it determines the developmental pathway of the plant cells (Slater *et al*, 2003). Typically, they were used to promote cell division and cell growth. Cytokinin is a group of PGR that has been widely used to stimulate multiple shoots formation from various species of plants (Slater, 2003). It works in combination with auxins in ratios to give the highest rate on multiple shoots

induction (Alizadeh, 1998; Koroch, 2001). However, cytokinin alone was sufficient to induce the highest numbers of multiple shoots formation as reported by Zaidah and Nazri (2004) in *Orthosiphon aristatus* Boldingh. where 6-BA alone greatly induced adventitious shoot formation.

A simple and efficient method using 6-BA alone was developed for direct multiple shoots induction from shoot tips culture of *C. willisii*. Shoots proliferation and elongation were achieved on the same shoot induction medium without the need of subculturing. Direct shoot organogenesis is often preferred in which it reduces somaclonal variations observed in transgenic plants (Alizadeh *et al.*, 2003).

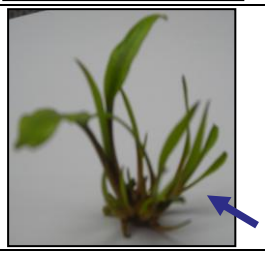
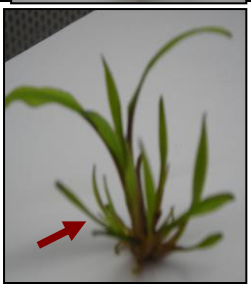
Media supplemented with different concentrations of 6 – BA	Average No. of Shoots Formed From *C. willisii* Shoot Tip Cultures ± Std. Dev	Plates
MS + 2 mg/ L 6–BA	**2.6 ± 1.14**	
MS + 4 mg/ L 6–BA	**3.2 ± 2.05**	
MS + 6 mg/ L 6–BA	**5.0 ± 2.00**	
MS + 8 mg/ L 6–BA	**3.6 ± 1.34**	
MS + 10 mg/ L 6–BA	**3.4 ± 0.89**	

Table 4.1: The effect of different concentration of 6-BA on *C. willisii* shoots induction. Axillary shoots formed (as indicated with dark indigo arrows) on media supplemented with 6 mg/ L 6-BA exhibited better growth in term of size. Smaller shoots indicated with dark red arrows

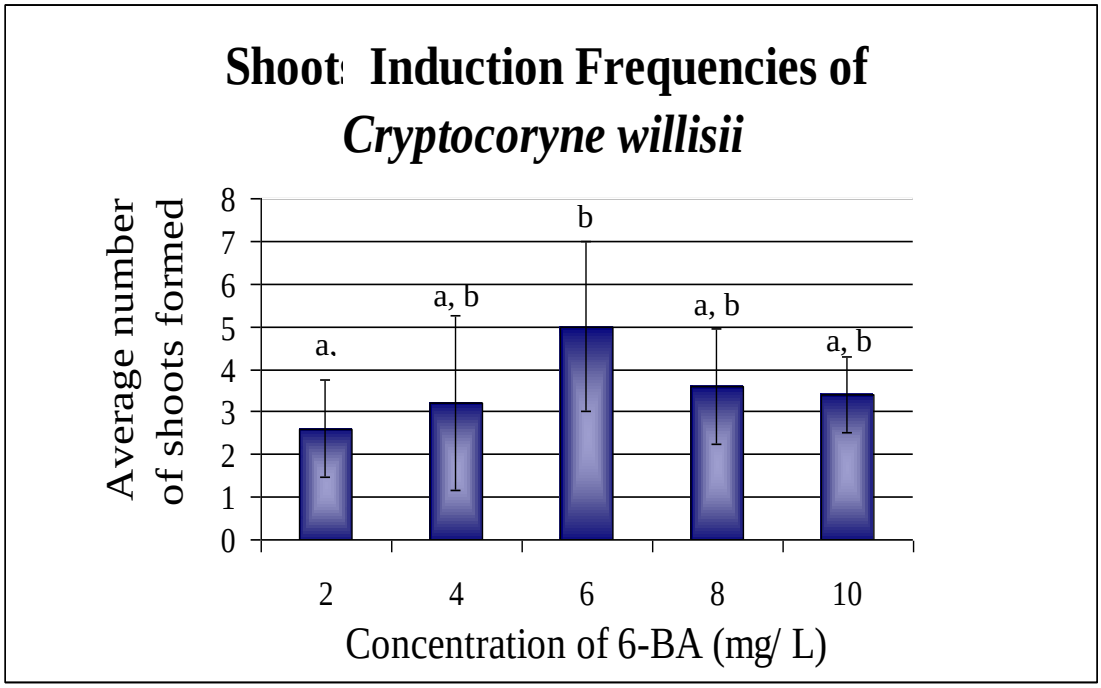


Figure 4.1: Effects of different concentration of 6-BA on multiple shoots formation frequencies of *C. willisii.* Error bars corresponding to standard deviation where n = 5. Different letters indicate values that are significantly different at 95 % confidence level using Duncan multiple comparison test.

Figure 4.2: Rooted plantlet of *C. willisii.*

4.2 *Agrobacterium tumefaciens* strain LBA4404 harbouring the binary vector pCAMBIA1304

4.2.1 Growth curve

Growth curve of *A. tumefaciens* strain LBA4404 harbouring the binary vector pCAMBIA1304 was constructed according to OD values obtained (**Appendix 10**). Growth curve presented on **Figure 4.3** showed three growth phases of the bacteria: lag phase (OD = 0 – 0.15), exponential/ log phase (OD = 0.15 – 1.25), and stationary phase (OD = 1.25 – 1.45). The bacterial cells grew slowly during the first 10 hours (lag phase). The cells grew vigorously during exponential phase of growth after adapting to permissive condition in the presence of adequate nutrients. Thus, exponentially grown cells are often desirable for *Agrobacterium*-mediated transformation. Exponential growth of *A.tumefaciens* LBA4404 harbouring pCAMBIA1304 used in transformation experiments was achieved after 15 hours of culture. The growth of bacterial reached a stationary phase of growth when the available nutrients were exhausted or when the toxic products of metabolism accumulated.

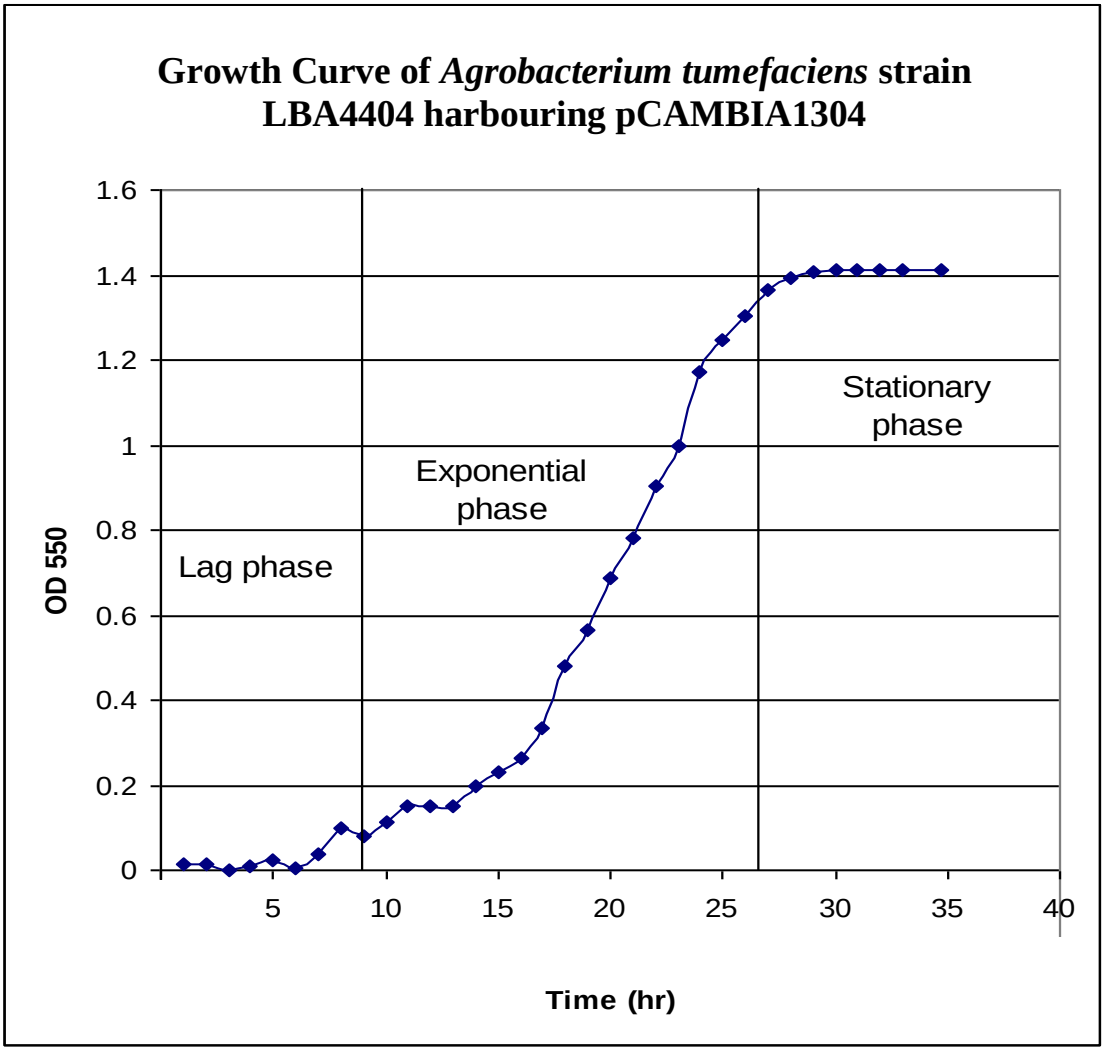


Figure 4.3: Growth curve of *A. tumefaciens* strain LBA4404 harbouring the binary vector pCAMBIA1304

4.2.2 *Agrobacterium* cell density count

Viable cell density was counted using serial dilution spread-plate method. Numbers of single colony forming unit (CFU) were then counted and recorded (**Appendix 11**). Graph was plotted against the incubation times at every 5 hour interval as shown in **Figure 4.4**.

