

***In vitro* propagation of *Manihot esculenta* Crantz in alternative substrate: Ammonium nitrate, potassium bi phosphate, *Zea mays* extracts and soil**

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Abstract. The study investigated an *in vitro* propagation of *Manihot esculenta* Crantz in a substituted substrate regime. The aim was to proffer and affordable alternative to the expensive high tech media formulations usually employed in tissue culture protocol. The experiment was conducted on laboratory bench, using standard tissue culture and micropropagation methods under aseptic conditions. The morphogenesis effect of the substrate was determined based on the integer number of explants' callus and adventitious shoot regeneration. Results showed that MS + Agar, supported embryogenic callus formation with 38% viability, NH₄NO₃ + KH₂PO₄ + Agar, supported same with 29%. MS + 2, 4-D + BAP + Agar supported shoot establishment with 32%. While NH₄NO₃ + KH₂PO₄ + *Zea mays* extracts + Agar, did same with 43.26%. MS + Soil, supported callus formation with 27% viability while NH₄NO₃ + KH₂PO₄ + Soil supported the callus establishment with 25%. MS + 2, 4 - D + BAP + Soil, supported shoot establishment with 38.41% viability while NH₄NO₃ + KH₂PO₄ + *Zea mays* Extracts + Soil supported same with 36%. The application of crude *Zea mays* seedling extracts can serve as potent alternative to the synthetic 2, 4 – D and BAP, in *in vitro* somatic cell morphogenesis. NH₄NO₃ + KH₂ + PO₄ can substitute for the MS salt in the same protocol. Loamy top soil can be a good alternative to agar powder as gelling agent in cassava somatic cell embryogenesis and shoot regeneration.

Keywords: Ammonium nitrate, Potassium biphosphate, MS salt, axillary meristem, morphogenesis.

INTRODUCTION

Alternative substrate in the biotechnology of tissue culture is a veritable tool; referred to as media formulations in which the conventional or standard colligative ingredients were substituted with alternative compounds in the menu recipes. Operations in *in vitro* regeneration of plants fully rely on standard basic salts. These salts, irrespective of its expensive nature, remained the only option in most tissue culture and genetic manipulation events (Alango *et al.*, 2018). In Agrobiolgy and Horticulture, novelty character had been successfully created, using MS salt and conventional

phyto hormones supplements in shoot regeneration culture of banana (*Musa sapientum* L) (Gebeyehu, 2015, Ngomuo and Ndakidemi, 2014; Kitimu *et al.*, 2015).

Similarly, medicinal plants and tuber crops like sweet potato (*Ipomoea batatas* (L) Lam.), cassava (*Manihot esculenta* Crantz.), etc had been raised in cultures hosted by MS salt supplemented with conventional phyto hormones (Muluke and Tileye, 2018; Tabins *et al.*, 2018). However, research workers in East Africa, succeeded in using local phyto fertilizer called easy grow to micro propagate cassava (Kwame *et al.*, 2012).

In vitro propagation of cassava is an aseptic test tube technique of growing and managing the crop in culture, under space, time and other resource economy. The regeneration process can either follow micropropagation pathway or via tissue culture.

Callus are unorganized mass of parenchyma cells derived from somatic tissues of explants in culture. They can differentiate to become meristematic and to give rise to other plasmic and blastic growth forms (Wang *et al.*, 2011).

In the biotechnology of plant tissue culture, callus induction and proliferation are of fundamental important. This is because embryogenic callus is the primordial stage in the cycle of *in vitro* somatic morphogenesis, extending to embryoid and organ establishment. This stage is also of pharmaceutical importance, because secondary metabolites can be extracted for biochemical industries.

Broad spectrum and minimal substrate formulations have been produced by several workers to aid *in vitro* regeneration and morphogenesis of totipotent cells in artificial cultural media. Among these are the Murashige and Skoog (1962) (MS), the White's medium (1963) (white), Nitsch medium (1951) (Nitsch) and Eriksson medium (2003) (Eriksson).

