Nigerian Journal of Biochemistry and Molecular Biology 2017: 32(1): 23-31

0189-4757/96 $3.0 + 0.00
Printed in Nigeria

Available online at http://www.nsbmb.com.ng

NJBMB/002/17

Bio-physicochemical Optimization of *Agrobacterium tumefaciens*-mediated Transformation of Maize

*[1]Matazu, N. U., [1]Matazu, I. K., [1]Abdullahi, A. S., [1]Abubakar, Z. S., [1]Muhammad, I., [2]Shahruddin, N. A. and [2]Mahmood, M.

[1]Biochemistry Department, Faculty of Natural & Applied Sciences, Umaru MusaYar'adua University Katsina, Nigeria
[2]Biochemistry Department, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra, Serdang, Malaysia

Abstract: *Agrobacterium tumefaciens* bacteria are pathogenic to a range of dicotyledonous plant species, causing crown galls or tumours at or close to infection sites. However, since *Agrobacterium* is not a natural pathogen of monocotyledonous plants (monocots) such as maize, artificial transformation of monocots by *Agrobacterium* has always been associated with several challenges due to their recalcitrant nature. Factors such as genotypes, strain of *Agrobacterium*, co-cultivation period among others have been known to affect transformation efficiency in maize. But the effects of the optimization of these factors on transformation efficiency have not been extensively studied. This study was conducted to optimize the biological and physicochemical parameters in order to improve transformation efficiency of selected maize inbred lines using *Agrobacterium tumefaciens* strains LBA 4404 and EHA 101 harbouring pCAMBIA1304 vector (CAMBIA, Canberra, Australia). Explants from *Zea mays* inbred lines CML 419 and CML 427 were used. Explants optimized were callus at different age (1, 2, 3, 4, and 5 weeks) and immature zygotic embryos (IZE) obtained 15 days after pollination (DAP). Different *Agrobacterium* concentrations (0.1-1.0) were optimized at OD_{600}. Period of infection of explants was optimized at 30, 60, 90, and 120 minutes. Co-cultivation periods were also optimized ranging from 1-7 days. Temperature of the co-cultivation and pH of the media were optimized at 25-28°C and 5.0-6.0 respectively. Acetosyringone (AS) optimum concentration (50-250 µM) was also investigated. The results showed that 3 weeks old callus was the preferred explant. *Agrobacterial* strain LBA 4404 at OD_{600} 0.4-0.5 was optimum for infection of both callus and IZE. The optimum inoculation and co-cultivation period were 30 minutes and 3 days respectively. Acetosyringone concentrations of 100 and 150 µM were optimum for callus IZE transformation. Optimum temperature and pH were 28°C and 5.6 respectively. It can be concluded that the combined effect of these factors dictates the transformation abilities of these lines. Therefore these optimized factors could be applied to other CMLs for optimum transformation.

KEYWORDS: *Agrobacterium tumefaciens, Zea mays*, Transformation, Biophysicochemical.

1.0 Introduction

Genetic transformation of plants and other organisms occurs naturally. Bacteria carry this out routinely. Viruses can also transfer DNA (or RNA) into an organism and cause desirable changes in their traits. Soil bacteria, such as *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, are common examples of natural transformation systems. *Agrobacterium tumefaciens* is a soil bacterium. It is pathogenic to a range of dicot plant species, causing the formation of crown galls or tumours at or close to infection sites (Zupan *et al.*, 2000). Opines, which are amine derivatives are synthesized as a result of tissue proliferation in the tumour. These opines provide the bacteria with essential carbon and nitrogen at the expense of the host plant. Genes needed for tumourigenesis and induction of biosynthesis of opines are transferred from *Agrobacterium*. For this reason, *Agrobacterium* is nicknamed nature's genetic engineer (Hellens *et al.*, 2000). Through this advanced parasitism, *agrobacterium* uploads a discrete segment of its DNA called t-DNA into nuclear genome of the host plant. This complex mechanism is executed by tumour-inducing plasmid (Ti plasmid) where the T-DNA itself resides. This plasmid is flanked by 25 bp imperfect repeats called left boarder (LB) and

*Corresponding Author
Tel.: +2348036446433
E-mail: nasruddeen.umar@umyu.edu.ng

right boarder (RB) that defined the frontiers of the T-DNA (Hoekema *et al.*, 1983). This plasmid has another virulence (vir) region which housed vir genes (Hellens *et al.*, 2000; Zupan *et al.*, 2000).

