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Transformation and Regeneration Protocol for Two Farmer Preferred Open Pollinated Tropical Maize (*Zea Mays*) Varieties

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Abstract: *In vitro* regeneration of open pollinated varieties (OPVs) Kakamega Striga Tolerant Population 94 (KSTP'94) and 'Namba Nane' alongside a tropical inbred line (CML144) was evaluated using immature zygotic embryos as explants. Four callus induction media (CIM) regimes; Murashige and Skoog (MS), Linsmaier and Skoog (LS), Chu (N6) and N6*(N6 medium fortified with 0.35 gL^{-1} L-proline and 0.8 mgL^{-1} $AgNO_3$) were evaluated for their potential to induce callus in the three genotypes. All the media were supplemented with sucrose and five levels of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) (0.5, 1.0, 1.5, 2.0 and 2.5 mgL^{-1}). Resulting calli were matured on MS and N6 basal media supplemented with 60 g/L sucrose and similar concentration levels (0.5, 1.0, 1.5, 2.0 and 2.5 mgL^{-1}) of 2, 4-D while the subsequent embryogenic calli were regenerated on hormone-free media. Transformability of these varieties was assessed via histochemical analysis of β-glucuronidase (GUS) reporter gene following *Agrobacterium*-mediated transformation. Statistical analyses were done using Statistical Analysis Software (SAS) and Graphpad Prism softwares with mean separations achieved at 95% confidence intervals. Of the 2 OPVs, KSTP'94 recorded the highest callus induction frequency (84.4%) while Namba Nane (45.6%) had the lowest. Similarly, KSTP, 94 had the highest mean of mature somatic embryos (59.7%) while Namba Nane recorded the lowest (16.4%). Assessment of regeneration frequencies from embryogenic calli revealed no significant differences among the 3 lines although CML 144 had the highest mean number of juvenile plantlets (36.7%). Analysis of transformation frequency (upon selection of calli on media with basta) showed that Namba Nane recorded the lowest transformation frequency (average 13.5%) some words missing. Transformation frequency (based on GUS positive calli) of these varieties ranged from 0.8 to 2.1%. This work therefore provides an empirical platform for potential introduction of useful genes into these varieties.

Keywords: Callus Induction, Embryogenesis, Plant Regeneration, Open Pollinated Varieties, *GUS*

1. Introduction

Maize is an important food crop and in Sub Saharan Africa (SSA), majority of resource-poor small-scale farmers largely depend on open-pollinated maize varieties (OPVs) for subsistence [1]. These varieties offer the economic advantage of seed recycling without the risk of a decline in yield as is the case with hybrid maize [1, 2]. Given the high price of certified maize seed, high input level and longer maturity period expected of hybrids, small-scale farmers in western Kenya predominantly cultivate OPVs especially during the short rains. Also, some of these varieties have Striga

tolerance attributes that are useful in Striga-infested regions [3, 4]. Furthermore, OPVs have been reported to have superior taste and better keeping quality than hybrids [4, 5]. Demand for maize in the region outstrips supply with farmers insisting on cultivating OPVs such as Kakamega Striga Tolerant Population 94 (KSTP'94)' and 'Namba Nane'. The risk of declining seed quality and farm yield due to on-farm seed production of these varieties calls for the need to use biotechnological approaches to improve these varieties for the benefit of resource poor rural famers. It is therefore important to set up platform for genetic improvement of the OPVs.

One way of achieving this is by developing a reliable *in vitro* regeneration and genetic transformation platform to enable rapid improvement of the farmer preferred OPVs [6, 7]. *In vitro* establishment of maize callus cultures through somatic embryogenesis has been reported for various maize lines but it is genotype dependent [8]. Currently, a variety of basal media have been used to initiate maize callus cultures and subsequent plant regeneration [9]. Generally, the incorporation of an auxin such as 2, 4-dichlorophenoxyacetic acid (2, 4-D) or dicamba (3, 6-dichloro-o-anisic acid) in callus induction media enhances callus formation [10]. However, the concentration levels of these auxins are reported to vary in maize culture media depending on genotype and type of explant used [10, 11]. Furthermore, it is reported that addition of silver nitrate ($AgNO_3$), and amino acids such as L-proline and casein hydrolysate enhances induction of regenerable type II embryogenic callus in maize [10].

In setting up a transformation protocol it is important that rapidly verifiable markers are used to distinguish transformed from non-transformed tissues. The β-glucuronidase (GUS) gene has been widely reported to be an important marker in evaluating transformation systems among various plants [12, 13]. The aim of this study therefore was to develop and optimize an *in vitro* regeneration and transformation protocol for KSTP'94 and Namba Nane alongside a commonly transformed tropical inbred line CML144 as a check.