In this study, a selected cell density of 7 X 10^9 cfu/ ml (OD ≈ 0.5) was used in the *Agrobacterium*-mediated plant transformation which correlated to a culture period of ≈ 15 hours. Viable cell density of *A. tumefaciens* is important parameter in plant transformation. Comparison between different concentrations of bacteria was often carried out. De bondt *et al.* (1994) showed that transformation on apple was dependent on *Agrobacterium* concentration. Highest transformation efficiency was achieved by 2.5 X 10^9 cfu/ ml of *Agrobacterium* strain LBA4404. In the study of Yong' s *et al*. study (2006), 1 X 10^7 cfu/ ml of *Agrobacterium* strain LBA4404 showed the highest percentage of GFP-positive transformants on *Melastomataceae* spp.

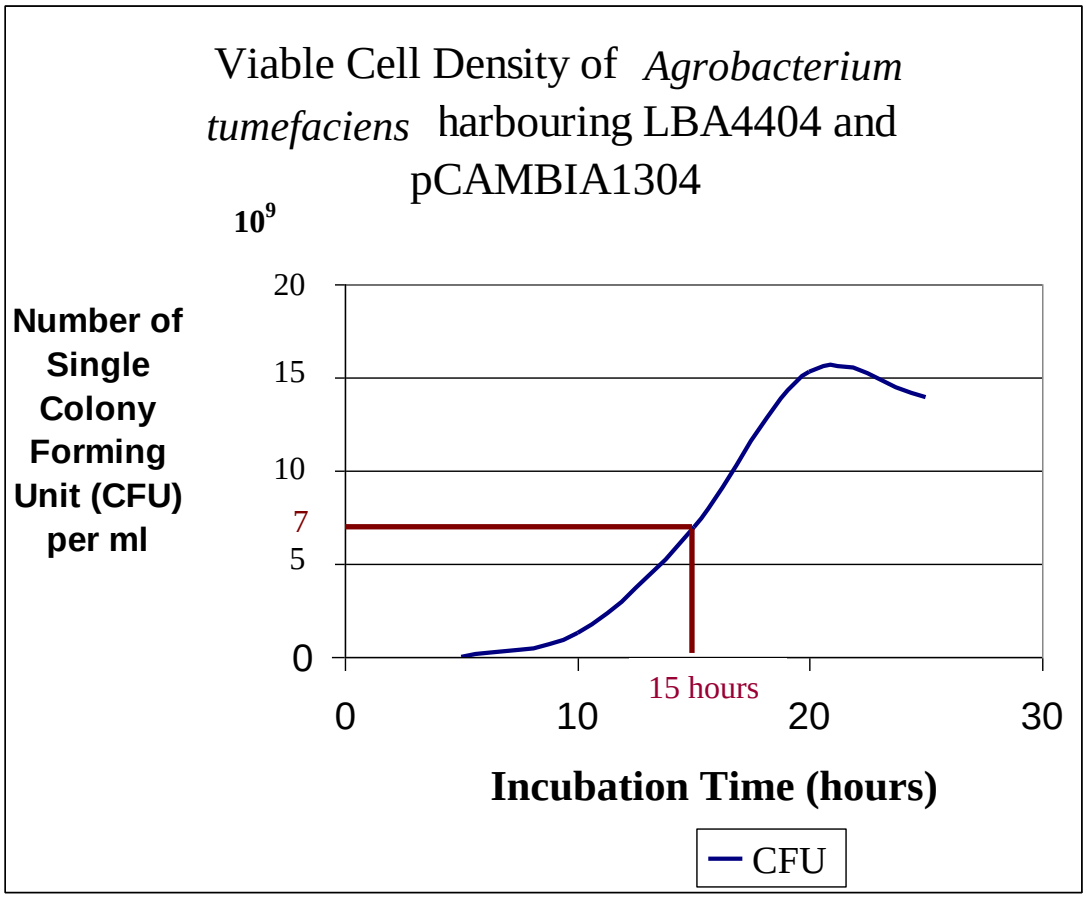


Figure 4.4: Viable cell count of *A. tumefaciens* strain LBA4404 harbouring pCAMBIA1304. Cell density was counted based on CFU per ml which represents the bacteria colony derived form a single ancestor of *A. tumefaciens* in one ml broth culture. Graph was plotted with average number of CFU where n=3

4.2.3 Minimal inhibitory concentration (MIC) of *Agrobacterium*

MIC is the minimal concentration of antibiotics that completely stops the visible bacterial cell growth. Twenty-five combinations of cefotaxime and carbenicilin in different concentrations were assessed for bacteriostatic and bactericidal effects on the *A. tumefaciens* as shown in **Table 4.2**.

The lowest concentration of antibiotics that suppressed the growth of bacteria in broth can be considered to be the MIC where the antibiotic is bacteriostatic. From the results obtained, 16 combinations of cefotaxime and carbenicillin concentrations (columns shown in yellow) can be considered as bacteriostatic to *A. tumefaciens* strain LBA4404 harbouring pCAMBIA1304. These broth cultures of *A. tumefaciens* which showed clear appearance without visible turbidity were then plated onto antibiotics-free YEB agar plate.

Five combinations (columns shown in pink) of cefotaxime and carbenicilin showed no growth of bacteria colony on the antibiotics-free agar plate which can be considered as bactericidal i.e. that killed the bacteria completely at that particular combination and concentration. Eleven combinations of the cefotaxime and carbenicillin were bacteriostatic but not bacteriocidal to the *A. tumefaciens* strain LBA4404 harbouring pCAMBIA1304 **(Table 4.2)**.

From the results obtained, a combination of 150 mg/L of cefotaxime and 200 mg/ L of carbenicilin was identified as the minimal concentration to eliminate the growth of *A. tumefaciens* in later plant transformation experiments.

Elimination of the *Agrobacterium* is vital to minimise false positive results that might arise due to contamination of *Agrobacterium*. False positive results emerged because of the leaky expression under the control of CaMV35S or *nos* promoters (Veluthambi *et al.*, 2003). In this study, leakiness of intron-less *gusA* gene expression driven by CaMV35S promoter in *Agrobacterium* could occur because this reporter gene is of bacterial origin (Jefferson *et al.*, 1987). Hence, putative transformant selection and analysis procedures must be carried out in *agrobacterium* – free condition.

Concentration of Cefotaxime (mg/ L) / Concentration of Carbenicilin (mg/ L)	0	50	100	150	200
0	+ + + +	+ + + +	+ + + +	+	+
50	+ + + +	+ + + +	+ + +	+	+
100	+ + +	+ + +	+ +	+	+
150	+ +	+ +	+	- -	- -
200	+	+	- -	- -	- -

Table 4.2: MIC of *A. tumefaciens* strain LBA4404 harbouring pCAMBIA1304

+ + + +	Vigorous growth of bacteria in broth
+ + +	Moderate growth of bacteria in broth
+ +	Large number of bacteria colony on antibiotic-free agar plate
+	Limited colony of bacteria on antibiotic-free agar plate
- -	No growth of bacteria on antibiotic-free agar plate

Bacteriostatic – Broth culture of *A. tumefaciens* without visible turbidity

Bactericidal – No growth of *A. tumefaciens* on antibiotics-free agar plate

4.2.4 Screening of *A. tumefaciens* strain LBA4404 harbouring the binary vector pCAMBIA1304

4.2.4.1 Preparation of plasmid DNA from bacteria culture

Plasmid DNA was extracted from the bacteria following alkaline lysis method described in **Section 2.2.4**. Extracted plasmids were visualised and checked on 0.8% agarose gel electrophoresis as shown in **Figure 4.5**. Extracted plasmids were then confirmed by Polymerase Chain Reaction (PCR) of *gus* and *mgfp5* reporter genes. (Results as shown in **Section 4.4.2**). Screening of *A. tumefaciens* strain LBA4404 harbouring the binary vector pCAMBIA1304 cultures is crucial prior to plant transformation experiments to verify the presence of the gene of interest in this case, the *gus* and *mgfp5* reporter genes. From the gel picture obtained, 3 bands with different migration rates represent the different conformation of the plasmids which migrate at different rates were observed. Closed circular plasmid DNA is known to migrate faster than nicked circular and linear form of plasmids DNA (Sambrook *et al.*, 1989; Brown, 2002)

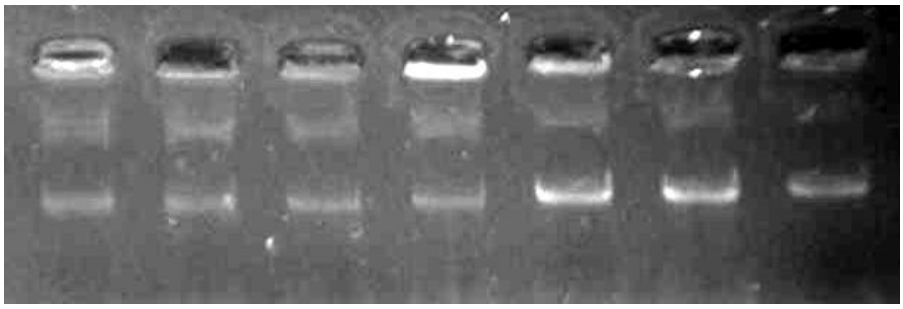


Figure 4.5: Agarose gel electrophoresis visualisation of plasmid DNA extracted from *A. tumefaciens* strain LBA4404 harbouring the binary vector pCAMBIA1304. The expected size of pCAMBIA1304 is 12361bp.