In maize genetic transformation, biolistic gun mediated-DNA transfer was the first successful methods used in producing fertile transgenic plants (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990). While biolistic delivery method possesses advantages for its ability to penetrate plant cell walls readily and competently, its major disadvantage is that it generates multiple copies of transgenes insertion in plant genome which increases the susceptibility of plant to multi-sequence-induced gene silencing (Frame *et al.*, 2006a; Torney *et al.*, 2007; Wang and Frame, 2009). *Agrobacterium*-mediated transformation on the other hand offers numerous benefits that include its simplicity and efficiency.

Although *A. tumefaciens* is not a natural pathogen of most monocots, it has been successfully used to transform many cereals, including maize (Ishida *et al.*, 1996), rice (Hiei *et al.*, 2006), wheat, barley and tricale (Hensel *et al.*, 2009). One of the most significant benefits of *Agrobacterium*-mediated transformation is formation of low transgenes copy number. This is advantageous because they are less prone to multi-sequence-induced gene silencing and have been shown to maintain higher and more stable transgene expressions over generations (Ishida *et al.*, 2007; Torney *et al.*, 2007).

In maize, both tissue culture and genetic transformation employ the embryo or embryo derived callus. Immature zygotic embryo is perhaps most extensively used in maize transformation (Frame *et al.*, 2006a; Ishida *et al.*, 2007; Vega *et al.*, 2008; Ombori *et al.*, 2013). Callus-derived from IZE is conceivably the second most suitable explant in genetic transformation. In general, in establishing transformation protocol for maize, the following factors should be considered: (i) effectiveness of gene delivery methods (ii) competence of the target tissue (iii) robustness of the selection systems used for recovery transformed events (Torney *et al.*, 2007). Despite the fact that several promising results (Ishida *et al.*, 1996; Hiei *et al.*, 2006; Hensel *et al.*, 2009), have been obtained, *Agrobacterium*-mediated genetic transformation protocols for maize can still be improved upon. The most critical factor in the transformation protocol is the co-cultivation of healthy immature embryos of the correct developmental stage with *A. tumefaciens*. Therefore, the aim of the investigation was to optimize transformation efficiency using some important parameters like inoculation time, co-cultivation period, type and age explant, *Agrobacterium* concentration and acetosyringone concentration.

2.0 Materials and Methods

2.0.1 Optimization of Explants

Two types of target tissues were explored. The first one was immature zygotic embryo (IZE) obtained at 15 days after pollination (DAP), the second one was callus derived from immature zygotic embryo at different developmental stage (1-6 weeks old).

2.0.2 Agrobacterium Preparation and Transformation Protocol

The protocol employed for this transformation was adopted from earlier methods (Frame *et al.*, 2006a; Ishida *et al.*, 2007; Sidorov and Duncan, 2009).

2.0.3 Agrobacterium tumefaciens Strains and Vector

Two types of *Agrobacterium tumefaciens* strains were used. These were *Agrobacterium tumefaciens* strain LBA 4404 and EHA 101. These strains were harboring pCAMBIA1304 vector (CAMBIA, Canberra, Australia). The vector contained bacterial kanamycin resistance, plant hygromycin selection, pUC18polylinker in lacZ, (mgfp5-gusA-His6 fusion), two reporter genes: mGFP5 or the modified version of the *Aequoria victoria* which encodes Green Fluorescent Protein (GFP) and GUS. GFP serves as visual selection marker (Siemering *et al.*, 1996). The GUS on the other hand serves as hygromycin B selectable marker in plants. The expressions of this fusion are driven by Cauliflower Mosaic Virus (CaMV) 35S

promoter. In addition, the vector contains a kanamycin resistance gene for bacterial selection

2.0.4 Preparation of Agrobacterium Culture

Agrobacterium strains were cultured as described by Wise *et al.* (2006). In brief, these strains were cultured in LB Agar (Merck, Kenilworth, U.S.A). The LB agar was composed of 5 g/L Yeast extract, 10 g/L peptone from casein, 10 g/L sodium chloride and 12 g/L agar. The solid LB media was prepared by dissolving 37 g L of ddH_20 and autoclaved at 121°C for 15 minutes. When the temperature dropped to 50°C, Kanamycin (50 mg/L) and Chloramphenicol (25 mg/L) were added. The media was solidified in Petri plates, sealed with paraffin tape and stored in a refrigerator until required. *Agrobacterium* from a glycerol stock was streaked on the solid media and incubated at 30 ± 2°C for 48 hours. Single colony of *Agrobacterium* cells were inoculated in 10 mL of LB broth supplemented with 5 mM glucose. The culture was grown overnight in an Incubator Shaker (Excella® E24, Eppendof, Hamburg, Germany) agitating at 200 rpm and maintained at 27°C in the dark until OD_{600} = 0.8-1.2 was reached.

2.0.5 Optimization of Transformation Parameters

Transformation efficiency in maize via *Agrobacterium* is dependent upon several physical, chemical and biological factors. These included the type of explant, bacterial concentration, inoculation time, co-cultivation period, pH, temperature and acetosyringone concentration.