2. Material and Methods

2.1. Plant Material and Explant Preparation

Seeds of OPVs 'Namba Nane' and KSTP'94 were acquired from Kenya Agricultural and Livestock Organization (KALRO) while tropical maize inbred line CML144 was acquired from International Maize and Wheat Improvement Center (commonly called by its Spanish acronym CIMMYT for *Centro Internacional de Mejoramiento de Maíz y Trigo*) and grown in a research field at Jomo Kenyatta University of Agriculture and Technology (JKUAT). Upon silking, ears were self-pollinated and then harvested between 9th and 11th day after pollination (DAP) to obtain immature embryos. Harvested ears were taken to Plant transformation Laboratory (PTL) at Kenyatta University where they were carefully de-husked. To sterilize the maize, de-husked ears were first dipped in 70% ethanol for two minutes and rinsed thrice using double-distilled water, followed by dipping for 30 minutes in sodium hypochlorite solution containing two drops of Tween 20. The ears were then rinsed three times with double-distilled water. The kernel crowns were aseptically excised and zygotic embryos (0.8 mm - 1 mm in size) of every variety isolated independently using a sterile blunt spatula into a sterile petri plate containing 10 ml of embryo suspension media (ESM).

2.2. Media Preparation

Excised embryos were separately cultured on four different callus induction media (CIM) denoted MS; comprising Murashige and Skoog (MS), Linsmaier and Skoog (LS); Chu (N6); and N6*(N6 medium fortified with 0.35 gL-1 L-proline and 0.8 mgL^{-1} $AgNO_3$ i.e silver nitrate);. The four media were fortified with 30 gL^{-1} sucrose, 2, 4-D at five concentration levels (0.5, 1.0, 1.5, 2.0 and 2.5 mgL^{-1}) and solidified using 3 g/L gelrite (Duchefa Biochemie, Haarlem, Netherlands, cat no. 004016). MS and N6 media were used for callus maturation (CMM) and were supplemented with five concentration levels of 2, 4-D (0.5, 1.0, 1.5, 2.0 and 2.5 mgL^{-1}), 60 gL^{-1} sucrose and 3 gL^{-1} gelrite (Duchefa Biochemie, Haarlem, The Netherlands, cat no. 004016). Regeneration media (RM) were similar to the callus maturation media but lacked 2, 4-D while the sucrose was reduced to 30 gL^{-1} and were solidified using 8 gL^{-1} phytoagar (Duchefa Biochemie Haarlem, The Netherlands, cat no. 025113). The pH of all the media was adjusted to 5.8 before autoclaving at 121°C for 15 min. $AgNO_3$ was filter-sterilized and added to autoclaved CIM and CMM media.

2.3. Callus Induction

To initiate callus, a method by Omer et al., [14] was used. Summarily, the embryos of the three varieties were separately cultured on four CIM fortified with five concentration levels (0.5, 1.0, 1.5, 2.0 and 2.5 mgL^{-1}) of 2, 4-D and replicated three times. In total One thousand, four hundred and forty embryos were used whereby four hundred and eighty immature embryos of each individual variety were used to set up primary callus induction experiment (40 embryos per treatment). Immature embryos with undamaged scutellar tissues were cultured with the embryo axis in contact with the medium and incubated in darkness at 25 ± 2°C. After two weeks of incubation, the number of immature embryos producing primary callus was recorded in each variety before subculturing onto new media.

2.4. Callus Maturation

For maturation of somatic embyros, primary calli from the setup above were sub cultured onto two CMM media (described earlier) fortified with five concentration levels of

2, 4-D (0.5, 1.0, 1.5, 2.0 and 2.5 mgL^{-1}. A total of 24 calli sets (36 embryos per set) were distributed in an experimental design defined as 2 CMM X 3 maize varieties X 4 replicates (calli sets) were setup with an individual set consisting of 36 calli. They were incubated in darkness at 25±2°C for two weeks, subcultured once and incubated for another two weeks. The calli were regularly screened (at three days interval) under microscope (Leica MZ10F/CLS150LED installed with Leica application suite V4) for embryogenic calli formation (globular and coleoplitar shapes) and the number of observed embryogenic calli recorded.

2.5. Plant Regeneration, Acclimatization and Growth of Regenerants to Maturity

Embryogenic calli were further subcultured to hormone-free regeneration media (RM) namely MS and N6 supplemented with 30 gL^{-1} sucrose and 8 gL^{-1} phytoagar (Duchefa Biochemie Haarlem, The Netherlands, cat no. 025113) for shoot regeneration. After CMM incubation period, All the calli were subculture on the two regeneration media (RM) in the same callus maturation experimental design. The cultures were maintained at 25 ± 2°C under a 16-h photoperiod using cool white fluorescent lights (40 mmol $m^{-2}s^{-1}$) for four weeks with one subculture (after two weeks of incubation). The number of embryogenic calli pieces that spontaneously produced shoots and roots was recorded. The plantlets were grown until they were about 10 cm tall and those with fully developed roots removed from culture, rinsed in water to remove media, and transplanted into a mixture of autoclaved peat moss and vermiculite (ratio of 1:1). They were then grown under humid conditions in a glasshouse, for acclimatization, for two weeks. Surviving plantlets were then transplanted into potted soil in the glasshouse where they were grown with regular watering until maturity.

