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# An optimized protocol for *in vitro* regeneration of tropical maize inbred lines through cell suspension and semi-protoplast cultures

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**Maize being an important staple food crop is widely consumed in Kenya but its production remains low due to biotic and abiotic challenges that have not been addressed sufficiently through conventional breeding. This study sought to optimize the establishment of cell suspension and protoplast cultures for 10 tropical inbred maize genotypes in order to identify genotypes that can readily establish in liquid cultures and those whose cell suspensions and protoplasts are capable of *in vitro* regeneration. Callus were induced using immature zygotic embryos on Murashige and Skoog (MS) medium supplemented with 3 mg/L 2, 4-D, 5 mg/L dicamba or 5 mg/L picloram. Dicamba and picloram induced friable calli that readily dispersed into cell suspensions in liquid MS medium supplemented with 0.1 g/L asparagine and either 0.4 or 0.8 g/L proline. Cell growth was determined by packed cell volume (PCV) every seven days. The highest PCV (240 µl/ml) was recorded in genotype EO4 followed by CML 216 (188 µ/ml). Optimal growth was observed in cells maintained in MS Amended with 0.4 or 0.8 g/L of proline in combination with 0.1 g/L asparagine compared to medium without proline. It was also observed that cells cultured in media with reduced Ammonium Nitrate (12 fold reduction) recorded higher PCV values than controls. Protoplasts were generated from the resulting cells using 2% cellulase and 0.5% pectolyase in an enzyme digestion cocktail containing Mannitol and Calcium Chloride (MaCa) and washed in MS with vitamins containing Mannitol (MSMa). Only cell clusters of genotype EO4 gave rise to plants with a regeneration frequency of 42.51%. In conclusion, success was achieved in callus initiation, formation of cell suspension cultures and their eventual regeneration into whole plants for selected tropical maize genotypes.**

**Key words:** Cell suspensions, callus, somatic embryos, packed cell volume, protoplasts.

## INTRODUCTION

Maize (*Zea mays* L) is an important staple food crop that is widely consumed in sub-Saharan Africa. It is also the third most cultivated cereal crop in the world after rice and wheat (Ram, 2011) and a principal crop that is grown in many parts of the world as human food and animal feed (Yadava et al., 2017). The average maize yield per hectare in Kenya is quite low relative to the demand owing to existing constraints to production including

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abiotic challenges such as drought and biotic stresses such as disease, pests, and weeds (Anami et al., 2008; Kariuki, 2015) in addition to poor agronomic practices that result in loss of soil fertility (Thomson, 2008). Management of these constraints has been unsuccessful, mainly because of the use of conventional breeding to develop resistant varieties. Despite a certain degree of success, some of the generated varieties result in undesired traits due to linkage drag and lack of appropriate well defined parental germ lines (Anami et al., 2009). There is need, therefore, to complement such breeding-based strategies with efficient and reliable genetic engineering manipulations for maize. This technology has previously been used in development of germplasm resistant to various pests, diseases such as maize streak virus (MSV) and fungi producing aflatoxin (Masanga et al., 2015).

Efficient use of genetic engineering techniques in the improvement of maize is hampered by the *in vitro* recalcitrance frequently seen in most cereals making it difficult to induce embryogenic callus and eventual regeneration of maize especially in elite varieties (Ge et al., 2016). Despite this challenge, transformation and regeneration of tropical maize lines and other genotypes including open pollinated varieties and hybrids (Ombori et al., 2008, 2013) has been achieved, albeit with some difficulty (Muoma et al., 2008; Oduor et al., 2006; Ombori et al., 2008; Omer et al., 2008). Successful transformation utilizes immature zygotic embryos as explants for indirect transgene delivery often mediated by *Agrobacterium tumefaciens* (Akoyi et al., 2013; Anami et al., 2010; Mgutu et al., 2011; Ombori et al., 2008). For direct transgene delivery however, only *in vitro* established tissues are suitable, but these still must be derived from immature zygotic embryos (Anami et al., 2010; Bedada et al., 2014; Seth et al., 2012; Shepherd et al., 2009). Despite prolonged efforts by many researchers, development of genetic transformation techniques for major cereal crops has not been fast enough. The recalcitrance of maize and most other monocot species to *Agrobacterium*-mediated transformation has necessitated advances in using direct DNA delivery methods (Bommineni and Jauhar, 1997; Gelvin, 1998). Unfortunately, preparatory procedures and the resultant transgenic lines possess redundant background genes that globally evoke biosafety and health concerns (Wang et al., 2000). The maize lines developed, therefore, require rigorous biosafety checks further delaying the rewards otherwise presented by biotechnology. Optimization and establishment of tissue culture protocols for tropical maize is imperative for genetic transformation in maize (Anami et al., 2010). In this paper, establishment of cell suspensions that provide a uniform donor explant material for *in vitro* manipulation of tropical maize genotypes was reported. Plant cell suspension cultures are dedifferentiated; hence, undergo much cell division and DNA synthesis availing the necessary cellular protein repertoire and conditions for easy integration of foreign naked DNA into the host genome. Most direct DNA transfer methods work efficiently when single cells are used as compared to use of differentiated explants (Schuurink and Louwerse, 2000). The cell population in suspension cultures is homogenous making it easy for cells to rapidly access nutrients, plant growth regulators (PGRs), and signals (Mustafa et al., 2011). The plant cells are totipotent, every cell contains all genetic information needed to grow into a new plant, hence any part of the plant can be used as an explant (Endress, 1967). Most maize tissue culture studies that have been done using cell suspensions and protoplast cultures for regeneration and transformation, are mainly for temperate genotypes (Boulton et al., 1993; Hu et al., 2020; Ortiz-Ramírez et al., 2018; Wang et al., 2000).