2.0.5.1 Agrobacterium Density, Inoculation and Co-cultivation Period

The optimization here was done using *Agrobacterium* concentration (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0) at specific optical densities (OD_{600}). Immersion time during inoculation/infection of explants was optimized at 30, 60, 90, and 120 minutes. Co-cultivation period in days were also optimized from 1-7days.

2.0.5.2 Acetosyringone Optimization

Acetosyringone optimization was conducted using concentrations ranging from 50-300 µM. During each stage of optimization, each factor effect was evaluated singly on transient GUS expression frequency which was carried out at the end of each co-cultivation period. Callus was scored GUS positive if there were blue spots.

2.0.5.3 GFP Visualization and Histochemical GUS Assay

Putatively transformed and untransformed (control) callus were histochemically assayed for β-glucuronidase (gus) activity as described by Jefferson (1987), modified by Kosugi *et al.* (1990). Briefly, the samples were incubated at 37°C overnight (14 hours) in 200 µL reaction buffer containing 50 mM NaH_2PO_4, 10 mM EDTA, 0.3 M mannitol (pH 7.0), 0.1% Triton X-100 and 1.0 mM 5-Bromo-4-Chloro-3-indolyl-β-D-Glucopyranoside (x-glu) with 20% methanol. Transient GUS activity was recorded as percentage of calli showing blue spots using a stereomicroscope Carl Zeiss (Stemi DV4, Jena, Germany) equipped with AxioCam ERc 5s camera.

2.6 Statistical Analysis

Data were analyzed using IBM SPSS Software (version 22). Mixed model analysis of variance (ANOVA) was used to determine the effect of agrobacterium strains, age of explant, temperature, acetosyringone concentration, inoculation time and co-cultivation period on transformation efficiency. The results were expressed as mean ± S.E.M. The differences were considered statistically significant if the P-value was less than 0.05.

3.0 Results and Discussion

Inoculation or infection period is as important as other factors in relation to the *Agrobacterium*-mediated transformation. Inoculation media and exposure time of explant to the media varies from strain to strain and explant to explant. Salt strength of infection media has also been reported to have an impact on maximum GUS

expression in wheat (Cheng *et al.*, 1997). In Figure 1, 20-40 minutes inoculation time was observed with 30 minutes being the optimum for both lines. When the explant was left in the inoculation media for more than an hour, the callus turned yellowish which could be due to multiplication of the bacterial cells. Inoculation time of 15 minutes caused high GUS expression in some tropical inbred lines (Valdez-Ortiz *et al.*, 2007). In some maize models, 30 minutes inoculation period was optimal (Ishida *et al.*, 2007). Generally, inoculation/infection period also depends on the medium, type of explant and age of the explants. Thus, optimization of all factors is imperative for optimum GUS expression in both transient and stable transformations.

Simultaneous cultivation of *agrobacterium* and explant to be infected is pre-requisite step in any gene transfer mediated by *agrobacterium* (Uranbey *et al.*, 2005). Generally, solid co-cultivation media was the most frequently used. However, liquid co-cultivation media displayed a higher transformation frequency than the solid one (Uranbey *et al.*, 2005). Combination of both liquid and solid media has also been reported by Requesens *et al.* (2010) for the transient expression of maize endosperm. Along with temperature, co-cultivation period plays a very crucial role in the level of gene expression. Their influences on T-DNA transfer have been previously reported (Ding *et al.*, 2009). Transient GUS expression studies in many monocots such as rice (Rahman *et al.*, 2013) and soybean (Santarem *et al.*, 1998). In this study, co-cultivation period evaluation suggests that the optimal number of days required for highest GUS expression was 3 days post infection (Figure 1) for both genotypes. The 3 days co-cultivation period was the most commonly reported in maize transformation (Frame *et al.*, 2002; Ishida *et al.*, 2007; Valdez-Ortiz *et al.*, 2007). Co-cultivation period of 3 days was also recorded in other plant species such as ginger (Suma *et al.*, 2008), barley and tricale (Hensel *et al.*, 2009) and wheat (Ding *et al.*, 2009). In soybean, 5 days were found to be the optimum co-cultivation period (Paz *et al.*, 2006). In rice, shorter co-cultivation period of 2 days were believed to be the optimum period for transformation (Jabeen *et al.*, 2009; Sahoo *et al.*, 2011).