2.6. Assessment of Transformability of the Maize Genotypes

To determine whether the maize genotypes under study are transformable, binary vector pTF 102 containing the *GUS* gene was used [15]. The binary vector, harbored in *Agrobacterium tumefaciens*, EHA 101, contains a *Gus* reporter gene, driven by 35S promoter and terminator. It also contains the *Bar* gene that confers resistance to the herbicide Basta. The bacterial culture was maintained on solid Yeast extract peptone (YEP) medium at 28°C supplemented with 50 mgL^{-1} kanamycin sulfate and 100 mgL^{-1} spectinomycin. *Agrobacterium* cultures on solid YEP media were grown at 28°C for 2 days prior to transformation experiments. Liquid infection media (MS, LS, N6 and N6*) were used for pre-culture, washing and *Agrobacterium* infection steps of embryos according to Frame *et al.* [15]. For transformation, 740 embryos of each individual maize variety prepared as earlier described were infected with *A. tumefaciens* harboring the binary vector and cocultivated on MS media according to Frame *et al.* [15]. Induced calli were transferred onto selection media supplemented with 1.5 mg/L Basta and later subjected to histochemical Gus analysis as described by Jefferson *et al.* [12]. Staining of the calli following Gus action was confirmed by microscopic observation of the stained calli and recorded as Gus transformants using Leica MZ10F/CLS150LED microscope (Leica application suite V4).

3. Statistical Analysis

Statistical analysis for determination of frequencies (%) of callus induction, somatic embryogenesis, regeneration and transient Gus expression was performed using SAS version 9.2. Tukey's HSD test at 95% confidence interval was used to determine significant differences among the parameters under study. Graphpad prism version 6.0 was used to generate the graphs.

4. Results

4.1. In Vitro Regeneration of Namba Nane, KSTP'94 and CML144

In this study, immature zygotic embryos (Fig. 1A) of CML144, KSTP'94 and Namba Nane, cultured on callus induction media showed primary callus formation by swelling of scutellum within 10 days of culture (Fig. 1B). Miniature globular somatic embryos were then observed at the surface of the friable callus in all genotypes by the 16th day of incubation on callus induction media. Transfer of these calli to maturation media resulted into two types of calli; non-embryogenic tissues that were soft, organized and slow-growing and embryogenic ones that were compact, friable and fast growing (Fig. 1C). Upon transfer of the coleoptilar-like embryogenic calli to hormone free regeneration media, juvenile shoots and roots emerged spontaneously after two weeks of culture (Fig. 1D). Well-rooted plantlets formed within two weeks of subculture on fresh regeneration media. They were transferred to vermiculite and peat moss mixture and hardened for one week (Fig. 1E). These plantlets were later transplanted in potted soil in the glasshouse (Fig. 1F), and allowed to develop to maturity. Some somaclonal variation was observed as evidenced by some of the harvested cobs (Fig. 1H).

Frequencies of callus induction in the three maize genotypes cultured on media supplemented with varying concentrations of 2, 4-D are shown in Figure 2. Generally, the frequency ranged from 45.6%±2.1 to 85.6±2.0%. In Namba Nane, the highest callus induction frequency (83.9 ± 3.8%) was recorded on N6* supplemented with 2.5 mg/L 2, 4-D while the lowest (45.6±2.0%) was recorded on MS supplemented with 0.5 mg/L of the auxin (Fig. 2a). In KSTP'94, N6* fortified with 1.5 mg/L 2, 4-D recorded the highest callus induction frequency (84.4 ± 3.1%) while the lowest (46.7±1.7%) was recorded on MS at 0.5 mg/L 2, 4-D (Fig. 2b). On the other hand, CML144 cultured on LS media recorded the highest callus induction frequency (85.6

± 2.1%) at 2 mg/L 2, 4-D while the lowest frequency (48.9±2.2%) was recorded on MS at 0.5 mg/L 2, 4-D (Fig. 2c). There were no significant differences (P≤0.05) in the 3 genotypes across all the media regimes used in this study (Fig. 2).