4.3 *Agrobacterium*-mediated plant transformation

4.3.1 Transformation of *Nicotiana benthamiana*

To verify the transformation capability of the vectors and constructs, tobacco plant (*N. benthamiana*) was used as a positive control. Tobacco plants served as model plants for plant transformation experiments because of its ease of regeneration, short generation time and high susceptibility towards *Agrobacterium* infection (Horsch *et al*, 1985).

Transformation was successfully done in leaf explants of *N. bethamiana.* GUS histochemical staining was carried out to investigate transient expression of *gus* reporter gene carried by pCAMBIA1304 in transformed *N. benthamiana* leaf explants (result as shown in **Section 4.3.1.1**). On the other hand, fluorescent visualisation was carried out to examine the *mgfp5* genes expression with fluorescent microscope (as in **Section 4.3.1.2**). The ability of the vectors and constructs to transform the tobacco plants were verified. Transformation of *C. willisii* was then carried out with *A. tumefaciens* strain LBA4404 harbouring the binary vector pCAMBIA1304.

4.3.2 Transformation of *C. wilisii*

Initially, the optimisation of *C. willisii* transformation was carried out using various parameters. This is important because there was no previous data concerning transformation on *C. willisii* yet. Several known factors greatly influence *Agrobacterium*-mediated plant transformation. These include the bacteria strain and concentration, infection time, co-cultivation period and pre-culture duration. Sometimes, the addition of acetosyringone and types of wounding were also taken into consideration for optimisation (Yong *et al.*, 2006; Veluthambi *et al.*, 2003; Aldemita and Hodges, 1996; Dillen *et al.*, 2000). In this study, the effects of infection time and co-cultivation period were investigated for the transformation of *C. willisii*. Infection time and co-cultivation period are known factors that play pivotal roles in determining transformation efficiency in many plant species (Yong *et al.*, 2006; Akbulut *et al.*, 2008). Infection time is important to enhance the attachment of *Agrobacterium* to the plant cells while co-cultivation period is required for the induction of virulence mechanism and gene transfer (Aldemita and Hodges, 1996; Wahlroos *et al.*, 2003).

Transformation of *C. willisii* takes place without any addition of phenolic signalling compound such as acetosyrigone (AS). The addition of AS during co-cultivation period of transformed explants has been routinely

practice in the transformation of recalcitrant crops such as rice (Hiei *et al*., 1994), maize (Ishida *et al.,* 1996), and wheat (Cheng *et al*., 1993). This might suggests that phenolic compounds were produced by the wounded shoot tips cultures of *C. willisii* and released into the culture medium and induce *Agrobacterium* virulence since sufficient amount of phenolic signalling compounds are necessary for the activation of *vir* gene induction of *Agrobacterium* (Li, *et al.,* 2000). This also throws light to the possibility that the aquatic plant *C. willisii* and its related species are being within the natural host range of *Agrobacterium*.

Experiment on the natural tolerance of *C. wiliisii* explants towards hygromycin was carried out. All explants regenerated shoots on media supplemented with hygromycin at concentration of 100, 500, 1000, 1500, 2000 and 5000 µg/ L. An alternative selection procedure was applied by using fluorescent microscopic examination of GFP. GFP-positive transformants were chosen for analysis.

Selection through GFP fluorescent screening served several advantages over antibiotics selection in this study. This alternative can facilitate in minimising false-negative results that occur due to non-specific endogenous resistance activity that may present. Non-specific endogenous resistance towards antibiotics in plants has been reported in chilli (Mok, 2007). Besides,

the influence of antibiotics upon regeneration of transformed plant tissues can be avoided. In the study of Seabra and Pais (1998), differentiation of transgenic chestnut cells were inhibited when higher concentrations of antibiotics selection agent were applied to eliminate non-transformed cells. Furthermore, lost of transgenic explants during antibiotics selection procedure due to the truncation of T – DNA can be evaded. Truncation that occurs during integration resulted in transgenic plants transformed with truncated or part of donor T – DNA (Offringa *et al.*, 1990). As antibiotics resistance gene is located at the left border of T – DNA, transgenic plants that only acquired the right border of T – DNA with reporter genes only will not survived in antibiotics selection procedure. Thus, GFP fluorescent screening of putative transformed plants is feasible and convenient.

4.3.2.1 Effects of infection and co-cultivation period

Expression of *gusA* reporter genes in transformed *C. willisi* was quantified using fluorometric assay after 4 weeks of culture on shoots induction medium. Hydrolysis activity of GUS enzyme towards the substrate MUG giving rise to fluorescent product (4MU) as a signal for GUS enzyme rate measurement. The amount of 4MU present is proportional to the quantity of enzyme expressed and present in transformed plant samples (Jefferson, 1987).

Although the endogenous GUS activity in plants is low (Wilkinson *et al.,* 1994), background noise remains in fluorometic assay of GUS enzyme activity. Hence, a background subtraction of 35 ± 4.30 pmol 4MU/mg/min obtained from control wild type *C. willisii* was deducted for each sample. The sources of background noise are mainly intrinsic fluorescent contributed by endogenous fluorescent compounds (Jefferson, 1987). Fluorometric assay for each parameter was carried out in triplicate and the fluorometric reading was normalised with total protein concentration in each sample. Data was recorded **(Appendix 12)**.

As shown in **Figure 4.6**, transformation of *C. willisii* with 6 mins infection time and co-cultivation period of 1 day showed the highest GUS enzyme activity based on fluorometric assay which was 582.09 ± 84.30 pmol 4MU/mg/min. The enzyme activity was approximately 10 times fold higher than the lowest activity observed which was only 50.57 ± 5.28 pmol 4MU/mg/min when *C. willisii* was infected for 2 minutes and co-cultivated for 2 days.

According to Men *et al.* (2003), two to three days of co-cultivation was required for standard protocol of *Agrobacterium*-mediated transformation. However, one day of co-cultivation period was sufficient to give the highest

GUS enzyme activity in *C. willisii*. This finding was in agreement with Rohini and Sankara (2000) whereby one day (24 hours) co-cultivation of *Carthamus tinctorius* (safflower) and *Agrobacterium* provided the best transformation efficiency. Although general opinion about longer co-cultivation period is superior to short period of time, prolonged co-cultivation might be deleterious to the plant explants and cause necrosis and cell death. De Bondt *et al.* (1994) suggested that a co-cultivation period of not greater than 4 days is favourable to encounter the difficulty in eliminating *Agrobacterium* from plant tissues.

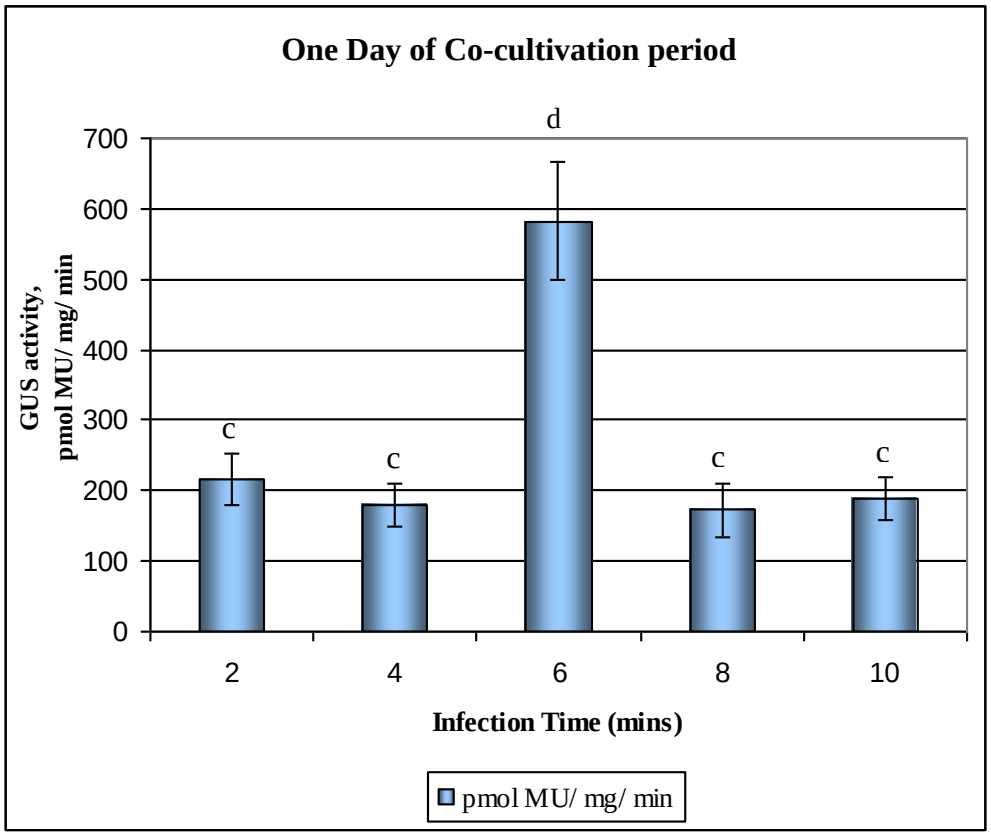


Figure 4.6: Fluorometric assay of GUS enzyme activity for transformed *C. willisii* with different infection times (2, 4, 6, 8, 10 min) and cocultivated with *A. tumefaciens* LBA4404 harbouring pCAMBIA1304 for 1 day. Different letter indicated that the mean values are significantly contrasted at 95 % confidence level. One way ANOVA analysis of the data obtained was summarised in **Appendix 13.** Error bars represent the standard deviations where n = 3.