Types of explant, physiological condition/state of explant as well as genotype of explant generally are known to affect not only the overall tissue culture phenomena but also transformation protocols. Several types of explants are being used today for efficient callusing response as well as transformation. The most commonly reported explants in different plants species transformed via *Agrobacterium* include epicotyl segment of pineapple, sweet orange and and *Swingle citrumelo* (de Oliveira *et al.*, 2009), hypocotyl of soybean (Wang and Xu, 2008), immature embryo from maize (Ishida *et al.*, 2007), mature embryo of wheat (Ding *et al.*, 2009), leaf-section and axillary shoot-meristem from pear (Matsuda *et al.*, 2005). The choice of a particular explant for transforming any tissue culture related work depends on the plants species (Frame *et al.*, 2006b).

Preliminary investigations on GUS expression between immature zygotic embryo and callus (Figure 3) unfold that blue spots formation were significantly ($p < 0.05$) higher on callus than on embryo. By this, it was assumed that GUS gene expression was correspondingly higher in callus; hence the use of callus. The physiological state of the callus too is dependent on its age (Yang *et al.*, 2006). Different ages of callus in weeks were evaluated for optimum GUS expression. Figure 3 display that in CML 427, two weeks old callus was the preferred age as indicated by the presence of higher average number of blue spots. While in CML 419, at three weeks, GUS staining revealed that the number of blue spots was significantly ($p < 0.05$) higher than in the other age groups. This could be related to their respective growth rates. These lines have maximum growth at week three with CML 427 displaying faster rate than CML 419. Coincidentally, their respective sub-culture time was deemed at two weeks when their cells were presumed to proliferate faster. Thus, two weeks old callus from both lines were subsequently used. Greater number of successful transformation carried out in monocots using callus as explants has reported in rice (Toki *et*

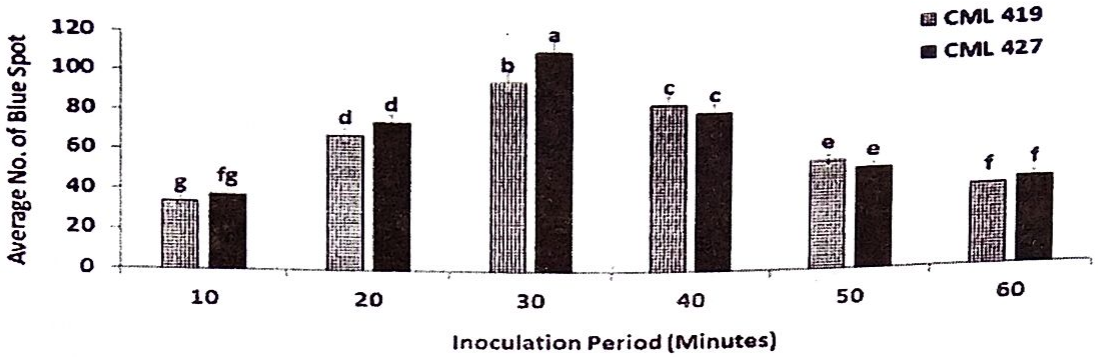

Figure 1: Optimization of inoculation/infection time for embryogenic callus lines from CML 419 and 427 for efficient transformation

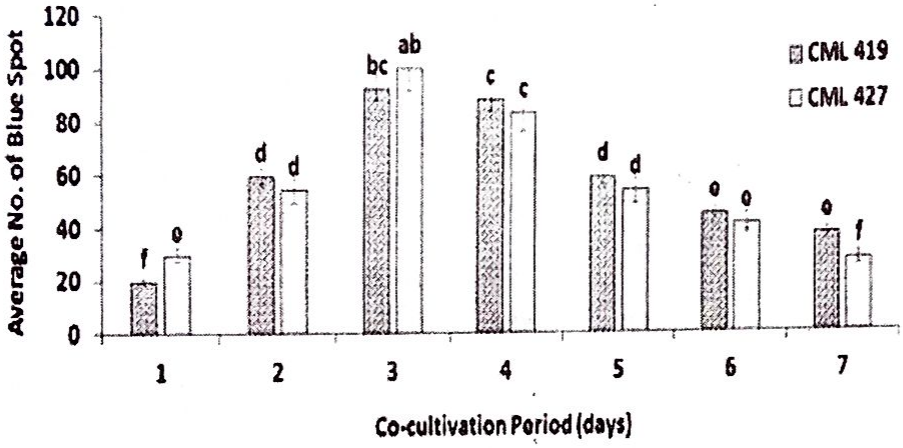

Figure 2: Transient GUS expression in CML 419 callus lines at various co-cultivation periods. Each test consisted of thirty pieces of embryogenic calli and repeated three times