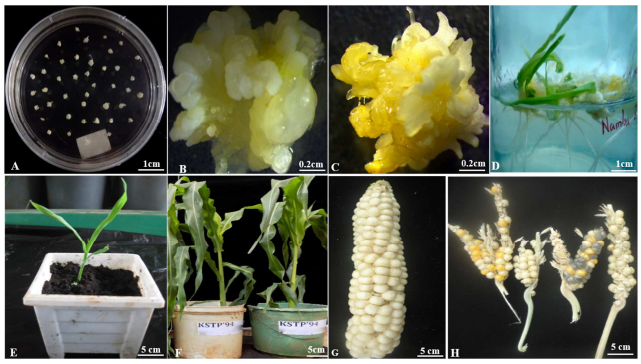
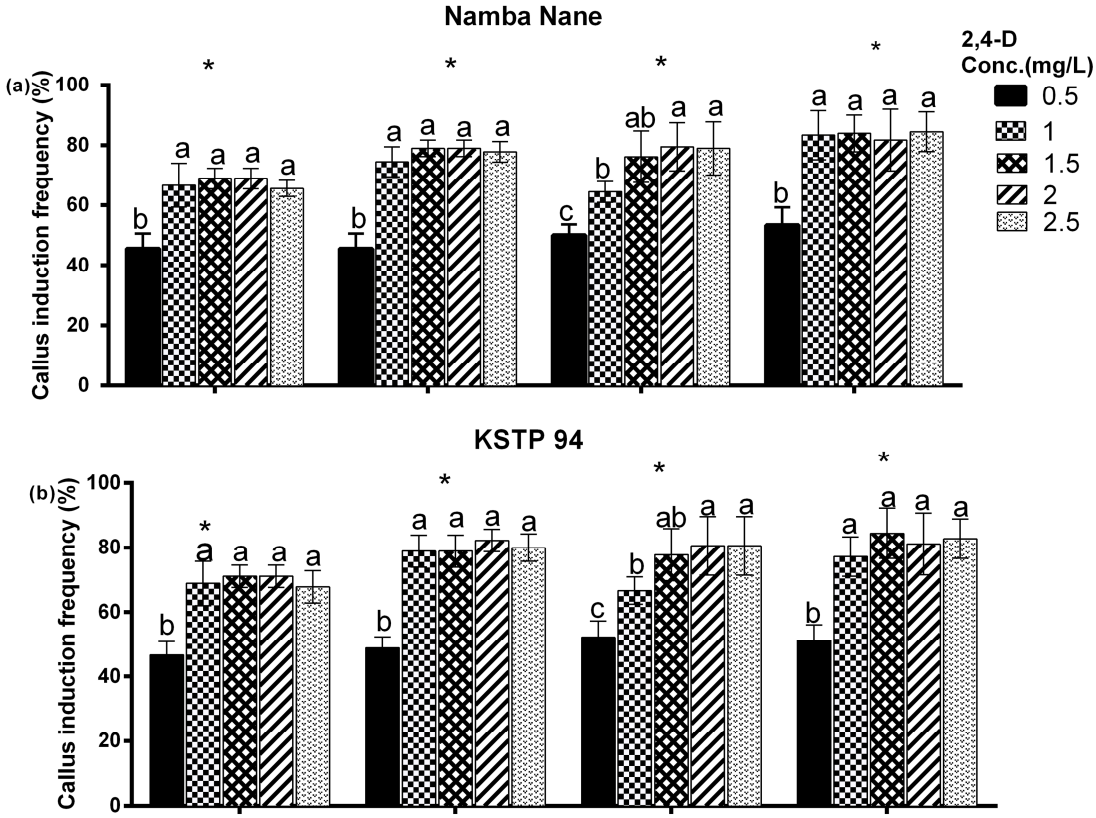


Fig. 1. *Profile of the developmental stages of maize regeneration through somatic embryogenesis using immature zygotic embryos. A. Immature zygotic embryos on callus induction medium. B. Induction of primary callus in CML 144 after 2 weeks of culture on medium with 2, 4-D. C. Development of embryogenic callus following 2 week culture on callus maturation medium in CML 144. D. Emergence of shoots and roots on regeneration medium in 'Namba Nane'. E. Acclimatization of a Namba Nane plantlet on peat moss in the glasshouse. F. Growth of KSTP' 94 maize on potted soil in the glasshouse. G. A repreventative picture of a normal mature maize cob with kernels of KSTP' 94. H. A repreventative picture showing somaclonal variation in Namba Nane.*

The frequencies of somatic embryo formation in the 3 maize genotypes using 2 media regimes supplemented with 2, 4-D are further shown in Figure 3. In Namba Nane, the highest frequency of embryogenic callus formation (54.7 ± 1.4%) was recorded on N6 media supplemented with 1.5 mg/L 2, 4-D while a significantly lower frequency (16.4±2.5%) was recorded on MS with 0.5 mg/L of the same auxin (Fig. 3a). In KSTP'94, the highest frequency of embryogenic callus formation (59.6 ± 1.0%) was recorded on N6 media enriched with 1.5 mg/L 2, 4-D while the lowest frequency (17.8±1.5%) was recorded on MS at 0.5 mg/L of the auxin (Fig. 3b). The highest frequency of embryogenic callus formation in CML 144 (59.7 ± 1.6%) was recorded on N6 at 1.5 mg/L concentration of 2, 4-D while the least (21.1±2.5%) was recorded on MS at 0.5 mg/L of 2.4-D (Fig. 3c). In general, there were significant differences (at P≤0.05) between 2, 4-D concentrations used however, there were no significant differences between the two media types used to induce embryogenic callus formation (Fig. 3).

An outline of shoot induction frequencies of the three genotypes cultured on MS and N6 media is presented in Figure 4. Overall, N6 medium induced higher numbers of plantlets than MS in all the 3 genotypes although these were not significantly different (P≤0.05). 'Namba Nane' recorded the lowest *in vitro* regeneration frequency. 28.2±4.8% of the embryogenic calli of 'Namba nane' on N6 resulted in shoot formation while a frequency of 24.5±4.2% was recorded on MS (Fig. 4a). In KSTP'94, 35.0±4.0% of embryogenic calli formed shoots on N6 medium while 29.6±6.4% was recorded when MS medium was used (Fig. 4b). Slightly similar results were observed for CML144 with an *in vitro* plant regeneration frequency of 36.7±5.9% and 34.3±4.3% recorded for N6 and MS respectively (Fig. 4c).