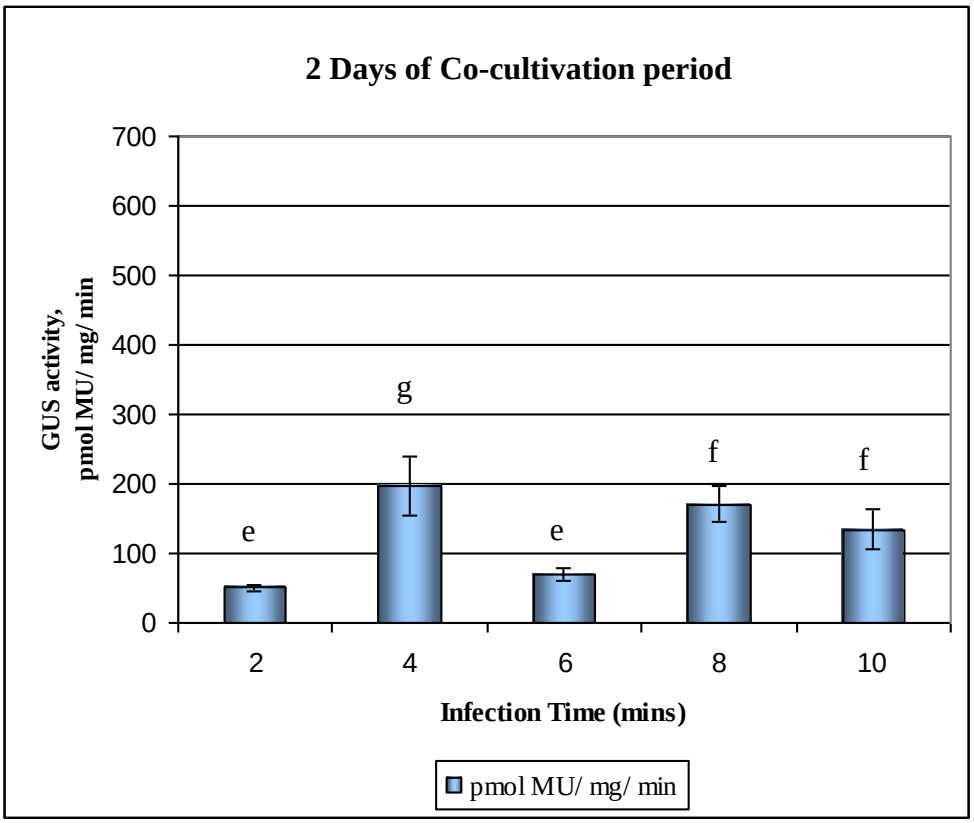


Figure 4.7: Fluorometric assay of GUS enzyme activity for transformed *C. willisii* with different infection times (2, 4, 6, 8, 10 min) and co-cultivated with *A. tumefaciens* LBA4404 harbouring pCAMBIA1304 for 2 days. Different letter indicated that the mean values are significantly contrasted at 95 % confidence level. One way ANOVA analysis of the data obtained was summarised in **Appendix 14**

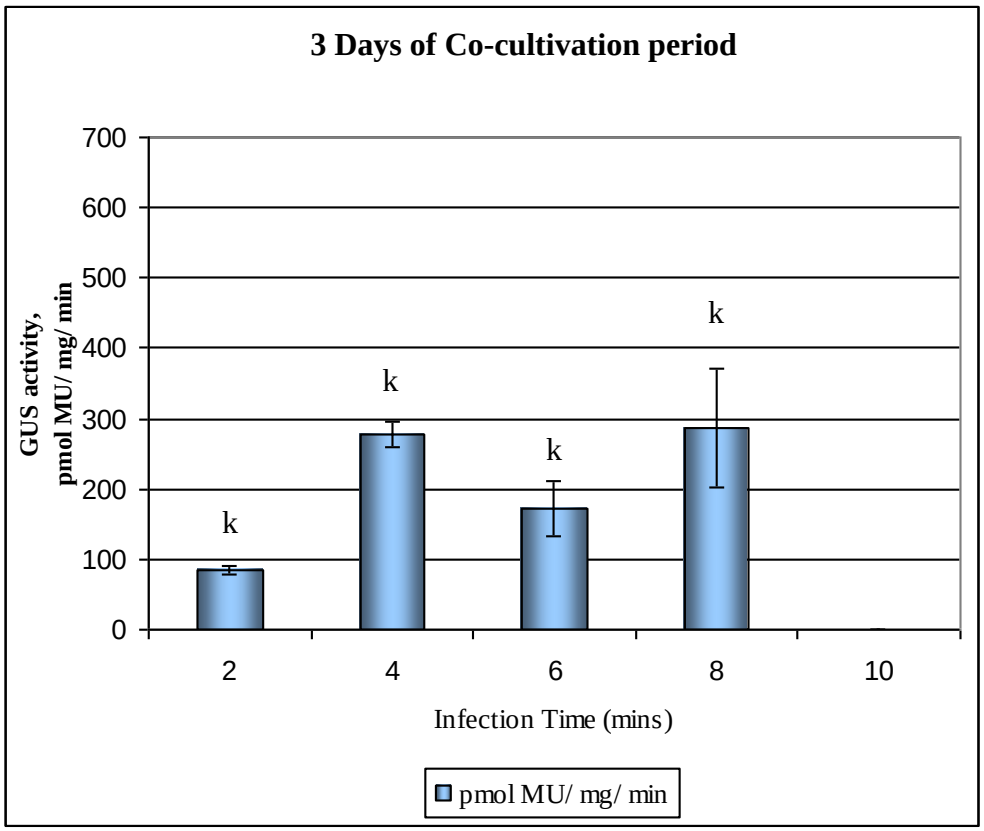


Figure 4.8: Fluorometric assay of GUS enzyme activity for transformed *C. willisii* with different infection times (2, 4, 6, 8, 10 min) and cocultivated with *A. tumefaciens* LBA4404 harbouring pCAMBIA1304 for 3 days. One way ANOVA analysis showed that the results obtained for this experiment was not significant at 95% confidence level **(Appendix 15)**

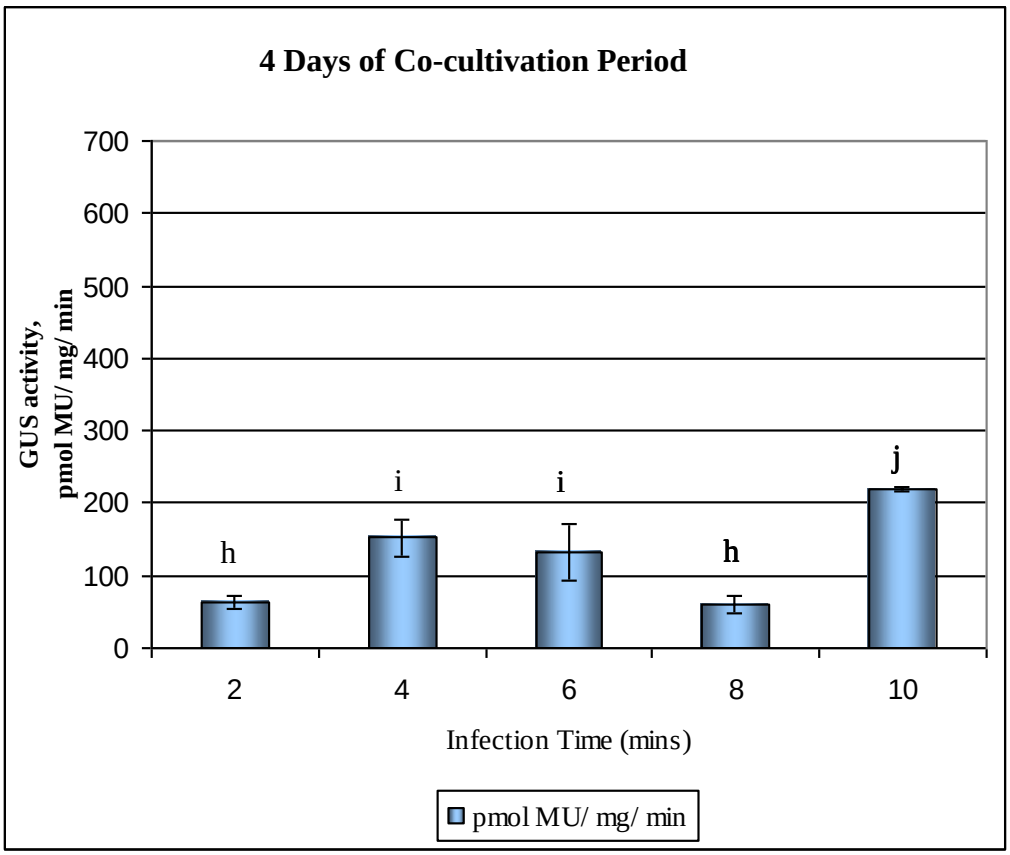


Figure 4.9: Fluorometric assay of GUS enzyme activity for transformed *C. willisii* with different infection times (2, 4, 6, 8, 10 min) and cocultivated with *A. tumefaciens* LBA4404 harbouring pCAMBIA1304 for 4 days. Different letter indicated that the mean values are significantly contrasted. One way ANOVA analysis of the data obtained was summarised in **Appendix 16**

4.3.3 Histochemical assessment of tobacco and *C. willisii*

GUS histochemical staining has been a widely used method to study plant transformation in a variety of plant cells and tissues for instance, leaf tissue of *Arabidopsis* (Lee and Schöffl, 1995); embryogenic callus of rice (Bilang,*et al.*, 1999), seeds, shoot tips and flower buds of Indian cowpea (Chaudhury *et al*., 2006); immature embryos of banana cultivar Mas (Wong *et al*., 2005), and suspension cells of tobacco (Kim *et al*., 2003) and etc. Localisation of the GUS enzyme activity in various plant tissues can be investigated precisely with the aid of histological and microscopical methods (Topping and Lindsey, 1997). It is mainly because GUS activity background is low in plants (Wilkinson *et al*., 1994). And in addition, the substrate is readily taken up by various types of plant cells and tissues.