These conventional substrates have been sustaining the business of tissue culture and micro propagation. But they also have their own limitations which include unavailability and seldom affordability by subsistent crop growers, especially, those from developing countries of Africa and Asia. This study therefore aims at providing crude alternative substrate recipes with highly affordable local content principles, and *in vitro* environment that is near natural.

MATERIALS AND METHODS

The study was conducted between the months of April and July 2017, at the Department of Biology and Biotechnology laboratory of the Federal University of Technology, Owerri, Imo state, Nigeria. Owerri is situated on latitude 5° 28' N and 5° 30' N and longitude 7° 01' E and 7° 03' E; within the Rain forest belt of South-Eastern Nigeria.

Substrate composition

Substrate formulation followed a modification of Duru and Opara (2009) procedure.

Substratum A: Twenty grammes (20 g) of Murashige and Skoog (1962) (MS) salt, 30 g of sucrose, 8 g of agar, 1000 mg of chloramphenicol, 1000 mg of ketoconazole and 1000 ml of de-ionized water.

Substratum B: Twenty grammes (20 g) of MS salt, 30 g

of sucrose, 0.5 mgL⁻¹ of 2,4-dichlorophenoxy acetic acid (2, 4 – D), 0.01 mgL⁻¹ benzyl amino purine (BAP), 8 g of agar, 1000 mg of chloramphenicol, 1000 mg of ketoconazole and 1000 ml of de-ionized water.

Substratum C: One mole of ammonium nitrate (NH₄NO₃), one mole of potassium biphosphate (KH₂PO₄), 30 g of sucrose, 8 g of agar, 1000 mg of chloramphenicol, 1000 mg of ketoconazole and 1000 ml of de-ionized water.

Substratum D: One mole of ammonium nitrate (NH₄NO₃), one mole of potassium biphosphate (KH₂PO₄), 30 g of sucrose, 2 tablets of *Zea mays* extracts, 8 g of agar, 1000 mg of chloramphenicol, 1000 mg of ketoconazole and 1000 ml of de-ionized water.

Substratum E: Twenty grammes (20 g) of Murashige and Skoog (MS) salt, 30 g of sucrose, 100 g of the top soil, 1000 mg of chloramphenicol, 1000 mg of ketoconazole, 1000 ml of de-ionized water.

Substratum F: Twenty grammes (20g) of MS salt, 30 g of sucrose, 0.5 mgL⁻¹ of 2,4-dichlorophenoxy acetic acid (2,4-D), 0.01 mgL⁻¹ benzyl amino purine (BAP), 100 g of the soil, 1000 mg of chloramphenicol, 1000 mg of ketoconazole and 1000 ml of de-ionized water.

Substratum G: One mole of ammonium nitrate (NH₄NO₃), one mole of potassium biphosphate (KH₂PO₄), 30 g of sucrose, 100 g of the soil, 1000 mg of chloramphenicol, 1000 mg of ketoconazole and 1000 ml of de-ionized water.

Substratum H: One mole of ammonium nitrate (NH₄NO₃), one mole of potassium biphosphate (KH₂PO₄), 30 g of sucrose, 2 tablets of *Zea mays* extracts, 100 g of the soil, 1000 mg of Ketoconazole and 1000ml of De ionized water.

Substrate sterilization

The pH of the agar base substrate was adjusted to 5.7 while the pH of the soil base substrate was left to its natural soil pH of 4.72. The substrate and the instruments were autoclaved for 30 min at 121°C and 103 × 10³ Pa. They were allowed to cool to 60°C before dispensing into the spacemen bottle.