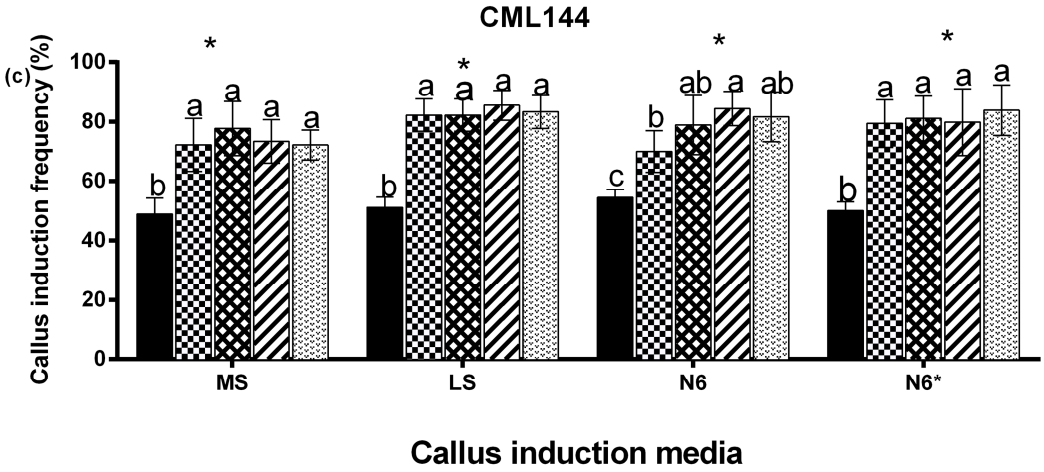


Fig. 2. *Frequency of callus induction in three maize genotypes (a) Namba nane, (b) KSTP'94 and (c) CML144 using four media regimes. Vertical bars indicate standard errors of the means while letters represent mean separations at P≤0.05. Asterisks (*) indicate mean separations among the media regimes at P≤0.05.*