Despite the enormous progress achieved in this area, several challenges still remain, the main one being the recalcitrance of some protoplast systems, thus inability to express their totipotency. While a lot of investigations have been carried out on tropical maize to develop efficient tissue culture techniques using immature zygotic embryos as the preferred ex plants (Anami et al., 2010; Bedada et al., 2014; Binott et al., 2008; Oduor et al., 2006; Ombori et al., 2008); the pace has been and still is agonizingly slow (Mgutu et al., 2011) and little has been carried out to optimize the culture conditions for cell suspensions and protoplasts for regeneration and transformation. One of the reasons for this predicament is the lack of high throughput optimization of tissue culture protocols for tropical white maize, techniques that should be locally developed or accustomed and therefore suitable for tropical adopted maize genotypes. This is in contrast to temperate maize genotypes, which have remained reference benchmarks for tropical maize tissue culture procedures (Anami et al., 2010). Despite a lot of research that has been done on the genetic transformation of temperate maize genotypes, there is limited information available on genetic manipulation of tropical maize genotypes using cell suspensions and direct gene delivery (Kausch et al., 2021; Yadava et al., 2017).

Protoplasts from the maize endosperm cells have been used for introduction of DNA, RNA and Ribonuclear proteins (RNPs) using protoplast technologies in genome editing (Hu et al., 2020; Kausch et al., 2019; Songstad et al., 2017). To the best of our knowledge, this is the first attempt to establish cell suspension cultures using the selected tropical maize inbred lines and therefore sets up a platform for use in genetic transformation which in turn will open up the use of more cell based technologies for increased productivity of maize in the tropics. The protocol developed from this study will make possible cell manipulation with an aim of increasing yields and management of various biotic factors that affect maize production in different parts of the world but especially

**Table 1.** Formulations of media regimes for determination of packed cell volume in maize.

| Auxin | Treatment | MS basal salts (g/L) | Proline (g/L) | Asparagine (g/L) |
|---|---|---|---|---|
| | D1 | 4.3 | 1.6 | 0.1 |
| 2,4-D | D2 | 4.3 | 0.8 | 0.1 |
| | D3 | 4.3 | 0 | 0.1 |
| | P1 | 4.3 | 1.6 | 0.1 |
| Picloram | P2 | 4.3 | 0.8 | 0.1 |
| | P3 | 4.3 | 0 | 0.1 |

D1: MS + 2 mg/L 2,4-D, 0.8 g/L proline + 0.1 g/L asparagine D2: MS + 2 mg/L 2,4-D, 0.4 g/L proline + 0.1 g/L asparagine D3: MS+ 2 mg/L 2,4-D+0.1 g/L asparagine P1: MS + 3 mg/L picloram, 0.8 g/L proline + 0.1 g/L asparagine P2: MS + 3 mg/L picloram, 0.4 g/L proline + 0.1 g/L asparagine P3: MS + 3mg/L picloram+0.1 g/L asparagine.

the tropics and Kenya in particular.

## MATERIALS AND METHODS

### Plant and explant generation

To generate immature zygotic embryos for use as explants, 10 tropical maize inbred lines, CML 216, CML 144, CML 395, acquired from the International Maize and Wheat Improvement Center (CIMMYT), and EO4, TL 21, TL 22, TL 26, TL 27, AO4 and TO4 obtained from Kenya Agricultural and Livestock Research Organization (KALRO), were planted at Kenyatta University research farm. At the flowering stage, tassels were covered with pollination bags (10 cm × 15 cm × 30 cm) while emerging silks were covered using clear polythene bags (15 cm × 15 cm) to prevent cross pollination. Controlled self-pollination was then done to obtain immature zygotic embryos.

### Establishment of callus cultures

Maize cobs were harvested between 12 and 16 days after pollination and immature zygotic embryos aseptically extracted for use to initiate callus cultures *in vitro* using a protocol described by Negrotto et al. (2000). This was done on a callus induction medium (CIM) comprising 4.3 g/L Murashige and Skoog (MS) basal salts (Murashige and Skoog, 1962), 3% (w/v) sucrose, 0.8 mg/L silver nitrate, 100 mg/L casein hydrolysate and 2 mg/L 2, 4-dichlorophenoxyacetic acid (2,4-D). The pH of the media was adjusted to 5.8 using dilute HCL and NaOH followed by addition of 8% (w/v) plant agar prior to autoclaving at 121°C for 15 min. The cultures were maintained at 26 ± 2°C in the dark for two weeks then sub-cultured onto callus maintenance medium (CMM), similar to CIM but without silver nitrate. To aid in induction and maintenance of friable embryogenic callus, 3 mg/L dicamba and 1.0 mg/L kinetin were added to the media (Akoyi et al., 2013).