Extraction of phytohormones from *Zea mays*

The grains of *Zea mays* were surface sterilized with 10% sodium hypochlorite (NaOCl). Solution, rinsed in three changes of distilled water, germinated on moist filter paper in petri dishes. Agar blocks were prepared according to manufacturer's specification, allowed to

solidify and cool. The tips of the coleoptiles and coleorhizae were sectioned out and placed contiguously with the cut surfaces on the solid media and allowed to extract for three hours. 8 mm diameter glass perforator was used to create the tablets.

Explant sterilization and implantation

The explants were collected from stems of 16 month old standing crops. The cut sections were thoroughly washed with 10% liquid detergent solution and rinsed properly with sterile distilled water. The sticks were soaked in 70% ethanol for five minutes. Axillary buds were excised and soaked in 10% sodium hypochlorite NaOCl solution for 10 min, then rinsed in 3 changes of sterile distilled water. The explants were trimmed and aseptically implanted and orientated vertically on the substrate, using sterile forceps. After implantation each substrate group was divided into three sets; one set was incubated in a dark cupboard, the second was incubated in an incubator at 27°C, photoperiod of 16 h at 1000 lux while the last set was kept on the laboratory bench at the room physical conditions. The cultures were incubated for 28 days and sub cultured for every 28 days passage periods. Substrate performance was evaluated using the relation:

$Pf_c = \text{No. of explants with callus} \times 100 / \text{viability}$; for callus and

$PF_s = \text{No. of explant with shoots} \times 100 / \text{Viability}$; for shoot induction

Shoot induction efficiency (%) = $\text{Mean no. of shoot} \times 100 / \text{Viability count}$.

Soil analysis

Soil samples were collected at 0 to 15 cm depth, from the garden stead of forestry department of the Federal University of Technology, Owerri. The samples were air dried at room temperature, sieved with a 2 mm mesh sieve. The parameters tested were particle size, air dry moisture, pH, organic matter, organic carbon, total nitrogen, available phosphorous exchangeable bases (K, Na, Ca, Mg) and base saturation.

Particle size

Particle size determination was done with an aerometer following the method of Mocek *et al.* (2000)

Air dry moisture

This followed the method of preliminary tests as described by Nwinuka *et al.* (2003). The air dried soil

were sieved with a 2 mm mesh sieve, 10 g were weighed into a dry crucible, placed in an air- circulating oven, set at 105°C and allowed to dry for 6 h, then reweighed till a constant weight was attained. They were then cooled to room temperature in desiccator. The air dry moisture was determined as percentage loss in weight, using the relation:

$$\% \text{ Moisture} = \frac{\text{Loss in weight (g)}}{\text{Initial weight of the air dry soil}} \times 100$$

Soil pH

The pH was determined with glass electrode pH meter at 1:5 soil/water content ratio (Thomas, 1996).

Organic matter content

The organic matter content was estimated as loss on ignition (Nwinuka *et al.*, 2003). 10 g of oven-dried soil was weighed into a crucible of known weight, then placed in a muffle furnace and the temperature was gradually raised to 450°C and allowed to remain for 4 h. The furnace is switched off, the crucible was transferred to a desiccator and allowed to cool to room temperature and reweighed. The loss was estimated using the relation:

$$\text{Loss on ignition} = \frac{\text{Weight loss (g)}}{\text{Weight of oven - dried soil (g)}} \times 100$$

Organic carbon content

This was determined by wet oxidation method following the procedure described by Nelson and Sommers (1996).

Available phosphorous

This was estimated using the method of Okalebo *et al.* (2002).

Available nitrogen

This was determined by Kjeldahl digestion and titration after distillation into boric acid, following the method of Bremner (1996).