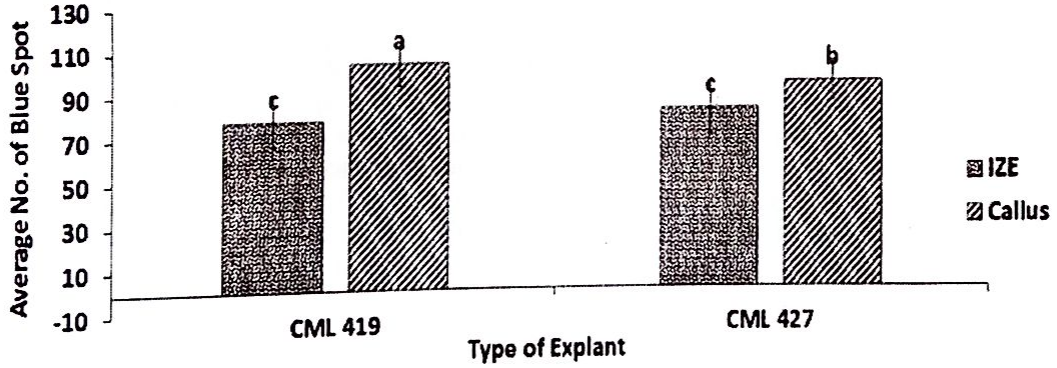

Figure 3: Influence of explant (IZE and embryogenic callus) for optimum transformation efficiency in CML 419 and CML 427

al., 2006; Saika and Toki, 2010). Ten day old callus derived from mature embryo (Hiei and Komari, 2008), 1-day-old rice seeds grown on a medium containing 2 mg/L 2,4-D have been reported show better competency for *Agrobacterium*-mediated transformation (Toki *et al.*, 2006). Saika and Toki (2010) have reported that 7-day old and 3-week-old calli were utilized for transformation as primary and secondary calli respectively. In maize, though immature zygotic embryo remains the preferred explant, some genotypes respond exceptionally better when callus was used as explant for transformation. For example, leaf-derived callus was used as an explant for transformation (Ahmadabadi *et al.*, 2007). Callus derived from IZE was also used as explant for *Agrobacterium*-mediated transformation (Yin *et al.*, 2004; Yang *et al.*, 2006).

Acetosyringone is a phenolic compound which when released by metabolically active wounded plant triggers the activation of *Agrobacterium tumefaciens* virulence (vir) gene expression (Delmotte *et al.*, 1991). In earlier studies, transformed *Arabidopsis thaliana* leaf explants in the presence of acetosyringone improved transformation frequency (Marion *et al.*, 2008; Ogawa *et al.*, 2008 and Li *et al.* 2009). However, the optimum concentration required depends on the plant species, explant type as well as the type of *Agrobacterium* strain (van Wordragen and Dons, 1992). In the present study, acetosyringone concentrations ranging from 0-300 µM were screened in order to obtain the needed optimum concentration. From the results (Figure 4), 150 µM of acetosyringone was the optimum concentration required for the highest GUS expression in CML 427.. CML 419 had optimum GUS expression when acetosyringone concentration was increased to 200 µM. This implies that the effect of acetosyringone on the optimum transformation is dependent on the genotype of plants in addition to agrobacterium strain.

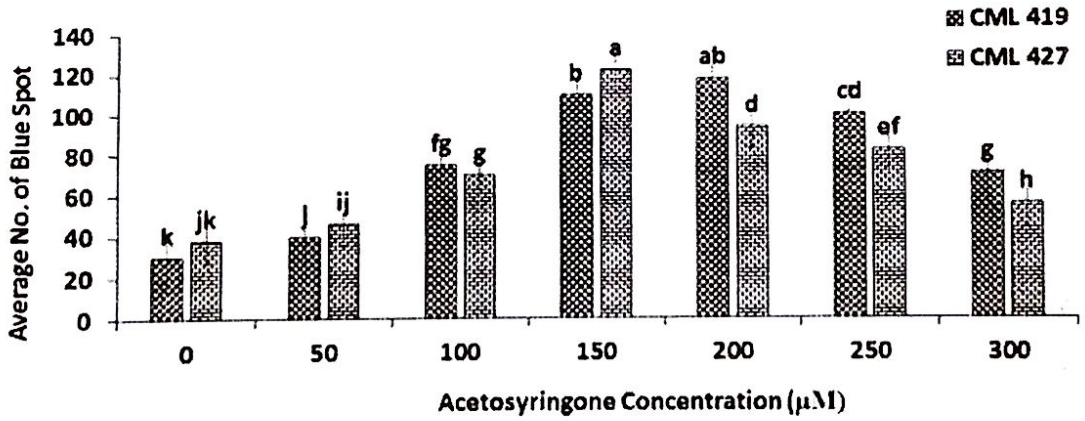

Figure 4: Effects of various acetosyringone concentrations on transformation efficiency in CMLs 419 and 527