### Establishment of cell suspensions

To establish cell suspension cultures, friable embryogenic calli initiated earlier were transferred into liquid medium comprising 4.3 g/L MS basal salts, 0.02 g/L thiamine, 1 g/L casein hydrolysate, 0.1 g/L myo-inositol, 3 mg/L picloram or 2 mg/L 2, 4 D, 1.16 g/L proline, 2 mg/L 2, 4-D, and 3% (w/v) sucrose (Wei and Xu, 1990). The pH of the media was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. 10 mL of the medium was dispensed into autoclaved 125 mL Erlenmeyer flasks and sealed with autoclaved cotton bulbs under aseptic conditions. Cell suspensions were initiated by placing 1, 2, 3, or 4 g of calli in the liquid medium, the flasks were sealed and incubated at 28°C on a rotary shaker at 100 to 120 revolutions per minute (rpm). Some of the cells developing in suspension were re-cultured onto solid CMM to form calli and somatic embryos that were subsequently used to regenerate clonal plantlets (Anami et al., 2010). Sub-culturing was done by refreshing the media after every 7 days and growth rate and general appearance of the cells monitored over time.

### Determination of cell growth

To investigate cell growth in culture, packed cell volume (PCV) of the cells was determined by measuring the cell volume per 1 ml of cell suspension culture after allowing the cells to settle upon removing them from the rotary shaker followed by centrifugation at 100 to 120 rpm (David et al., 1989; Mustafa et al., 2011). Growth was first set at a PCV of 10 ± 2% of the total volume using fresh medium and then incubated at 28°C on a rotary shaker at 100 to 120 rpm. After every seven days, 1 mL of the cell suspension was aliquoted into microcentrifuge tubes and centrifuged at 6000 rpm, at room temperature, for 5 min and the supernatant discarded. The PCV was estimated using a graduated microcentrifuge tube and the data used to decipher the trend in growth rate of the cells in suspension. The PCV was determined using three replicates for each inbred line and media formulation. Several medium regimes (Table 1) were investigated to identify which formulations in the medium support the best growth of cells in suspension.

The medium was refreshed after every 7 days and growth rate of the cells monitored over time. For visual determination of growth, cells were observed under a bright-field microscope (Leica-Wetzlar, Germany) and photographed using a canon digital camera (Oita, Japan). To investigate the effect of ammonia on cell growth, different levels of ammonium nitrate were investigated. The amount of ammonium nitrate was reduced to 4, 8 and 12 fold (during media reconstitution) before initiation of cell suspensions. The PCV was then measured as earlier described and the results compared with those obtained using media with the normal ammonium nitrate ($NH_4NO_3$) concentration of 1650 mg/L (Jones, 2009).

### Establishment of semi-protoplast cultures

Four-day old cells were used to generate semi-protoplasts through partial degradation of the cell wall using different combinations of cellulase and pectolyase enzymes. Cellulase was varied in a range of 1 to 3%, while pectolyase was maintained at 0.5% w/v

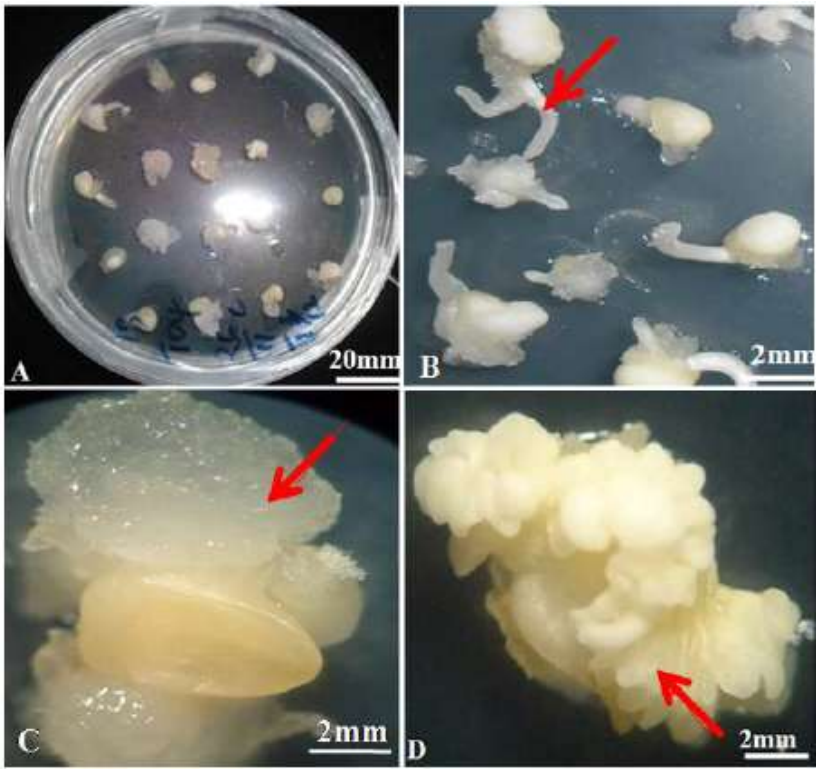


**Figure 1.** Establishment of friable embryogenic calli in tropical maize. (A) Immature zygotic embryos starting to form callus tissue on CIM. (B) Established callus showing precocious germination 14 days after callus initiation. (C) Nascent callus formation on CIM as seen under a light microscope (X 40 magnification). (D) Appearance of friable embryogenic callus on CMM with dicamba and kinetin.

concentration in the enzyme cocktail. The enzymes were first dissolved in 60 mM mannitol and 80 mM calcium chloride (MaCa) solutions at pH 4 (Armstrong et al., 1990; Boulton et al., 1993)

Ten milliliters of the enzyme solution was added to the cells in 50 mL sterile centrifuge tubes and the mixture incubated for 4 h at 28°C in the dark at 50 rpm. Cells were then harvested by centrifugation at 6000 rpm for 5 min (26 ± 2°C) and washed with fresh MS medium supplemented with 60 mM mannitol (MSMa). To ensure an equal amount of protoplasts was used, PCV of the cultures was set at 10% using fresh MSMa and the cells subsequently plated on semi-solid CMM containing 60 mM mannitol. Excess media was allowed to dry and the cultures were incubated in the dark at 26 ± 2°C to allow cell wall development. Samples of the cells were observed under a bright-field microscope (Leica Wetzlar, Germany) and photographs taken.