Exchangeable acidity and Basicity

Exchangeable acidity was determined following the protocol of Melean (1965) while the exchangeable bases

Table 1. Callus establishment of *Manihot esculenta* on the test substrate on the bench (%f).

| Substrate | Mean no. explants | % Viability | % Sf. | % Cf. | %Sub. performance. | |
|---|-------------------|-------------|-----------|-----------|--------------------|-------------|
| | | | | | Callus | Shoot |
| MS + Agar | 20 | 58 ± 0.12 | 20 ± 0.18 | 38 ± 0.00 | 66 ± 1.34 | 34 ± 0.06 |
| MS + 2,4-D + BAP + Agar | 20 | 40 ± 10.48 | 20 ± 0.58 | 20 ± 0.00 | 50 ± 6.08 | 50 ± 1.18 |
| NH ₄ NO ₃ + KH ₂ PO ₄ + Agar | 20 | 50 ± 1.38 | 21 ± 0.36 | 29 ± 8.08 | 58 ± 1.06 | 42 ± 0.00 |
| NH ₄ NO ₃ + KH ₂ PO ₄ + <i>Z. mays</i> + Agar | 20 | 43 ± 0.18 | 24 ± 1.38 | 19 ± 2.36 | 44 ± 0.08 | 55.8 ± 1.39 |
| MS + Soil | 20 | 40 ± 0.36 | 13 ± 0.06 | 27 ± 0.04 | 68 ± 1.39 | 32.5 ± 0.29 |
| MS + 2, 4 D + BAP + Soil | 20 | 44 ± 1.07 | 24 ± 0.45 | 20 ± 3.41 | 45.5 ± 6.18 | 54.5 ± 0.04 |
| NH ₄ NO ₃ + KH ₂ PO ₄ + Soil | 20 | 47 ± 3.01 | 22 ± 0.25 | 25 ± 3.00 | 53 ± 6.11 | 46.8 ± 1.23 |
| NH ₄ NO ₃ + KH ₂ PO ₄ + <i>Zmays</i> + Soil | 20 | 40 ± 1.34 | 24 ± 0.53 | 16 ± 1.02 | 40 ± 6.65 | 60 ± 6.81 |

Legend: %f - Percentage frequency; Sf - Shoot formation; Cf - Callus formation; Sub. Performance - substrate performance.

Table 2. Somatic shoot establishment of *Manihot esculenta* on the test substrate on bench.

| Substrate | MNE | NEV | NSF | SIE (%) | CIE (%) |
|---|-----|--------------|-------------|--------------|-----------|
| MS + Agar | 20 | 11.6 ± 1.53 | 2.32 ± 0.34 | 20 ± 0.27 | 38 ± 0.05 |
| MS + 2, 4 D + BAP + Agar | 20 | 8.00 ± 2.89 | 2.56 ± 7.63 | 32 ± 1.05 | 20 ± 0.34 |
| NH ₄ NO ₃ + KH ₂ PO ₄ + Agar | 20 | 10.00 ± 6.72 | 1.36 ± 1.45 | 13.6 ± 4.16 | 29 ± 1.53 |
| NH ₄ NO ₃ + KH ₂ PO ₄ + <i>Z. mays</i> + Agar | 20 | 8.6 ± 0.81 | 3.00 ± 0.18 | 34.88 ± 1.54 | 19 ± 1.83 |
| MS + Soil | 20 | 8.00 ± 0.89 | 1.04 ± 7.83 | 13 ± 0.58 | 27 ± 5.07 |
| MS + 2,4-D + BAP + Soil | 20 | 8.80 ± 4.16 | 3.38 ± 1.34 | 38.41 ± 0.63 | 20 ± 1.05 |
| NH ₄ NO ₃ + KH ₂ PO ₄ + Soil | 20 | 9.40 ± 0.34 | 2.68 ± 0.87 | 28.51 ± 5.31 | 25 ± 0.04 |
| NH ₄ NO ₃ + KH ₂ PO ₄ + <i>Z. mays</i> + Soil | 20 | 8.00 ± 0.49 | 2.88 ± 1.43 | 36.00 ± 1.32 | 16 ± 6.18 |

Legend: MNE - mean number of explants; NEV - number of explants viable; NSF - number of shoot formed; SIE - shoot induction efficiency; CIE - callus induction efficiency; *Z. mays*.... *Zea mays*.

were estimated using the method of Thomas (1996). Summation was used to estimate the effective cation exchange capacity.