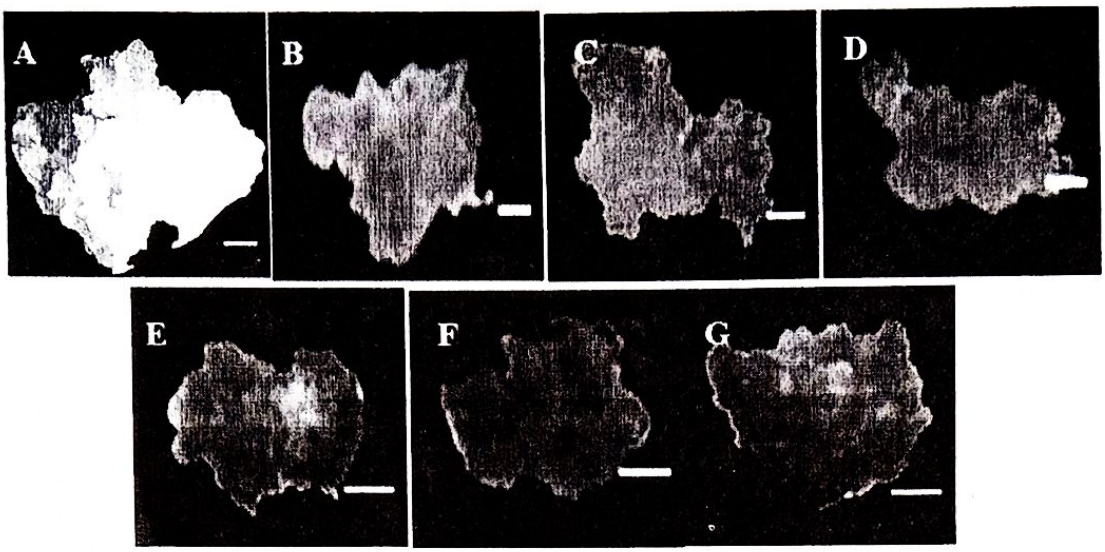

Figure 5: Putatively transformed callus lines expressing gus. (A) Control (B-D) CML 419 (E-G) CML 427

In general, the supplementation of acetosyringone to both inoculation and co-cultivation media greatly increased transient GUS expression by more than 50%. In maize transformation mediated by *agrobacterium*, several different concentrations of *Agrobacterium* have been reported (Frame *et al.*, 2006b). The common concentration of acetosyringone was 100 µM for maize model Hi II and its parents A188xB73 (Frame *et al.*, 2006b; Hiei *et al.*, 2006; Horn *et al.*, 2006) and other elite inbred lines (Yang *et al.*, 2006). Yet, 200 µM acetosyringone was required in these non proprietary lines H99, LH198, HiII, PHA, which is a triple hybrid (Pa91xH99) xA188 and some CIMMYT maize lines (Ombori *et al.*, 2013).

Conclusion

It can be concluded that these lines are transformable with *agrobacterium* and their transformation abilities could be influenced by a combination of factors such as type of agrobacterium strain, inoculation time and co-cultivation period among others.

References

Ahmadabadi, M., Ruf, S. and Bock, R. (2007). A leaf-based regeneration and transformation system for maize (*Zea mays* L.). Transgenic Research 16 (4): 437-448.

Cheng, M., Fry, J. E., Pang, S., Zhou, H., Hironaka, C. M., Duncan, D. R., Conner, T. W. and Wan, Y. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiology 115 (3): 971-980.

de Oliveira, M. L. P., Febres, V. J., Costa, M. G. C., Moore, G. A. and Otoni, W. C. (2009). High efficiency *Agrobacterium*-mediated transformation of citrus by sonication and vacuum infiltration. Plant Cell Reports 28 (3): 387-395.

Delmotte, F. M., Delay, D., Cizeau, J., Guerin, B. and Leple, J. C. (1991). *Agrobacterium* vir inducing activities of glycosylated acetosyringone, acetovanillone, syringaldehyde and syringic acid derivatives. Phytochemistry 30 (11): 3549-3552.

Ding, L., Li, S., Gao, J., Wang, Y., Yang, G. and He, G. (2009). Optimization of *Agrobacterium*-mediated transformation conditions in mature embryos of elite wheat. Molecular Biology Reports 36 (1): 29-36.

Frame, B. R., Shou, H., Chikwamba, R. K., Zhang, Z., Xiang, C., Fonger, T. M., Pegg, S. E. K., Li, B., Nettleton, D. S., Pei, D. and Wang, K. (2002). *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard

binary vector system. Plant Physiology 129 (1): 13-22.

Frame, B., Paque, T. and Wang, K. (2006a). Maize (*Zea mays* L.). In *Agrobacterium Protocols*, 2nd edn. (Wang, K. ed.), 43-54. Totowa, New Jersey: Humana Press, Chapter 15.