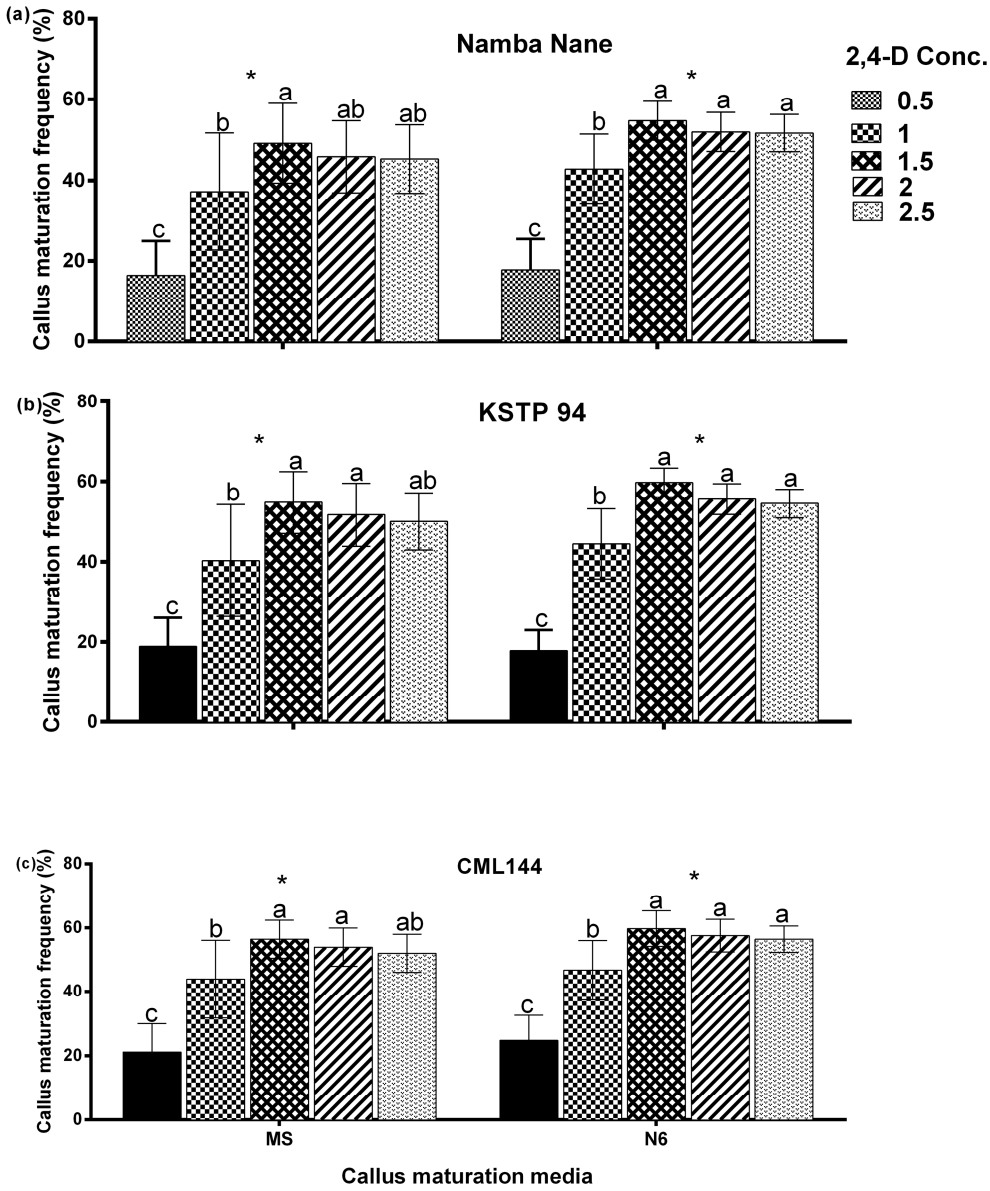


Fig. 3. *Frequency of embryogenic callus in the 3 maize genotypes (a) Namba nane, (b) KSTP'94 and (c) CML144 using two media regimes. Vertical bars indicate standard errors of the means while letters represent mean separations at P≤0.05. Asterisks (*) indicate mean separations between the media regimes at P≤0.05.*

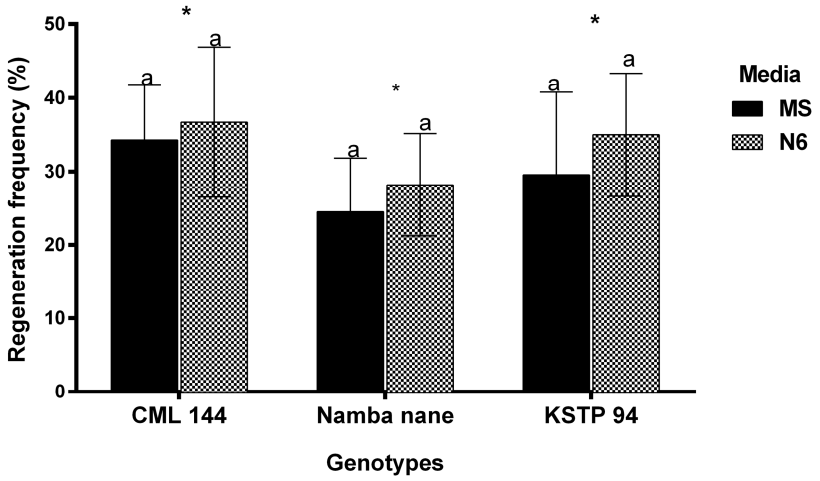


Fig. 4. Shoot induction frequency of three maize genotypes on MS and N6 media. Letters represent mean separations within the genotypes while asterisks (*) indicate mean separations between the media regimes (P≤0.05).

4.2. Transient Transformation Analysis

In a separate experiment, immature embryos of CML144, KSTP'94 and 'Namba Nane' maize genotypes were co-cultivated with *Agrobacterium tumefaciens* harboring binary vector pTF102 with a GUS reporter gene according to Frame *et al.,* (2002) [14]. Selection of putative transformed tissues was achieved using Basta (Fig. 5A) with the compact calli surviving herbicide action used for histochemical GUS assay to assess the expression of the *uid*A gene according to Jefferson *et al.,* [12]. Untransformed callus used as a negative control showed no blue coloration characteristic of GUS expression (Fig. 5B). Putative transformed calli of KSTP'94 (Fig. 5C), Namba Nane (Fig. 5D) and CML144 (Fig. 5E) stained blue following histochemical assay. The frequency of transformation of maize (expressed as the percentage of transformed callus tissues surviving selection) significantly varied across the 3 maize genotypes. Particularly, CML144 recorded the highest frequency (18.2±0.9%) followed by KSTP'94 (14.15±1.3%) and Namba Nane (13.46±1.5%) and these were significantly different at P≤0.05 (Table 1). Based on expression of GUS (blue colouration) in putatively transformed tissues, CML 144 once again recorded the highest frequency of transformation (2.14±0.2%). Namba Nane and KSTP'94 had significantly lower frequencies with 1.35±1.5% and 0.8±0.2% respectively (Table 1).

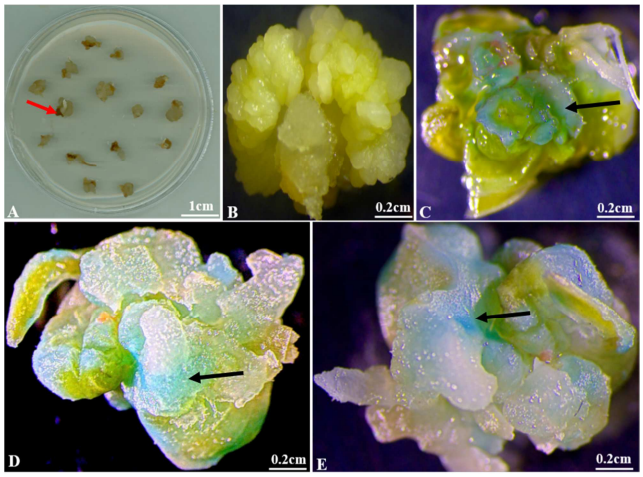


Fig. 5. Profile of maize transformation and histochemical GUS assay. A. Selection of putative transformed maize calli on media with 3 mg/L Basta after two-week incubation period. The arrow indicates a dying callus tissue due to herbicide action. B. CML 144 callus used as a negative control for GUS showing no staining. Positively stained calli in KSTP'94 (C), Namba Nane (D) and CML 144 (E). The black arrows indicate transformed sections of the callus.