### Whole plant regeneration from cell cultures

Cultures in suspension were first centrifuged at 6000 rpm for 10 min. The supernatant was then discarded and the collected cells and cell clusters plated on protoplast regeneration media (PRM) overlaid with autoclaved lens paper (Whatman No. 4, Sigma USA). The PRM comprised 4.4 g/L of MS salts with a fourfold ammonium nitrate reduction plus vitamins, 30 g/L sucrose, 2 mg/L 2, 4 D, 0.7 g/L proline, and 1.0 g/L casein hydrolysate. Cultures were incubated at 26±2°C in the dark for 3 to 4 weeks to allow for callus induction. Calli were transferred to callus maturation media (CMM), comprising 4.4 g/L MS with vitamins, supplemented with 60 g/L sucrose, 0.7 g/L proline and 1 mg/L NAA for 14 days. Callus showing the presence of somatic embryos were transferred onto regeneration medium (half strength MS salts and vitamins (2.2 g/L), 20 g/L sucrose and 8 g/L plant agar) for a further 2 weeks to allow for shoot formation and rooting. The number of shoots formed per callus were counted and recorded. *In vitro* regenerated plantlets with well-developed roots were transferred into 250 g pots containing autoclaved peat moss (Sungro horticulture, Amiran, Kenya), covered with wet/moisturized clear polyethylene bags and acclimatized for 10 days. Plants were then transferred to 10 kg potted soil and allowed to grow to maturity till seed formation in the green house at Kenyatta University.

One-way analysis of variance (ANOVA) of the frequencies (%) of callus induction, somatic embryogenesis and regeneration was performed using SAS version 9.2 and Tukey's HSD test at 95% confidence interval was used to determine significant differences among the means.

## RESULTS

### Initiation of friable embryogenic callus

Appearance of callus was varied for each genotype, notably callus appeared from the 5 to 7th day (Figure 1A). There was appearance of root-like structures on

**Table 2.** Frequencies of primary callus formation on CIM and friable embryogenic callus on CMM in maize inbred lines.

| Maize ID | No. of embryos cultured | Callus induction frequency | Friable embryogenic callus frequency |
|---|---|---|---|
| CML 144 | 920 | 75.62±3.39$^{a}$ | 20.40±3.33$^{c}$ |
| CML 216 | 1953 | 58.00±3.76$^{c}$ | 27.28±2.24$^{c}$ |
| CML 395 | 355 | 45.07±1.57$^{d}$ | 0.00±0.00$^{d}$ |
| A04 | 583 | 27.44±2.67$^{f}$ | 0.00±0.00$^{d}$ |
| E04 | 1126 | 69.45±2.05$^{ab}$ | 85.42±3.05$^{a}$ |
| T04 | 301 | 60.47±4.03$^{c}$ | 45.60±1.06$^{b}$ |
| TL21 | 185 | 42.16±1.99$^{de}$ | 0.00±0.00$^{d}$ |
| TL22 | 186 | 64.52±2.79$^{bc}$ | 0.00±0.00$^{d}$ |
| TL26 | 166 | 49.40±.91$^{d}$ | 0.0±0.00$^{d}$ |
| TL27 | 210 | 34.28±.88$^{ef}$ | 0.0±0.00$^{d}$ |

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

callus (Figure 1B). The removal of these structures enhanced growth of the nascent callus (Figure 1C) resulting in fully developed (type II) calli after 21 days of culture (Figure 1D). These well-developed calli were later used to initiate cell suspensions.

Primary callus formation was observed in all the genotypes under the current culture conditions of CIM and CMM. The rate of callus induction and friable embryogenic callus formation is outlined in Table 1. The highest frequency of callus induction was recorded in CML 144 at 75.62% and the lowest frequency was observed in A04 at 27.44% (Table 2). Significant differences (P≤0.05) in callus induction were observed among the inbred lines.

Friable embryogenic calli were observed in CML 144, CML 216, E04 and T04 genotypes. The rest of the genotypes gave rise to non-embryogenic callus that showed browning as another salient observation. The number of embryos forming callus were counted and the frequency of callus induction calculated as a percentage of the total embryos cultured. Similarly, calli showing the presence of somatic embryos were counted and frequency of embryogenic calli calculated as a percentage of the total calli transferred to CMM. Frequency of shoot formation was calculated as the total number of shoots formed as a percentage of the total calli transferred to PRM. The highest frequency of friable embryogenic callus was recorded in E04 (85.42%) while the lowest was in CML 144 (20.4%) (Table 2). Incorporation of dicamba and kinetin led to formation of soft and friable callus (Figure 2).