The substrate used in GUS histochemical staining is X-Gluc which gives rise to a blue precipitate at the site of enzyme activity (Jefferson, 1987). This result from the indoxyl derivative produced undergo an oxidative dimerisation to form insoluble and highly colored blue precipitates. This oxidative dimerisation was enhanced with the addition of K^+ ferricyanide in histochemical staining reagent (Lojda, 1970).

Transient expression of *gusA* gene in tobacco was successfully performed with histochemical staining assessment. Results were shown in **Figure 4.** . Transient GUS activity was found at the edge of transformed tobacco callus tissues **(Figure 4. Ia,Ib, IIa, and IIb)** where injuries were induced and also on root formed from the explants **(Figure 4. III)**. As observed, GUS enzyme activity was uneven in transformed explants of tobacco even though cuts were made surrounding tobacco leaf explants. Untransformed control explants of tobacco did not show any blue coloration when stained with X-Gluc.

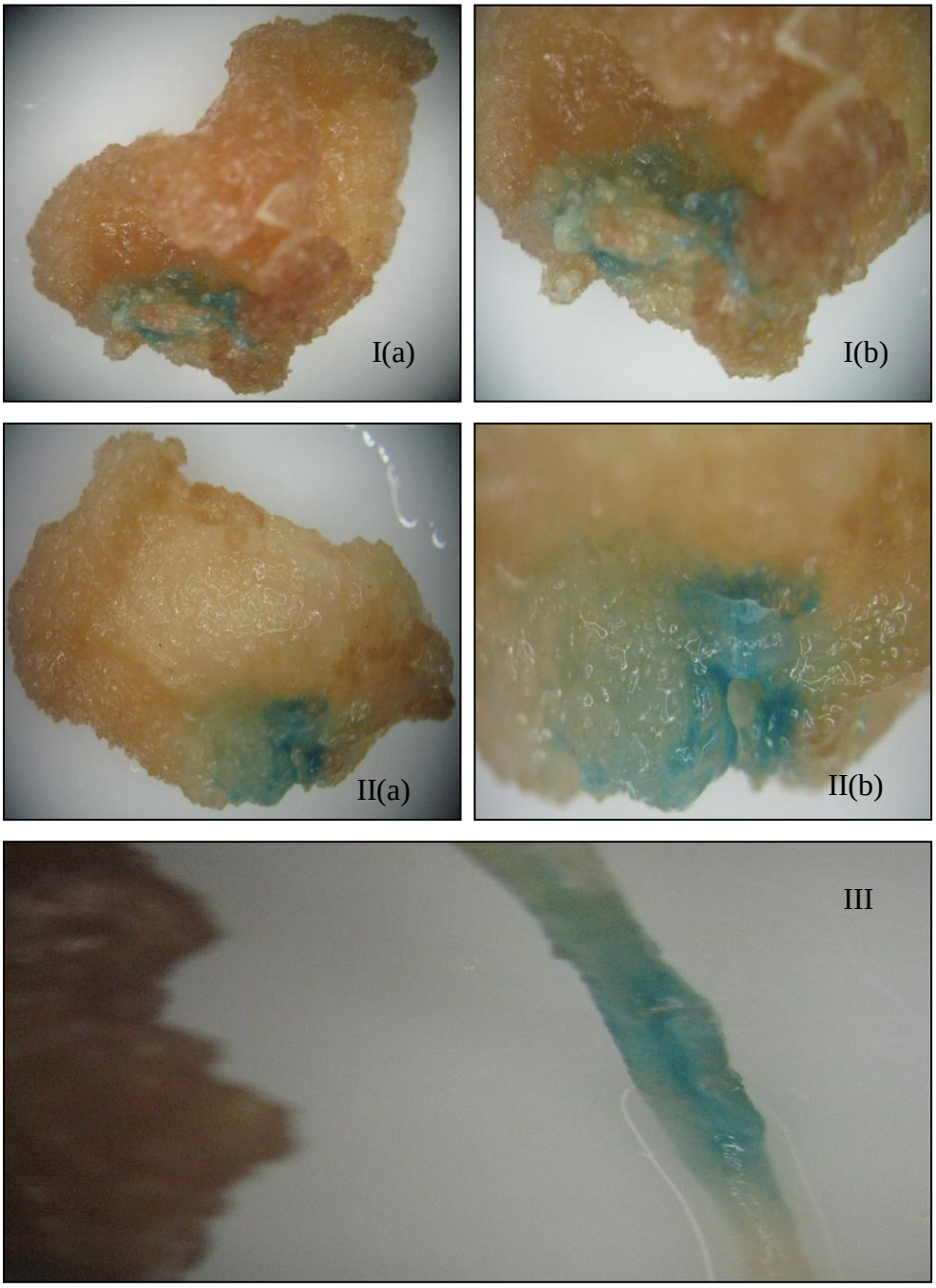


Figure 4.10: Pictures showing GUS histochemical staining in tobacco explants transformed with *A. tumefaciens* strain LBA4404 harbouring the binary vector pCAMBIA1304

Transformed plantlets of *C. willisii* were stained for histochemical analysis. Results were shown in **Figure 4.11**. Blue stains were indicated by arrows. Expression of *gusA* gene in *C. willisii* based on the analysis with histochemical staining was inconsistent with results from quantitative assay. From the quantitative assay, one day co-cultivation and six mins infection time showed the highest GUS enzyme activity but the qualitative assay result was incongruous. This is because quantitative fluorometric assay is more sensitive than qualitative histochemical assay. Low GUS gene expression is detected at a level where it was not visible or stained blue in qualitative assay. However, GUS qualitative histochemical assay is useful and important for localisation of GUS gene expression in transformed tissues despite of the lack of sensitivity.

GUS enzyme activity was observed mainly in petioles of transformed *C. willisii* although *gusA* gene was driven by a constitutive CaMV35S promoter. Chimerism was observed in all transformed *C. willisii*. This is expected as shoot tip cultures were used as transformation target tissue in the experiment. Chimeric plants often recovered in organogenesis which involved more than one cell in the shoot initiation process. Nevertheless, since there is no published report on regeneration route of *C. wiliisii* other than organogenesis, chimerism in transformed *C. willisii* remains to be investigated.

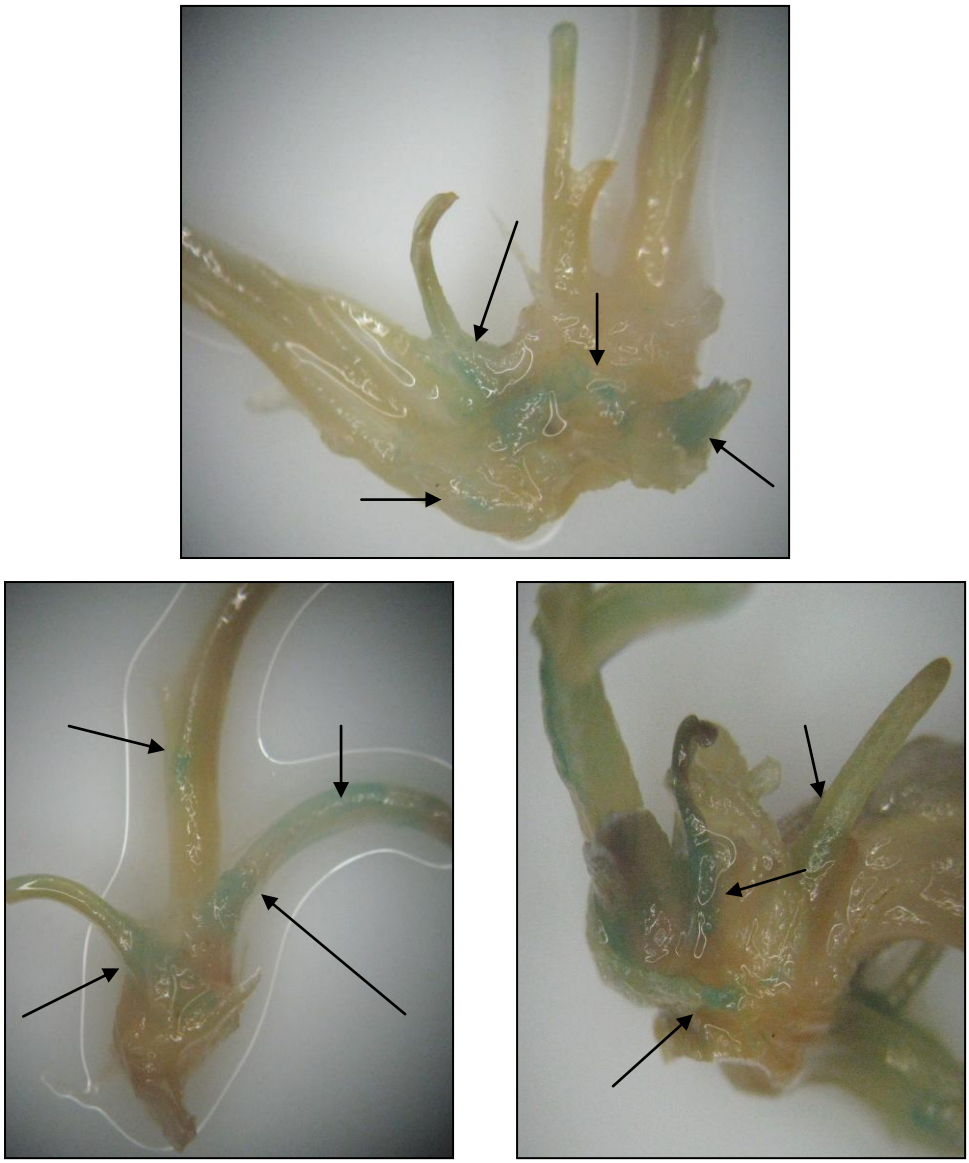


Figure 4.11: Histochemical staining of transformed *C. willisii*. Blue-stains were indicated by arrows.