Statistical analysis

Statistical evaluation was carried out on all the data collected, using statistical analysis system (SAS). Treatment significance were assessed at $P < 0.05$ and means differentiated by Duncan's multiple range test (DMRT).

RESULTS

Results of callus induction and shoot establishment on the MS agar base, MS soil base and MS agar and soil base supplemented with 2, 4 - D and *Z mays* extracts, ammonium nitrate (NH₄NO₃) plus potassium bi Phosphate (KH₂PO₄) agar base and NH₄NO₃ + KH₂PO₄ soil base, supplemented with *Zea mays* extracts were shown in Tables 1 and 2, Figures 2, 3 and 4 while the propagule establishment profile was shown in Figure 1. The test soil agro nutrient composition was shown in Table 3.

As shown in Table 1, all the substrates are viable. For callus formation, the standard medium (MS + agar) recorded 38 ± 0.00 out of 58 ± 0.12, showing 66 ± 1.34% performance. The soil substituted (MS + soil) recorded 27 ± 0.04 out of 40 ± 0.36, showing 68 ± 0.39% while NH₄NO₃ + KH₂PO₄ + Soil recorded 25 ± 3.00 out of 47 ± 3.01, showing 53 ± 6.11% performance.

Adventitious shoot establishments were shown in Table 2. The standard medium supplemented with 2,4-D and BAP in agar as the gelling agent (MS + 2,4-D + BAP + Agar) recorded 2.56 ± 7.63 out of 8.00 ± 2.89 that were viable. This showed induction efficiency of 32 ± 1.05%. The soil base substitute (MS + 2,4-D + BAP + Soil) recorded 3.38 ± 1.34 out of 8.80 ± 4.16 viable count, showing 38.41 ± 0.63 % while the crude local alternative (NH₄NO₃ + KH₂PO₄ + *Z mays* extract + Soil) recorded 2.88 ± 1.43 out of 8.00 ± 0.49 viable count, showing 36 ± 1.32% efficiency.

The viability profile in the three sets had no significant difference between them. Set 'A' incubated in the dark cupboard produced more calli than set 'B' and 'C' incubated in the conventional incubator and that kept on the laboratory bench respectively. There was no significant difference in the production profile between the set 'B' and 'C' that is, those in the incubator and those kept in the open laboratory bench. Shoot production