## Establishment of cell suspension cultures and determination of packed cell volume

Cell suspension cultures were successfully initiated in two genotypes CML 216 and EO4 using six liquid media regimes designated D1, D2, D3, P1, P2 and P3 (Table 1). Upon determining their packed cell volume (PCV), E04 in media regime D2 showed the highest initial PCV at day 1 with regimes D3 and P2 recording the lowest PCVs (Figure 3). Overall, the highest PCV (average 240 µl) was recorded in media regimes D1 and D2 after 14 days of incubation. On the other hand, media regime D3 recorded the lowest PCV (82 µl) after 21 days (Figure 3). There was an initial increase in the PCV values in most media regimes for the first 7 days in E04. Contrastingly, media regime P1 recorded an initial decrease before increasing from day 7 to day 14 (Figure 3). The levels of change (increase and decrease) varied significantly throughout the time points tested. Inbred line CML 216 had the highest PCV value (188 µl) in media regime D1 on the 7th day. On the other hand, media regime P3 recorded the lowest PCV (68 µl) after day 21. Media regime D1, P2 and P3 showed an initial increase in PCVs with D2, D3 and P1 showing reduced levels within the same time point after day one (Figure 4). Overall, there were significant reductions in PCVs across all the media regimes after day 7 (Figure 4).

A profile of the trend in cell growth in E04 inbred line using media regimes with reduced levels of ammonium nitrate over a period of time is outlined in Figure 5. The PCVs in the 3 treatments and the control decreased until the 4th day of culture with a subsequent increase until day 8 although the increments varied with time (Figure 5). Medium M12 with a 12-fold ammonia reduction recorded the highest PCVs increment than M4 and M8. The control media treatment M1, recorded significantly lower PCV levels as compared to M12 but significantly higher levels than M4 and M8 (Figure 5).

## Production of semi-protoplast cultures in tropical inbred lines

Digestion with enzyme regimen containing 2% cellulase produced semi-protoplasts (Figure 6). The enzyme

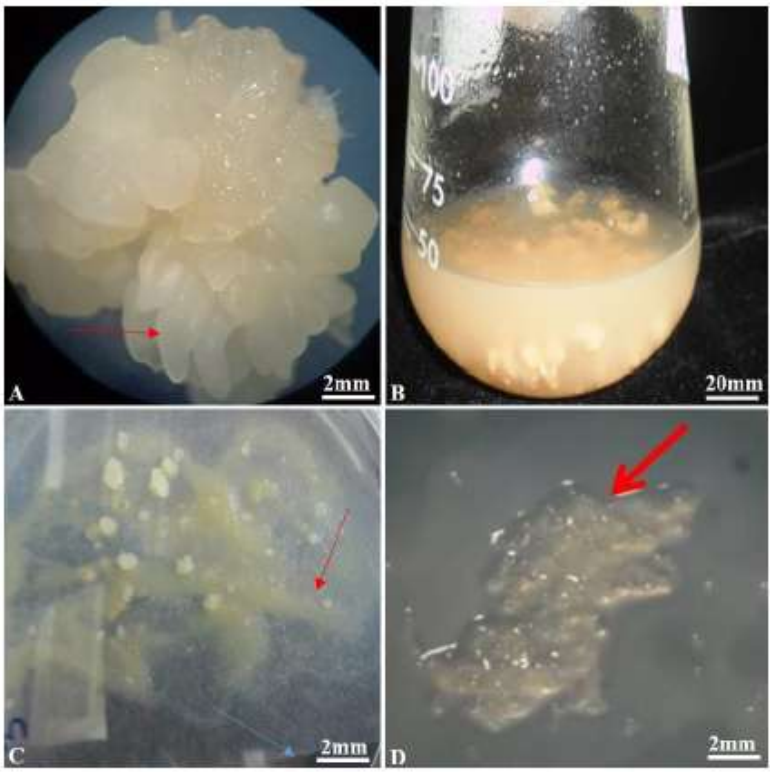


**Figure 2.** Establishment of cell suspension cultures in tropical maize. (A) Friable embryogenic callus after culture on callus maintenance media. The red arrow shows somatic embryo on the callus. (B) Establishment of cell suspensions in liquid medium in Erlenmeyer flasks. (C) Maize cells and cell clusters growing on Protoplast Regeneration media (PRM) overlaid with a lens paper. The red arrow shows cell cluster starting to form. (D) Cell cluster on solid medium after 21 days of growth in liquid medium (X 40 magnification on a stereo microscope under normal white light).

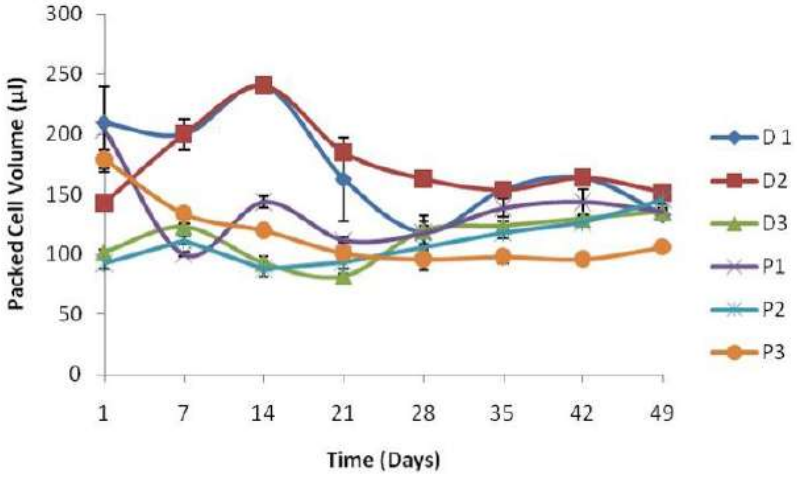


**Figure 3.** Effect of various media regimes on packed cell volume in cell suspensions of E04: MS + 2 mg/L 2,4-D, 0.8 g/L proline + 0.1 g/L asparagine; D2: MS + 2 mg/L 2,4-D, 0.4 g/L proline + 0.1 g/L asparagine; D3: MS+ 2 mg/L 2,4-D+0.1 g/L asparagine; P1: MS + 3 mg/L picloram, 0.8 g/L proline + 0.1 g/L asparagine; P2: MS + 3 mg/L picloram, 0.4 g/L proline + 0.1 g/L asparagine; P3: MS + 3 mg/L picloram+ 0.1 g/L asparagine.