4.3.4 GFP visualisation in putative transformants

4.3.4.1 Tobacco

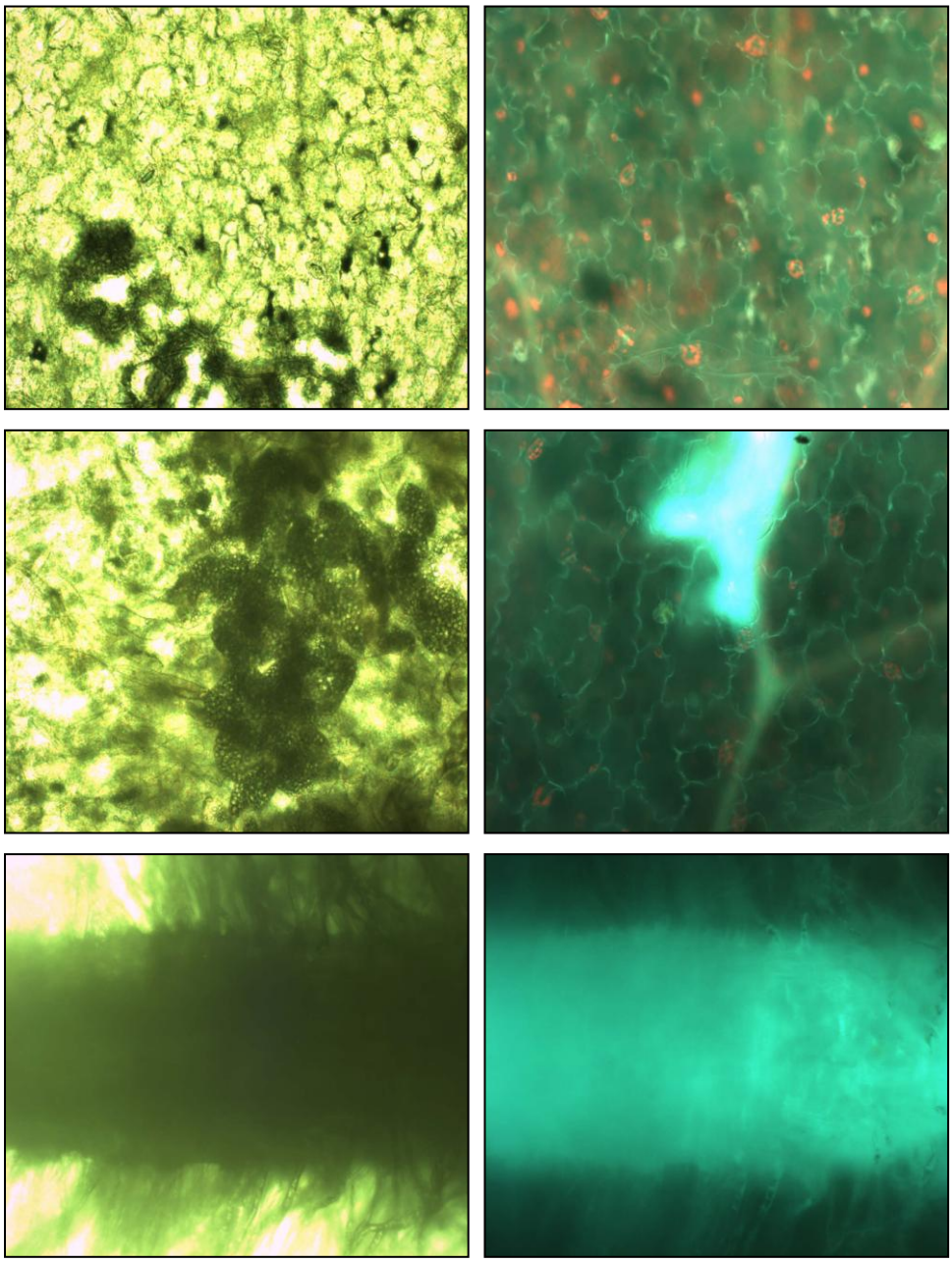


Figure 4.12: GFP visualisation on transformed tobacco explants. Left: Explants viewed in normal light. Right: Explants viewed in UV light (blue-light)

4.3.4.2 GFP visualisation in transformed *C. willisii* explants

GFP visualisation transformed *C. willisii* explants was shown in **Figure 4.13**. Transformed explants of *C. willisii* were excited with blue UV and showed green fluorescence as in pictures at the right. Pictures at the left showing the explants exposed to normal light. Non-transformed *C. willisii* explant appeared red due to auto-fluorescence from chlorophyll without green fluorescent spot as shown in **Figure 4.14**.

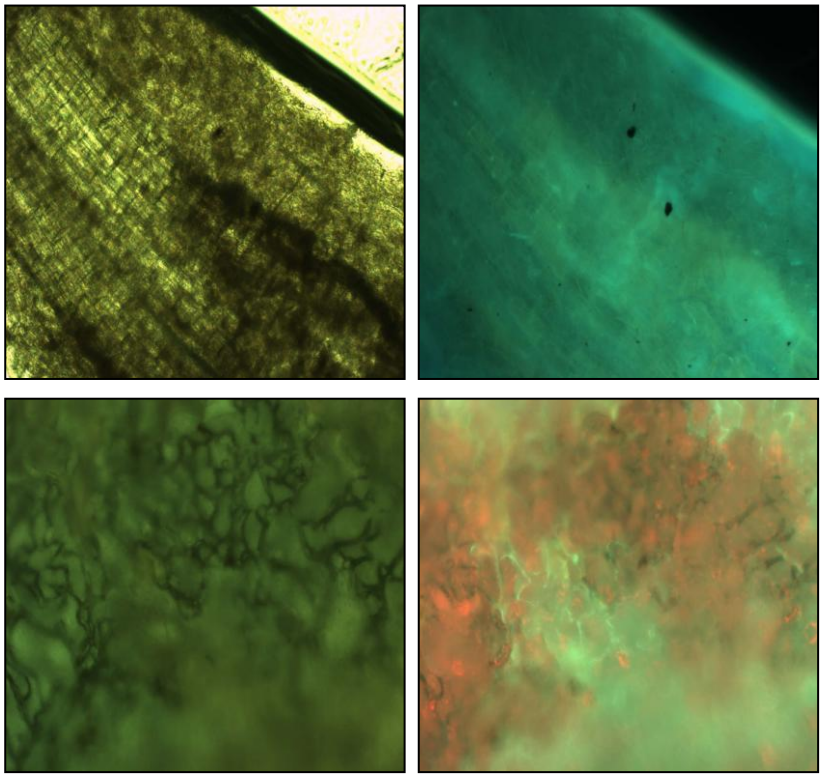


Figure 4.13: GFP visualisation on transformed *C. willisii* explants. Left: Explants viewed in normal light. Right: Explants viewed in UV light (blue-light)

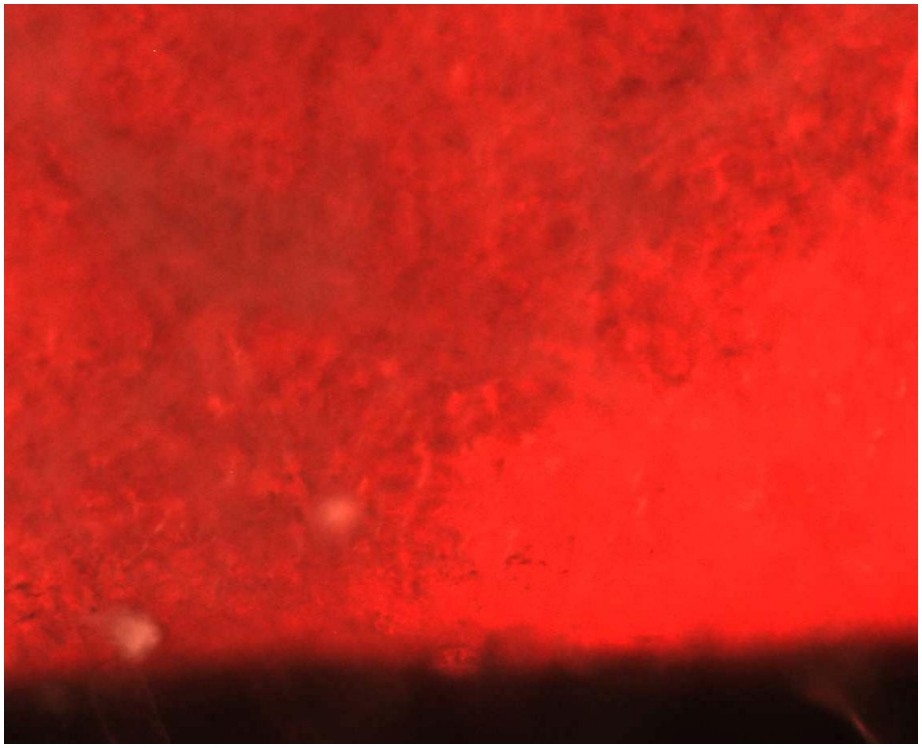


Figure 4.14: Picture showing the negative control of non-transformed *C. willisii* leaf explant viewed under UV (blue) light. Leaf explant appeared red as a result of auto-fluorescence from chlorophyll

4.4 Molecular assessments

4.4.1 Primer design and optimization of PCR condition

A pair of primers flanking *mgfp5* gene in pCAMBIA1304 was designed using the software Primer3 input 0.4.0. Sequences and details of the primers are as in **Table 4.3**.