Frame, B. R., McMurray, J. M., Fonger, T. M., Main, M. L., Taylor, K. W., Torney, F. J., Paz, M. M. and Wang, K. (2006b). Improved Agrobacterium-mediated transformation of three maize inbred lines using MS salts. Plant Cell Reports, *25*(10), 1024-1034.

Fromm, M. E., Morrish, F., Armstrong, C., Williams, R., Thomas, J. and Klein,T. M. (1990). Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Nature Biotechnology 8 (9): 833-839.

Gordon-Kamm, W. J., Spencer, T. M., Mangano, M. L., Adams, T. R., Daines, R. J., Start, W. G., O'Brien, J. V., Chambers, S. A., Adams, W. R. Jr., Willets, N. G., Rice, T. B., Mackey, C. J., Kruegar, R. W., Kausch, A. P. and Lemaux, P. G. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. The Plant Cell *2*(7): 603-618.

Hellens, R., Mullineaux, P. and Klee, H. (2000). Technical Focus: A guide to *Agrobacterium* binary Ti vectors. Trends in Plant Science 5 (10): 446-451.

Hensel, G., Kastner, C., Oleszczuk, S., Riechen, J. and Kumlehn, J. (2009). *Agrobacterium*-mediated gene transfer to cereal crop plants: current protocols for barley, wheat, triticale, and maize. International Journal of Plant Genomics 2009: 1-9.

Hiei, Y. and Komari, T. (2008). *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. Nature Protocols 3 (5): 824-834.

Hiei, Y., Ishida, Y., Kasaoka, K. and Komari, T. (2006). Improved frequency of transformation in rice and maize by treatment of immature embryos with centrifugation and heat prior to infection with *Agrobacterium tumefaciens*. Plant Cell, Tissue and Organ Culture 87(3): 233-243.

Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J., and Schilperoort, R. A. (1983). A binary plant vector strategy based on separation of vir-and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179-180.

Horn, M. E., Harkey, R. L., Vinas, A. K., Drees, C. F., Barker, D. K. and Lane, J. R. (2006). Use of Hi II-elite inbred hybrids in *Agrobacterium*-based transformation of maize. *In Vitro* Cellular & Developmental Biology-Plant 42 (4): 359-366.

Ishida, Y., Hiei, Y. and Komari, T. (2007). *Agrobacterium*-mediated transformation of maize. Nature Protocols 2 (7): 1614-1621.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kumashiro, T. (1996). High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnology 14 (6): 745-750.

Jabeen, N., Mirza, B., Chaudhary, Z., Rashid, H., Gulfraz, M. (2009). Study of the factors affecting *Agrobacterium* mediated gene transformation in tomato (*Lycopersicon esculentum* mill.) cv. Riogrande using rice chitinase (cht-3) gene. Pakistan Journal of Botany 41 (5): 2605-2614.

Jefferson, R. A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Molecular Biology Reporter 5 (4): 387-405.

Kosugi, S., Ohashi, Y., Nakajima, K. and Arai, Y. (1990). An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Science 70 (1): 133-140.

Li, J. F., Park, E., von Arnim, A. G., and Nebenführ, A. (2009). The FAST technique: a simplified Agrobacterium-based transformation method for transient gene expression analysis in seedlings of Arabidopsis and other plant species. Plant Methods *5*(1), 1-15.

Marion, J., Bach, L., Bellec, Y., Meyer, C., Gissot, L. and Faure, J. D. (2008). Systematic analysis of protein subcellular localization and interaction using high-throughput transient transformation of Arabidopsis seedlings. The Plant Journal 56(1): 169-179.

Matsuda, N., Gao, M., Isuzugawa, K., Takashina, T. and Nishimura, K. (2005). Development of an *Agrobacterium*-mediated transformation method for pear (*Pyruscommunis* L.) with leaf-section and axillary shoot-meristem explants. Plant Cell Reports 24 (1): 45-51.

Ogawa, Y., Dansako, T., Yano, K., Sakurai, N., Suzuki, H., Aoki, K., Noji, M., Saito, K. and Shibata, D. (2008). Efficient and high-throughput vector construction and Agrobacterium-mediated transformation of *Arabidopsis thaliana* suspension-cultured cells for functional genomics. Plant and Cell Physiology 49(2), 242-250.

Ombori, O., Muoma, J. V. O. and Machuka, J. (2013). *Agrobacterium*-mediated genetic transformation of selected tropical inbred and hybrid maize (*Zea mays* L.) lines. Plant Cell, Tissue and Organ Culture (PCTOC) 1-13.

Paz, M. M., Martinez, J. C., Kalvig, A. B., Fonger, T. M. and Wang, K. (2006). Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-

mediated soybean transformation. Plant Cell Reports 25 (3): 206-213.