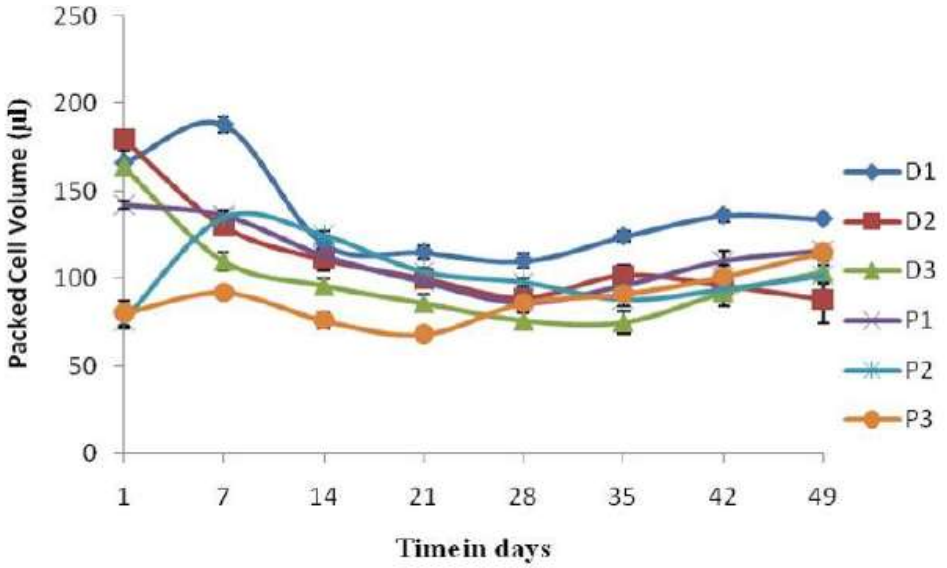


**Figure 4.** Effect of various media regimes on packed cell volume in CML 216. D1: MS + 2 mg/L 2,4-D, 0.8 g/L proline + 0.1 g/L asparagine; D2: MS + 2 mg/L 2,4-D, 0.4 g/L proline + 0.1 g/L asparagine; D3: MS+ 2 mg/L 211 2,4-D+0.1 g/L asparagine P1: MS + 3 mg/L picloram, 0.8 g/L proline + 0.1 g/L asparagine; P2: MS + 3 mg/L picloram, 0.4 g/L proline + 0.1 g/L asparagine; P3: MS + 3 mg/L picloram + 0.1 g/L asparagine.

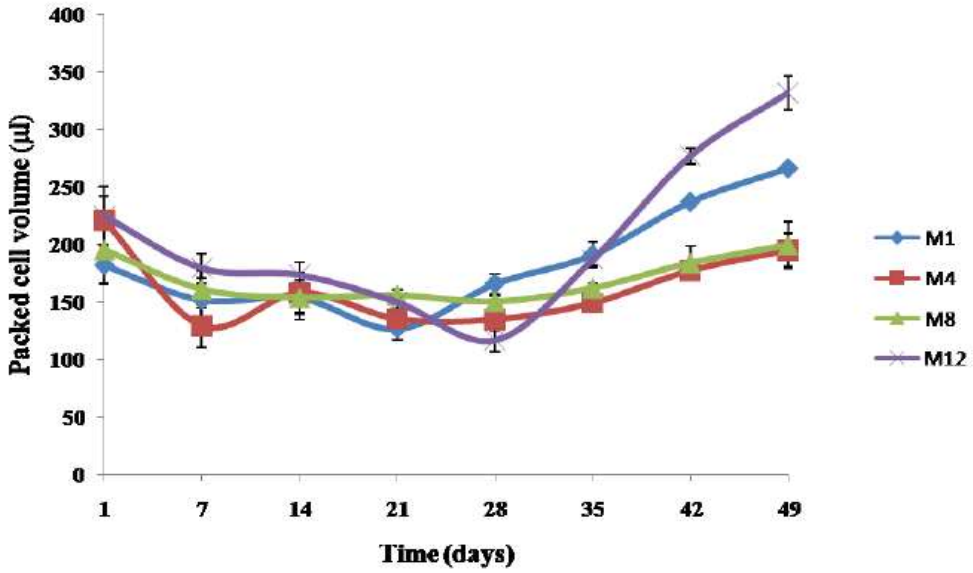


**Figure 5.** Effect of reduced Concentrations of Ammonium nitrate on packed cell volume (PCV) in EO4. Media M1 had 20.61 mM/L $NH_4NO_3$ (the normal ammonium concentration in MS used in culture media). The amount of ammonium nitrate reduced in different proportions in M4 (four-fold); M8 (eight-fold); M12 (twelve-fold).

cocktail containing 2% cellulase produced optimal digestion results based on the spherical appearance of the generated protoplasts. Cells treated with digestion regimen containing 1% cellulase yielded low numbers of protoplasts while those digested with enzyme regimen containing 3% cellulase appeared over-digested (Figure 6). Semi-protoplasts produced after digestion with 3% cellulase appeared deformed with characteristic large-