Primers name	Sequences	Length	GC contents	Melting Temperature (Tm)
mgfp5 FWD	5' – AAG GAG AAG AAC TTT TCA CTG GAG – 3'	24 mer	41.7 %	54.0 ^{o}C
mgfp5 REV	5' – AGT TCA TCC ATG CCA TGT GTA – 3'	21 mer	42.9 %	52.4 ^{o}C

Table 4.3: List of primers designed for *mgfp5* gene amplification and the details of each primer

Gradient PCR of plasmid DNA was carried out to obtain the optimum annealing temperature for the new primers. Result is shown in **Figure 4.15**.

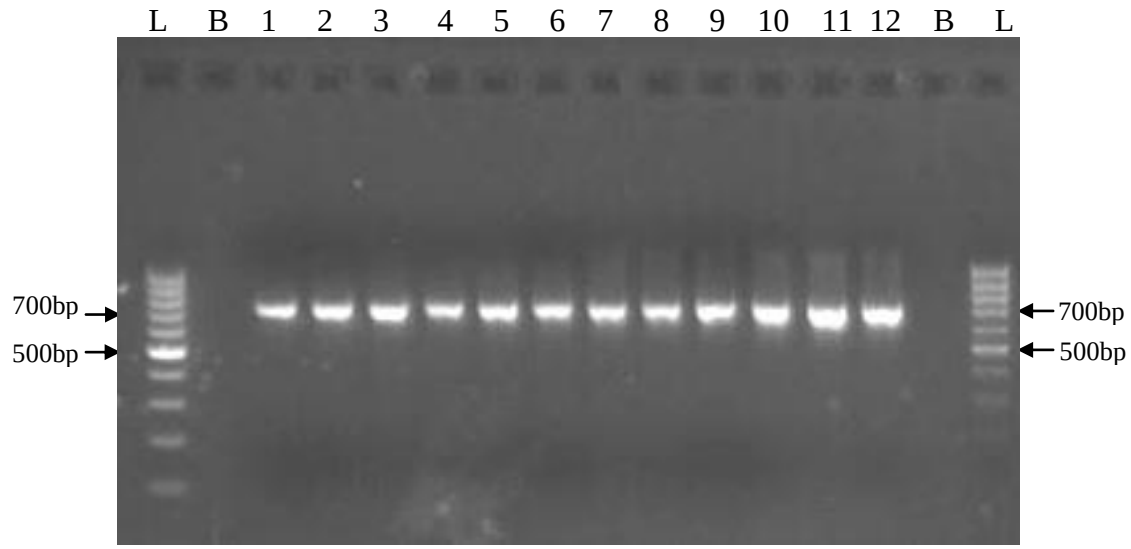


Figure 4.15: Gradient PCR of *mgfp5* reporter gene of plasmid DNA at different annealing temperature

L = 100 bp DNA Ladder B = Blank

1 = 40.7 ℃	2 = 40.7 ℃	3 = 41.7 ℃
4 = 43.5 ℃	5 = 45.9 ℃	6 = 48.7 ℃
7 = 51.6 ℃	8 = 54.4 ℃	9 = 56.9 ℃
10 = 58.9 ℃	11 = 60.1 ℃	12 = 60.3 ℃

From **Figure 4.15**, the optimum annealing temperatures for amplification of *mgfp5* gene identified were 58.9 ℃, 60.1 ℃, and 60.3 ℃ as appeared as thick bands at approximately 700 bp of the molecular weight on agarose gel. Annealing temperature, 58.9 ℃ was randomly chosen and applied for PCR confirmation of putative transformed tobacco and *C. willisii* in the subsequent analysis.

4.4.2 Screening of *Agrobacterium* and confirmation of plasmid DNA by PCR

Screening of *Agrobacterium* was carried out prior to transformation experiments. The plasmid pCAMBIA1304 extracted from the bacteria **(Section 4.2.4.1)** was subjected to PCR for confirmation of *mgfp5* reporter gene. Amplification of *mgfp5* reporter gene **(Figure 4.16)** was successful and it indicated the presence of the gene in the *Agrobacterium* used for transformation experiments.

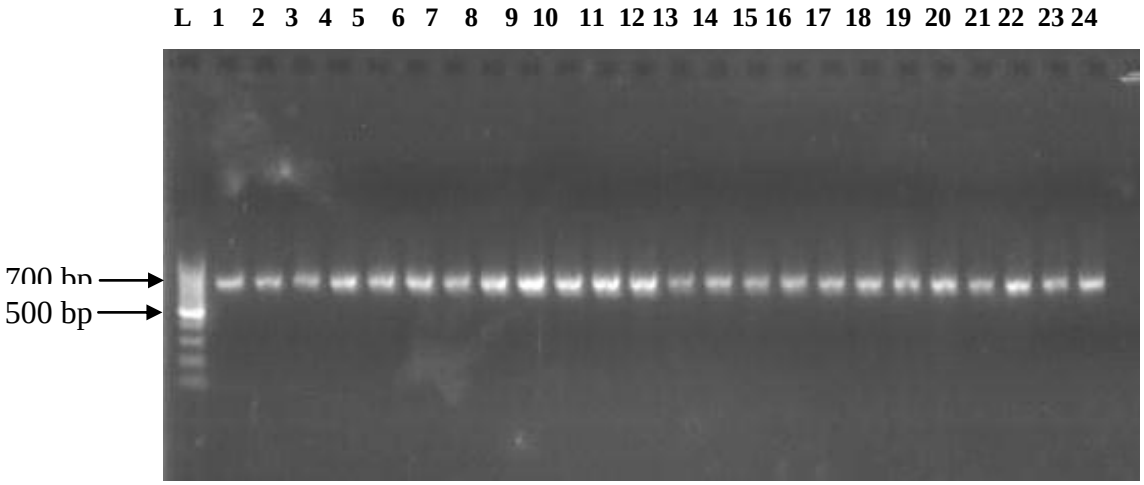


Figure 4.16: Screening of *A. tumefaciens* LBA4404 harbouring pCAMBIA1304 prior to plant transformation experiments

L = 100 bp DNA Ladder

Lanes 1 – 24 = PCR of *mgfp5* gene for screening of *A. tumefaciens*

4.4.3 PCR assessment on putative transformed plants

PCR was carried out on putative transformed plants that survived on selective medium and showed positive results in GFP visualisation.

4.4.3.1 Tobacco

Transformed callus explants of tobacco were assessed for molecular confirmation by PCR of *mgfp5* gene. Result is shown in **Figure 4.17**. Sample no. 1, 3, 4, 6, 7 and 8 showed the band of ≈ 700bp MW amplification indicating presence of *mgfp5* gene. Meanwhile, samples no. 2 and 5 showed negative results although these two samples showed green fluorescence under UV excitation as well.

The negative results here could be due to degradation of non-integrated *mgfp5* gene which showed transient expression in GFP visualization. This occurred because PCR assessment was not carried out on the regenerant but the whole explant itself. Non-integrated transgenes often degraded in the cytoplasm by nucleases and lead to transient expression in transgenic plants. Transient expression in the transformed explants generated escapes in antibiotics selection and also reporter genes assessment. These escapes could lead to false-positive results in PCR assessment.

With the results obtained from the transformation of tobacco with pCAMBIA1304, it has strongly suggested us to avoid transgene assessment on the explants but must be done on the regenerants of *C. willisii*.

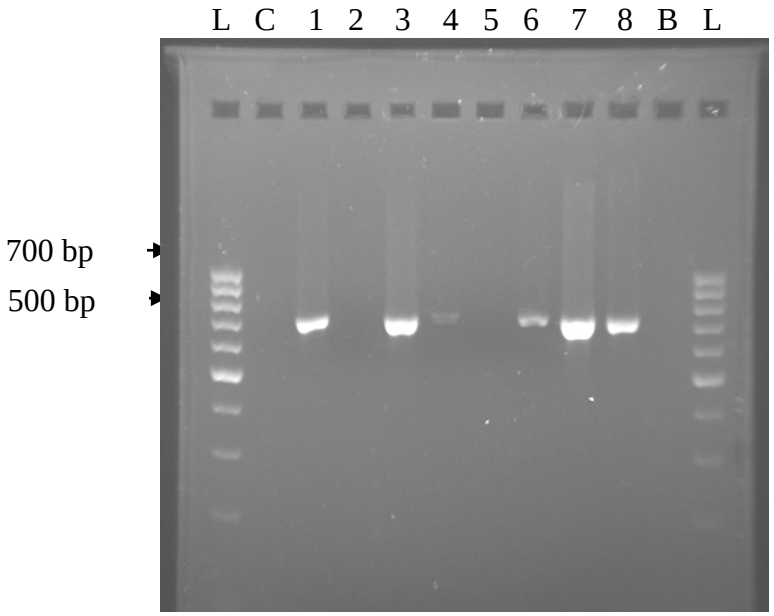


Figure 4.17: PCR confirmation of the presence *mgfp5* gene in transformed tobacco callus explants.