Table 1. *Transformation and transient Gus frequency (%) in maize.*

Maizem Genotype	No. of embryos infected	Bar-resistant calli	GUS positive calli
Namba Nane	727	13.46±1.5[b]	0.82±0.2[b]
KSTP 94	730	14.15±1.3[ab]	1.37±0.2[b]
CML 144	739	18.18±0.9[a]	2.14±0.2[a]

Values are means and their standard errors. Means in each column followed by the same letter are not significantly different according to Turkey's test (P≤0.05).

5. Discussion

The findings of this study indicate that open pollinated maize varieties (Namba Nane and KSTP'94) are regenerable and amenable to *Agrobacterium*-mediated transformation. Several studies have reported *in vitro* regeneration of maize genotypes important in agriculture that aimed at creating platforms for the genetic improvement of the maize germplasm [14, 16]. In the present study, CML144 significantly differed from KSTP'94 and Namba Nane in

terms of callus induction and maturation frequencies. Maize has been reported to be recalcitrant to *in vitro* regeneration [17] and it has been demonstrated that *in vitro* regeneration of maize is genotype-dependent, therefore, requiring optimization of protocols for efficient regeneration of each variety [17, 18, 19]. In the current study, this was clearly demonstrated by significant differences observed across the maize genotypes.

In vitro regeneration of maize has also been shown to be dependent on various factors such as the type of media, plant growth regulators (PGRs) and polyamines (e.g L-proline) as well culture conditions. These parameters have been shown to significantly influence the *in vitro* regeneration process of maize [20]. Among the four types of media used, N6* medium not only recorded the highest primary callus induction frequency but also had a high frequency of the embryogenic calli formed. Addition of L-proline and silver nitrate ($AgNO_3$) to the nutrient medium has been found to increase the formation of Type II callus, ideal for transformation [6, 10]. Immature zygotic embryos used as explants in this study are vital to any cereal regeneration exercise since numerous competent cells for somatic embryogenesis with high potential of producing fertile plantlets are present at this stage compared to any other explant material [18]. The immature embryos were cultured with their axis in contact with the medium and the scutellum exposed. This placement technique together with the high sucrose levels aided high callus induction frequencies with limited direct precocious embryo germination as previously reported [21].

Plant growth regulators such as 2, 4-D and Dicamba play a critical role in maize callus cultures, and their effect on *in vitro* regeneration of maize is well documented [19, 22]. Particularly, earlier studies revealed that the use of 2, 4-D to induce callus from immature maize embryos is a critical factor [23]. Other plant growth regulators such as kinetin and Abscisic acid have on contrary been reported to inhibit regeneration in maize callus cultures [24]. This study established that 1.5 mgL^{-1} 2, 4-D was the best concentration level for induction of primary and embryogenic calli in the 3 maize genotypes. Higher concentrations beyond this level had negative effect on induction of embryogenic calli. Bronsema *et al* [24] reported that at least 0.2 mgL^{-1} of 2, 4-D is needed to cross the threshold level for the transition from germination to callus induction while a concentration range of 0.994 mg L^{-1} to 3.006 mg L^{-1} (4.5-13.6 μM) has been shown to be essential for the formation of embryogenic callus in cereals [11]. The mature maize kernels from in vitro regenerated maize that grew in the glasshouse showed somaclonal variation that was possibly due to the tissue culture conditions and the use of 2, 4-D. Similar findings have been observed in maize and flowering stage of groundnuts during regeneration [19, 25].

To evaluate transformation potential of the three maize genotypes, immature embryos were co-cultivated with *Agrobacterium tumefaciens* harboring the β-glucuronidase gene. This reporter system has widely been used for evaluating transformability potential of plants [12]. Expression of the GUS gene as detected by the colour-staining assay is a reliable indicator of transformability of the maize genotypes under this study. The GUS reporter system can only be expressed efficiently in plant cells but not in *Agrobacterium* [12]. Results of transformation efficiency of the tested maize genotypes provide a statistical estimate of the potential of introducing useful genes into these maize genotypes.

6. Conclusion

Derivative medium N6 (N6* medium) was the best medium for *in vitro* regeneration of the three maize genotypes. The best 2, 4-D concentration level for indirect organogenesis in the selected open pollinated tropical maize genotypes was 1.5 mgL^{-1}. Further, assessment of transformation of these genotypes revealed that they are amenable to transformation therefore setting up a platform for their potential improvement with novel genes. This developed regeneration and transformation protocol when optimized will provide a critical foundation for future genetic improvement of these genotypes.

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References

[1] Warburton ML, Setimela P, Franco J, Cordova H, Pixley K, Banziger M, Dreisigacker S, Bedoya C, MacRobert J (2010) Toward a Cost-Effective Fingerprinting Methodology to Distinguish Maize Open-Pollinated Varieties. Crop Sci 50: 467–477.