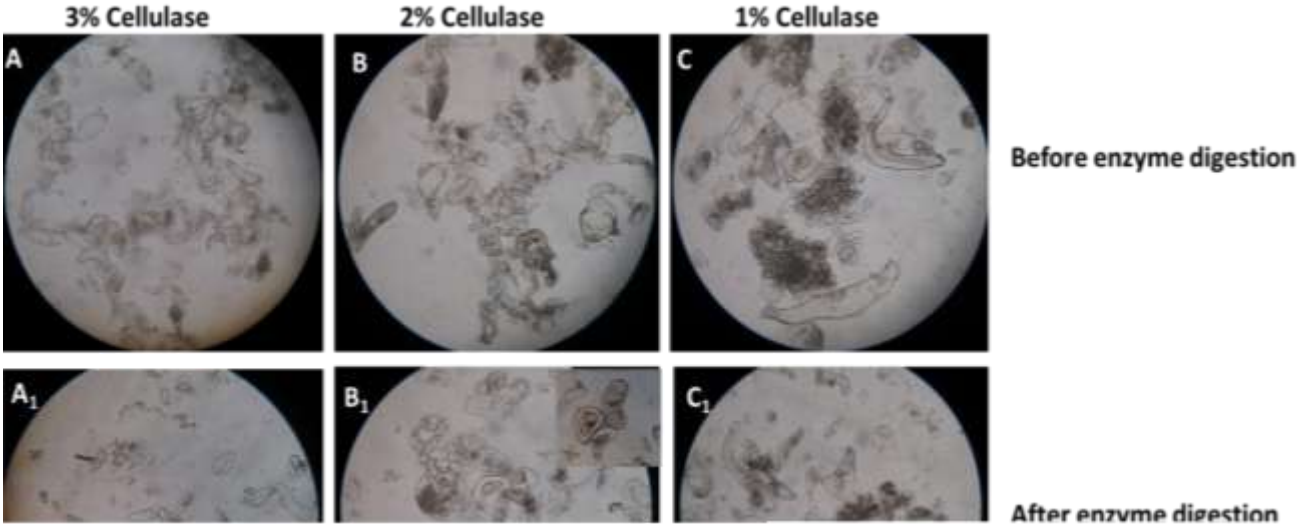


**Figure 6.** Profiles of Semi-protoplast cells of maize genotype EO4 derived from cell suspensions following digestion using enzymes. The cells are shown before (A, B, C) and 5 hours ($A_1$, $B_1$, $C_1$) of cocktail enzyme digestion. Pictures were taken using a digital camera following observation under a light stereo microscope at X40 magnification and normal white light filter.


**Figure 7.** Regeneration profile of maize from cell suspension clusters. (A) Cell clusters on Protoplast regeneration media (PRM), (B) Greening callus on CMM showing shoot development. (C) Maize plantlets on regeneration media showing a fully developed shoot with roots. (D) Hardening of plantlets on peat moss in the glasshouse. (E) Acclimatization of the plants in potted soil. (F) Mature plants after the regeneration process.

**Table 3.** Effect of media regimes on shoot formation and number of shoots per callus in E04 inbred line.

| Treatment | Frequency of shoot formation (%) | Average shoots per callus |
|---|---|---|
| P1 | 15.31±3.86[b] | 0.81±0.83[a] |
| P2 | 28.95±3.45[a] | 1.23±0.90[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). P1: MS + 3 mg/L picloram, 0.8 g/L proline + 0.1 g/L asparagine used in culturing cells used to form callus P2: MS + 3 mg/L picloram, 0.4 g/L proline + 0.1 g/L asparagine used in culturing cells used to form callus.

sized cells; a sign of unsuitable cell types or quality of donor cell suspensions.

### Regeneration of whole plants from cell clusters

Callus clusters successfully developed into plantlets while the single cells failed to regenerate. The profile of stages in whole plant regeneration from cell suspensions is summarized in Figure 7. Only genotype E04 was successfully regenerated in this study. The frequency of shoot formation in this genotype varied across media regimes with P2 producing the highest regeneration frequency (28.95%) while P1 recorded a significantly lower frequency (15.31%) (Table 3). In regard to the number of shoots, P2 again produced the highest average shoots per callus (1.23) although this was not significantly different from P1 (0.81) at P≤0.05 (Table 3). On the other hand, callus recovered from cell suspensions maintained on media with 2,4-D did not form shoots. Fully formed plantlets were obtained at the 15th day. The plants were subjected to rooting by being cultured on autoclaved peat moss. These plants acclimatized well on peat moss and were later transferred to large soil filled pots in the open field.

## DISCUSSION

In agreement with this study, Anami et al. (2010) used immature embryos to produce friable embryogenic callus. Success is described in the initiation of friable embryogenic calli in selected maize inbred lines, use of these calli to induce cell suspension cultures, and their eventual regeneration into whole plants in maize. Success in the induction of primary callus tissues was achieved in all the genotypes tested using 2, 4-D. However, conversion of these tissues into friable embryogenic structures was observed in only four genotypes, under the current conditions. Conversion of primary callus into friable embryos is genotype dependent, that is why all primary callus did not form friable embryos which is in line with the study by Binott et al. (2008). Only friable embryogenic (Type II) callus cultures can be used in formation of embryogenic cell suspensions for whole plant regeneration (Gordon-Kamm et al., 1990; Songstad, 2010). Genotype dependency in callus induction during *in vitro* maize regeneration has previously been reported (Akoyi et al., 2013; Armstrong et al., 1992). In this study, some of the genotypes formed callus that turned brown, became necrotic, and eventually died which was in line with studies from other tropical maize genotypes (Binott et al., 2008; Ombori et al., 2008).