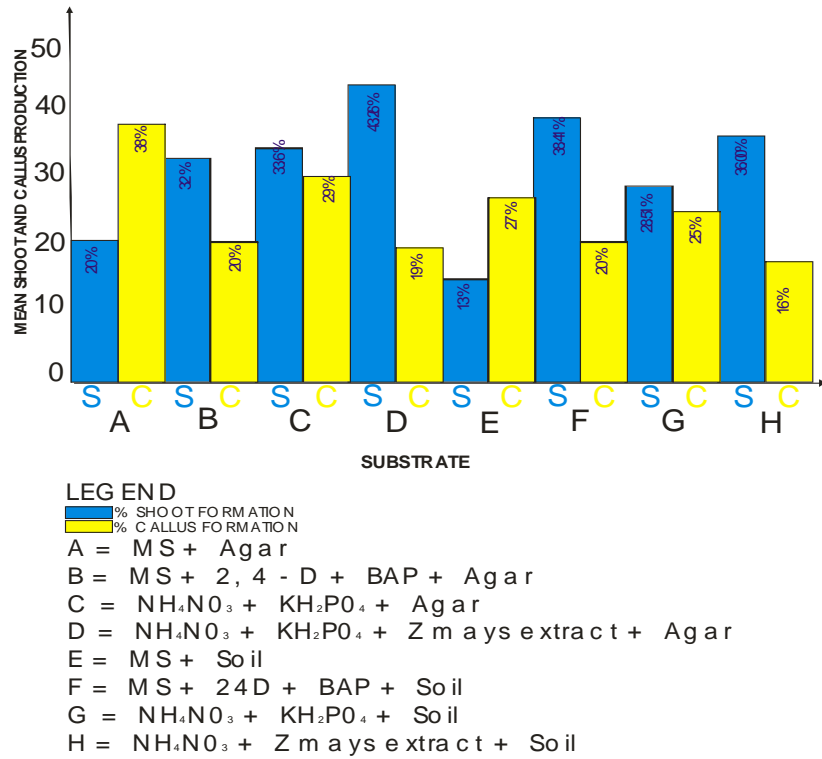


Figure 1. The establishment profile of callus and shoot in the test substrate.



Figure 2. Adventitious shoot of *M. esculenta* in soil base NH₄ NO₃-KH₂PO₄ + *Zea mays* extracts substratum.

differentials show more shoot in B and C than in A.

In the individual substratum performance, calli were more on both the agar and soil base basal substrate of

MS than NH₄NO₃ – KH₂PO₄ (Tables 1 and 2). Shoot - emergent performance showed that MS and NH₄NO₃ – KH₂PO₄, supplemented with 2,4-D and *Z mays* extracts



Figure 3. Adventitious shoot of *M. esculenta* in MS + 2,4-D + BAP + soil substratum.

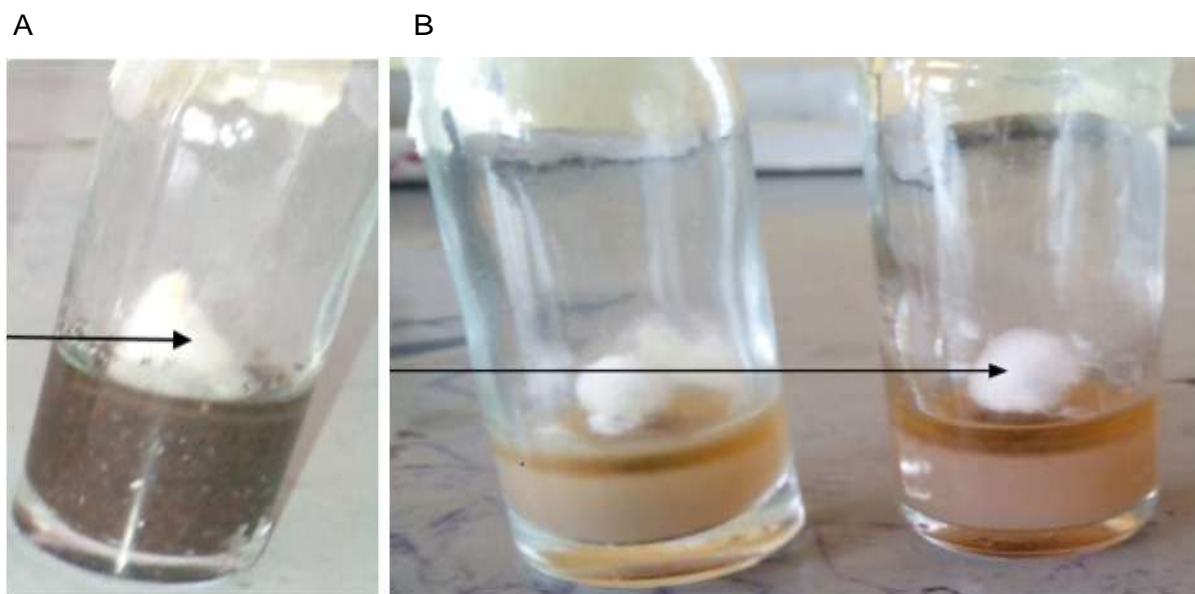


Figure 4. Callus of *M. esculenta* formed on the test soil base and agar base substrate. A = Soil – base substratum; B = Agar – base substratum.

ran neck and neck in both callus induction and direct shoot establishment (Tables 1 and 2, Figure 1).