Rahman, Z. A., Seman, Z. A., Basirun, N., Julkifle, A. L., Zainal, Z. and Subramaniam, S. (2013). Preliminary investigations of *Agrobacterium*-mediated transformation in Indica rice MR219 embryogenic callus using gus A gene. African Journal of Biotechnology 10 (40): 7805-7813.

Requesens, D. V., Egelkrout, E., Devaiah, S. and Hood, E. E. (2010). A method for transient expression in maize endosperm. In Vitro Cellular & Developmental Biology-Plant 46 (6): 485-490.

Sahoo, K. K., Tripathi, A. K., Pareek, A., Sopory, S. K. and Singla-Pareek, S. L. (2011). An improved protocol for efficient transformation and regeneration of diverse Indica rice cultivars. Plant Methods 7 (1): 49.

Saika, H. and Toki, S. (2010). Mature seed-derived callus of the model Indica rice variety Kasalath is highly competent in *Agrobacterium*-mediated transformation. Plant Cell Reports 29 (12): 1351-1364.

Santarem, E., Trick, H., Essig, J. and Finer, J. (1998). Sonication-assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: Optimization of transient expression. Plant Cell Reports 17 (10): 752-759.

Sidorov, V. and Duncan, D. (2009). Agrobacterium-mediated maize transformation: immature embryos versus callus. Transgenic Maize: Methods and Protocols, 47-58.

Siemering, K. R., Golbik, R., Sever, R. and Haseloff, J. (1996). Mutations that suppress the thermosensitivity of green fluorescent protein. Current Biology 6 (12): 1653-1663.

Suma, B., Keshavachandran, R., Nybe, E. V. (2008). *Agrobacterium tumefaciens* mediated transformation and regeneration of ginger (*Zingiber officinale* Rosc.). Journal of Tropical Agriculture 46: 38-44.

Toki, S., Hara, N., Ono, K., Onodera, H., Tagiri, A., Oka, S. and Tanaka, H. (2006). Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. The Plant Journal 47 (6): 969-976.

Torney, F., Frame, B. and Wang, K. (2007). Maize. In: *Transgenic Crops IV* Springer Berlin Heidelberg. pp. 73-105.

Uranbey, S., Sevimay, C. S, Kaya, M. D., Ipek, C., Sancak, D., Basalma, C., Er., and ¨Ozcan, S. (2005). Influence of different co-cultivation temperatures, periodsand media on *Agrobacterium tumefaciens*-mediated gene transfer. Biologia Plantarum 49 (1): 53-57.

Valdez-Ortiz, A., Medina-Godoy, S., Valverde, M. E. and Paredes-Lopez, O. (2007). A transgenic tropical maize line generated by the direct transformation of the embryo-scutellum by *A. tumefaciens*. Plant Cell, Tissue and Organ Culture 91(3): 201-214.

van Wordragen, M. F. and Dons, H. J. (1992). Agrobacterium tumefaciens-mediated transformation of recalcitrant crops. Plant Molecular Biology Reporter 10(1): 12-36.

Vega, J. M., Yu, W., Kennon, A. R., Chen, X. and Zhang, Z. J. (2008). Improvement of Agrobacterium-mediated transformation in Hi-II maize (*Zea mays*) using standard binary vectors. Plant Cell Reports 27(2): 297-305.

Wang, G. and Xu, Y. (2008). Hypocotyl-based *Agrobacterium*-mediated transformation of soybean (Glycine max) and application for RNA interference. Plant Cell Reports 27 (7): 1177-1184.

Wang, K. and Frame, B. (2009). Biolistic gun-mediated maize genetic transformation. Methods in Molecular Biology (Clifton, N.J.) 526: 29-45.

Wise, A. A., Liu, Z. and Binns, A. N. (2006). Culture and maintenance of *Agrobacterium* strains. In *Agrobacterium. Protocols*, 2nd edn. (ed. K. Wang), Totowa, New Jersey: Huamana Press, Chapter 1, pp. 3-14.

Yang, A., He, C. and Zhang, K. (2006). Improvement of *Agrobacterium*-mediated transformation of embryogenic calluses from maize elite inbred lines. *In Vitro* Cellular & Developmental Biology-Plant 42 (3): 215-219.

Yin, X. Y., Yang, A. F., Zhang, K. W. and Zhang, J. R. (2004). Production and analysis of transgenic maize with improved salt tolerance by the introduction of At NHX1 gene. ACTA BotanicaSinica-English Edition 46 (7): 854-861.

Zupan, J., Muth, T. R., Draper, O. and Zambryski, P. (2000). The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. The Plant Journal 23(1): 11-28.