L = 100 bp DNA Ladder

B = Blank

C = Negative control

4.4.3.2 PCR confirmation of *C. willisii* transformation

Putative transformed *C. willisii* plantlets were assessed for molecular confirmation by PCR of *mgfp5* gene (**Figure 4. 18**). Regenerated plantlets of *C.willisii* from transformed shoot tips cultures were assessed individually. All *C. willisii* plantlets subjected to PCR analysis showed positive results with a band corresponding to ≈ 700 bp in MW. Southern blot analysis was then performed to investigate integration of *mgfp5* gene in transformed *C. willisii*. Southern hybridization is more informative than PCR analysis in transgenic plant studies because it provides evidence for integration and reveals the patterns of inheritance. Besides, PCR analysis might confers false positive results due to contamination, mis-match and non-specific amplification. Most importantly, it couldn't differentiate the template DNA being amplified is of *Agrobacterium* or plant origin. Moreover, expression studies on RNA and proteins must also be carried out because the presence of the transgene does not equate to the functionability of the transgene in the transgenic plants.

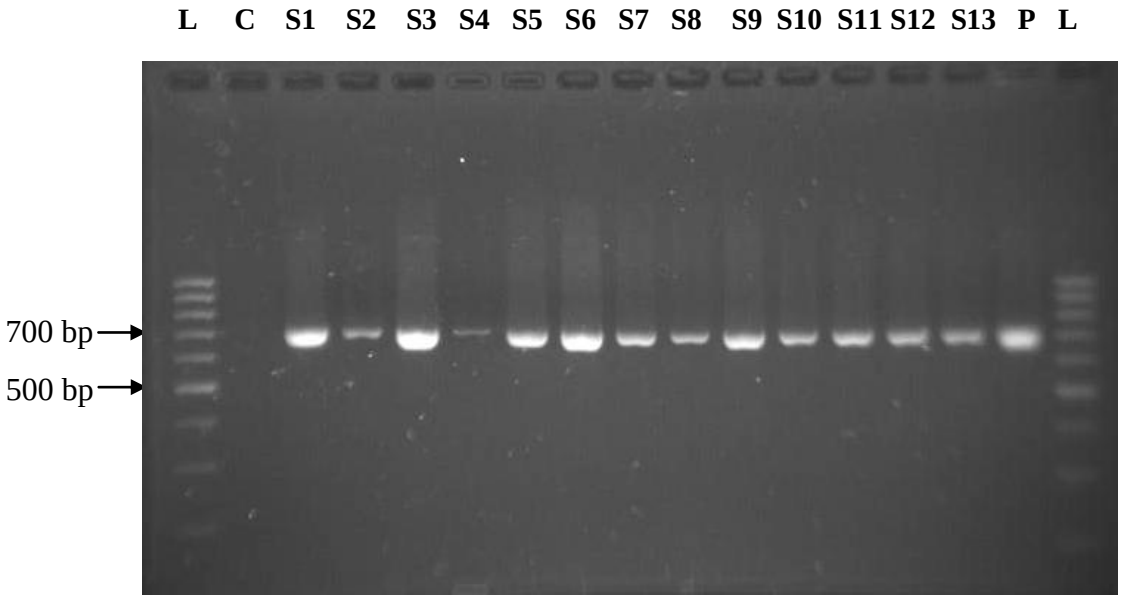


Figure 4.18: PCR confirmation of *C. willisii* transformation

L = 100 bp DNA Ladder

B = Blank

C = Negative control

S1 – S13 = Samples plantlets of *C. willisii*

P = pCAMBIA1304 plasmid as positive control

4.4.4 Restriction digest of genomic DNA and Southern blotting

Southern Blot analysis is important in transgenic plant studies as it facilitate the detection of stable integration of the transgene in the plant genome and also helps to trace its' inheritance in the subsequent segregating generation of the transgenic plants. Stable integration of transgene is desirable to prevent the loss of the transgene in subsequent segregating generation (Veluthambi, 2003). Together with the genomic DNA digestion with restriction enzymes technique, copy number of integrated transgenes could be determined. High copy number of transgene integrated in plant genome was reported to be correlated with higher incidence of "transgene silencing" in transgenic plants (Stam *et al.*, 1997; Metzlaff and Dell, 1997). Thus, low copy number of transgene integrated is preferred in transgenic plant studies. Furthermore, plants with one or two transgenes are usually more desirable for high level of transgene expression.

In order to detect stable integration of the *mgfp5* genes in transformed *C. willisii*, Southern blot analysis of *HindIII* restriction enzyme digested genomic DNA was carried out in this study. *HindIII* used cuts only once in the pCAMBIA1304 construct helped in revealing the integration pattern of *mgfp5* gene in transformed plants. Digested genomic DNA was blotted onto

nitrocellulose membrane. Result of hybridization with labelled GFP probes is shown in **Figure 4.19**. There is only one sample (S5) found to have stable integration events with two bands observed (Arrows shown in red, **Figure 4.19**). This indicated that there were two copies of *mgfp5* genes in sample S5. This is in contrast to de la Riva's *et al.* (1998) opinion, in which *Agrobacterium*-mediated transformation usually granted transgenic plants with single copy of integrated transgene which would reduces chances of transgene silencing. In Aswath's *et al*. (2006) studies, *Agrobacterium*-mediated transformation has generated transgenic onion with single copy number of *pmi* gene at a single locus. While, integrated *pmi* gene showed polymorphism banding of hybridization patterns when particle bombardment using gene gun was applied on the onion callus.

The integration frequency of the *mgfp5* gene in *C. willisii* was found to be low. This could be explained by the chimeric nature of the plant obtained from shoot tips culture wherein the transformants were regenerated from a group of cells. Similarly for Horn's *et al.* (1988) works, chimeric plant regenerated from protoplast culture of orchardgrass failed to provide evidence of transgene integration in Southern hybridization analysis. According to Kar *et al.* (1996), chimeric plants often resulted when the transgenic plant was regenerated by organogenesis. Contrary, somatic embryogenesis is the best route for regenerating non-chimeric transgenic plant (Slater *et al.*, 2003).

Particle bombarded embryogenic callus of papaya showed stable integration of *mgfp5* gene in regenerated papaya plant (Zhu *et al.*, 2004). Wong *et al.* (2006) also successfully developed non-chimeric transgenic *Musa acuminata* cv. Mas using embryogenic suspension culture via *Agrobacterium*-mediated transformation.

Besides, non-integrated fragments of DNA tend to be degraded in the plant genome by autonomous plant defence system. This could explain false positive results obtained in PCR analysis where the presence of *mgfp5* gene was detected in some samples but failed to verify its' integration via Southern hybridization analysis. Nonetheless, contamination with traces of *Agrobacterium* could not be ruled out using PCR analysis since it could not differentiate the template DNA being amplified originated from *Agrobacterium* or plant.

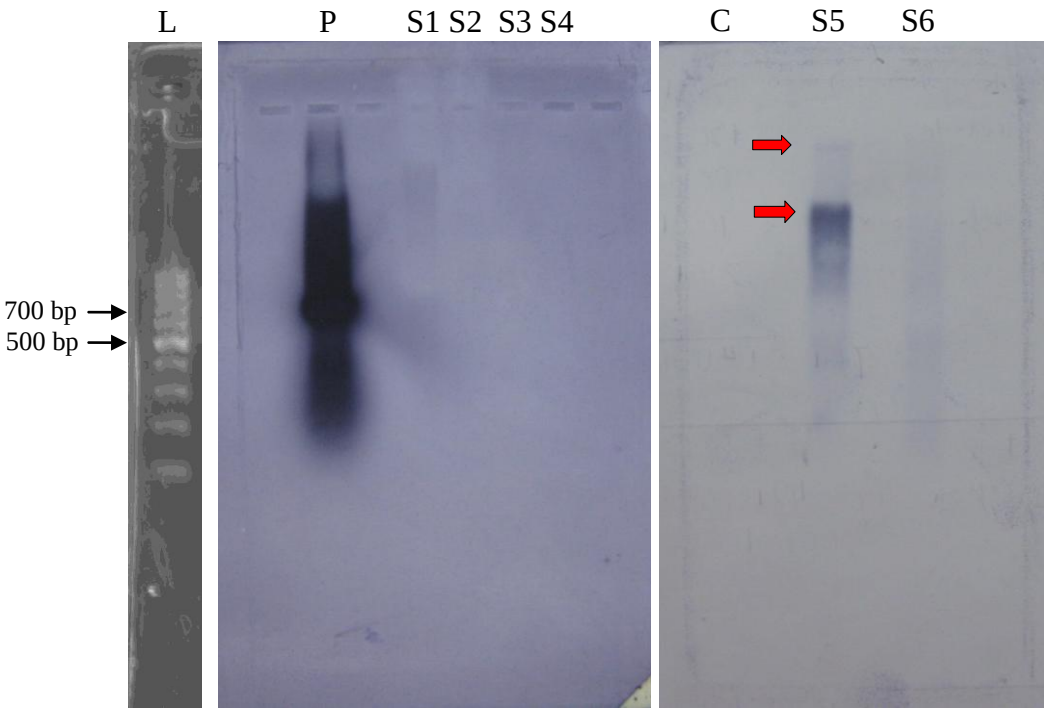


Figure 4.19: Digested genomic DNA (Sample S1, S2, S3, S4, S5 and S6) separated on 1% (w/v) agarose gel and blotted onto nitrocellulose membrane. Figure showed membrane after Southern Hybridization being carried out with *mgfp5* gene specific probes.

L = 100 bp DNA Ladder

P = PCR products of *mgfp5* gene positive control

S1 – S6 = Digested genomic DNA of *C. willisii* samples

C = Non-transformed control *C.willisii*