In maize, type I callus can only be maintained for a short period of time and is therefore unsuitable for generation of suspension cultures which require friable and rapidly growing tissues (Armstrong and Green, 1985; Frame et al., 2000; Vasil and Vasil, 1986). To generate cell suspension cultures, the friable calli need to be cultured in a suitable liquid medium (Dixon, 1985; Gamborg and Phillips, 1995). Under the current study, lack of formation of friable embryogenic calli (FEC) in the six genotypes is attributed to their lack of amenability to tissue culture under the current conditions. Significant differences (P<0.05) in FEC frequencies recorded among the four genotypes confirmed our hypothesis that regeneration of these lines is genotype-dependent and this is in line with a previous study where similar observations were made (Akoyi et al., 2013). Dicamba was instrumental in maintenance of the FEC as this auxin was found to be superior to 2, 4-D. The latter PGR caused precocious germination and produced non-embryogenic calli. Previous studies have also implicated 2,4-D in negative somaclonal variations in regenerants (Akoyi et al., 2013; Omer et al., 2008).

The present study further reports success in establishment of cell suspensions in two genotypes. Continuous agitation of cultures in liquid media was instrumental in obtaining cell suspensions and as expected, cell clusters/clumps were also recovered owing to the often required degree of aggregation (Hulst et al., 1989). Various methods for determination and measurement of growth of cells in culture have been described (Mustafa et al., 2011). Just like initial descriptions (Dixon, 1985; Hahlbrock, 1975), the current study employed the packed cell volume (PCV) technique. Here, growth is determined as the packed cell volume of

pelleted cells and expressed as a function of the volume of culture over time. This has been shown as a fast and simple method for growth measurement of cell suspensions (Mustafa et al., 2011).

The growth of cells in suspension was initially exponential for the first four to eight days before decreasing. This suggests that the factors inhibiting continued exponential growth were effective by the fourth day of culture, becoming more potent thereafter. Alternatively, growth-rate limiting components in the medium were depleted fast by the fourth day of culture leaving the cells without nourishment. A continuous culture system is currently an option for exploration, while the responsible component(s) limiting and/or inhibiting growth are also being identified by optimizing each component of the medium formulation while holding the others constant.

The observed variation in growth of cells in liquid media depended on components incorporated therein. Media containing proline yielded higher levels of PCV than that without confirming the importance of proline in maintenance of friable embryogenic calli for a long time in culture media (Armstrong and Green, 1985; Jones, 2009). Despite the failure of 2, 4 D to produce friable embryogenic callus, it proved to be more superior in yielding higher levels of PCV compared to picloram. Similar studies on effect of auxins on embryogenesis during cell growth in culture have been reported (Ombori et al., 2008). Generally, the use of MS media with 20 mM ammonium nitrate yielded lower PCV values in all the genotypes across media treatments. Reducing these levels, however, resulted in a marked increase in PCV over time. Ammonium nitrate is essential in promoting cell division but is toxic to cells and protoplasts in concentrations used in most tissue culture media. To enhance cell division and reduce toxicity, its concentration needs to be reduced. This is based on reports demonstrating that reducing nitrogen was effective in maintenance of friable embryogenic callus (Jones, 2009). In the current study, a 12-fold reduction of ammonium nitrate resulted in the highest PCV in maize compared to the control. We have also optimized a protocol for generation of tropical maize from semi-protoplasts. An optimum enzyme cocktail for production of semi-protoplasts used in this study was comparable to that used by Armstrong et al. (1990) during production of protoplasts used in PEG-mediated DNA delivery. The slight differences observed could be due to the genotypes studied. The earliest reports on successful maize transformation involved the use of protoplasts to introduce transgenes through microprojectile particle bombardment (Fromm et al., 1990; Gordon-Kamm et al., 1990; Rhodes et al., 1988). Unlike *Agrobacterium*-mediated technique, this method is not host specific (Davey et al., 2000). Protoplasts offer an advantage during transformation, via PEG treatment or electroporation, in that they allow penetration of DNA into the cells with much ease (Schuurink and Louwerse, 2000). These processes, however, require homogeneous cell populations for release of protoplasts and enzyme treatment (Davey et al., 2000; Tyagi et al., 1999).

Genotypes CML 216 and T04 did not regenerate into whole plants despite having formed friable embryogenic calli. This can be attributed to genotype dependence during regeneration in most cereals with similar results being reported in maize regeneration (Ge et al., 2016). Furthermore, lower regeneration efficiencies have been reported for tropical maize inbred lines as compared to temperate ones (Anami et al., 2010; Ombori et al., 2008), thereby necessitating exploration of ways to improve the efficiencies. The media components used during maintenance of the cells in suspension influenced the regenerability of the maize cells and the fact that cells and protoplasts are dedifferentiated and respond less drastically to changes during tissue culture manipulations, has been exploited in this study to obtain plants.

## Conclusion

The potential of tropical maize inbred genotypes to induce friable embryogenic callus and sustain growth through cell suspensions has been evaluated. All the maize genotypes tested were capable of inducing callus, although only a few were able to induce friable embryogenic callus that was used to initiate cell suspensions. Embryogenic cells and callus are a prerequisite for maize regeneration and transformation, and their formation is genotype dependent. The embryogenic callus cultures used effectively produced semi-protoplasts and only the cells in clusters were able to produce plants. Therefore, this optimized protocol has the potential for further improvement of these tropical maize genotypes through genetic engineering.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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