[2] Pathi KM, Tula S, Huda KMK, Srivastava VK, Tuteja N (2013) An efficient and rapid regeneration via multiple shoot induction from mature seed derived embryogenic and organogenic callus of Indian maize (Zea mays L.). Plant Signal Behav 8: doi: 10.4161/psb.25891.

[3] Wambugu P., Mathenge P., Auma EO, VanRheenen H. (2012) Constraints to On-Farm Maize (Zea mays L.) Seed Production in Western Kenya: Plant Growth and Yield. Int Sch Res Netw ISRN Agron 2012: 1–7.

[4] Omondi E., Norton J., Ashilenje D. (2014) Performance of a local open pollinated maize variety and a common hybrid variety under intensive small-scale farming. African J Agric Res 9: 950–955.

[5] Macharia C., Njeru C., Ombakho G., Shiluli M. (2010) Comparative performance of advanced generations of maize hybrids with a local maize variety: Agronomic and financial implications for smallholder farmers. J Anim Plant Sci 7: 801–809.

[6] Sairam R, Al-Abed D, Johnson J, Muzynski M, Raab M, Reddy T, Goldman S (2008) Maize. In: Kole C, Hall T. (eds) A Compend. Transgenic Crop Plants. pp 49–82.

[7] Oneto C, Germán G, Lewi D, González G (2010) Biolistic maize transformation: Improving and simplifying the protocol efficiency. African J Agric Res 5: 3561–3570.

[8] Jimenez V, Bangerth F (2001) Hormonal status of maize initial explants and of the embryogenic and non-embryogenic callus cultures derived from them as related to morphogenesis in vitro. Plant Sci 160: 247–257.

[9] De-yi Z, You-yin Z, Qian L, Ti Z, De-gang Z (2011) Production of Embryogenic Callus and Plant Regeneration from Elite Guizhou Waxy Maize Inbred Lines. Agric Sci China 10: 490–498.

[10] Songstad DD (2010) Genetic Modification of Plants. In: Kempken F, Jung C (eds) Biotechnol. Agric. For. 64. Springer-Verlag Berlin Heidelberg, Berlin Heidelberg, pp 349–352.

[11] Cheng-hao Z, Li-jun Z, Chao G, Kai H (2008) Establishment and Optimization of the Regeneration System of Mature Embryos of Maize (*Zea mays* L.). Agric Sci China 7: 1046–1051.

[12] Jefferson R., Kavanagh TA, Bevan M. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907.

[13] Witcher D., Hood E., Peterson D, et al (1998) Commercial production of β-glucuronidase (GUS): a model system for the production of proteins in plants. Mol Breed 4: 301–312.

[14] Omer R., Ali A., Matheka J and Machuka J. (2008). Regeneration of Sudanese maize inbred lines and open pollinated varieties. *African J. of Biotech.* 7 (11), 1759-1764.

[15] Frame B., Shou H, Chikwamba R, et al (2002) Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol 12: 13–22.

[16] Zhong DY, Zhu YY, Liu Q, Zhou T, Zhao DG (2011) Production of Embryogenic Callus and Plant Regeneration from Elite Guizhou Waxy Maize Inbred Lines. Agric Sci China 10: 490–498.

[17] Abebe D Zerihun, Teffera Wondyifraw MJS, Abebe DZ, Teffera W, Machuka JS (2008) Regeneration of tropical maize lines (Zea mays L.) from mature zygotic embryo through callus initiation. African J Biotechnol 7: 2181–2186.

[18] Ombori O, Gitonga NMN, Machuka J, Ombori O, Gitonga NM and MJ (2008) Somatic embryogenesis and plant regeneration from immature embryos of tropical maize (Zea mays L) inbred lines. Biotechnology 7: 224–232.

[19] Omer RA, M. Matheka J, Runo S, M. Ali A, Kuria E, Masiga C, Mugoya C, Machuka J (2012) Effects of Auxin and Source of Explants on Callus Induction of Tropical Maize. Biotechnology (Faisalabad) 11: 225–231.

[20] Anami S., Mgutu A., Taracha C, Coussens G, Karimi M, Hilson P, Van Lijsebettens M, Machuka J (2010) Somatic embryogenesis and plant regeneration of tropical maize genotypes. Plant Cell Tiss Organ Cult 102: 285–295.

[21] Vasil IK (2005) Tissue cultures of maize. Maydica 50: 361–365.

[22] Xu L, Huang H (2014) Genetic and Epigenetic Controls of Plant Regeneration. pp 1–33.

[23] Bohorova NE, Zhang W, Julstrum P, et al (1999) Production of transgenic tropical maize with crylAb and crylAc genes via microprojectile bombardment of immature embryos. Theor Appl Genet 99: 437–444.

[24] Bronsema F, Oostveen W, Van Lammeren A (2001) Influence of 2, 4-D, TIBA and 3, 5-D on the growth response of cultured maize embryos. Plant Cell Tissue Organ Cult 65: 45–56.

[25] Masanga J, Ommeh S, Kasili R, Alakonya A (2013) An optimized protocol for high frequency regeneration of selected groundnut (Arachis hypogaea L) varieties from East Africa using cotyledons. Int J Agric Crop Sci 6: 1421–1425.