DISCUSSION

The agro-geography of crop diversity and propagation dynamics had shown that subsistent croppy culture, dominated the agro-stead of Tropical Africa and Asia. This is evident in the low level of mechanized farming, near zero high tech applications and high ratio of agro-input to output. In this study, *in vitro* propagation is seen as a high speed delivery option in crop development and genetic diversity (Ubi, 2013). Improvising and applying

local contents alternatives in the protocol recipe can be as viable as the conventional ones, such as Murashige and Skoog (1962) (MS). This was the position of Ngomuo and Ndakidemi, (2014), Kitimu *et al.* (2015), Muluken and Tileye (2018) and Tabins (2018) which was demonstrated in this study.

Within one passage period, callus production in agar base MS basal substratum was 38% out of 58% viability count while agar based $\text{NH}_4\text{NO}_3 - \text{KH}_2\text{PO}_4$ substratum produce 29% out of 50% viability count (Table 1). This showed a performance ratio of 66:58. Somatic shoot regeneration was 20% out of 58% viability in MS and 21% out of 50% viability in $\text{NH}_4\text{NO}_3 - \text{KH}_2\text{PO}_4$, corresponding to the ratio of 34:42 respectively. This

Table 3. Nutrient properties of the soil used in the study.

| Soil parameters | Values |
|---|-------------|
| Sand (g.kg ⁻¹) | 645 ± 2.40 |
| Silt (g.kg ⁻¹) | 248 ± 4.28 |
| pH | 5.66 |
| Organic matters (%) | 1.50 ± 0.18 |
| Organic carbon (%) | 0.74 ± 0.01 |
| Available phosphorous (mgkg ⁻¹) | 8.00 ± 0.38 |
| Total Nitrogen (mg L ⁻¹) | 0.07 ± 0.01 |
| Exchangeable acidity (cmolk ⁻¹) | 1.56 ± 0.06 |
| Exchangeable bases (cmolk ⁻¹) | 3.68 ± 0.58 |
| Potassium (cmolk ⁻¹) | 0.03 ± 0.00 |
| Calcium (cmolk ⁻¹) | 1.60 ± 0.58 |
| Effective cation exchange capacity (cmolk ⁻¹) | 4.61 ± 0.06 |

implied that for friable callus induction and proliferation projects, either the combinations of NH₄NO₃ – KH₂PO₄ or MS salts can substitute for each other (Figure 4).

The cytokinins and the auxins are growth regulators required in tissue culture media (Kwame *et al.*, 2012). The same were proved in this study. The synthetic cytokinins used were BAP (benzyl –amino-purine) and the natural equivalent is the *Z. mays* coleorhizae tip extract tablets. The synthetic auxins used was the 2,4–D (2,4–dichlorophenoxy acetic acid) in accordance with the report of Tabins (2018) and Wang *et al.*, (2011) while natural equivalents is the crude diffusion extract of *Z. mays* coleoptile tip in the agar block tablets. These growth regulators and their equivalents are required in appropriate ratios in substrate, to facilitate cell division leading to callus proliferation, somatic embryogenesis and shoot formations establishments. This was demonstrated in this study. The results as shown in Figures 1 to 4 proved that the *Z. mays* extracts is as efficient as the 2,4-D.

Soil as a substrate is a whole solid medium for both seed and vegetative propagation. Agar is a substratum gelling agent depending on formulation. But in this study, both substances were used as chelating agents. The performance profile showed that one can substitute for the other.

Ketoconazole and chloramphenicol are employed in this study as add-on against fungi and bacteria, respectively

CONCLUSION

To the extent of this study, it is evident that ordinary top soil collected at 15 cm depth, from the earth surface can substitute for the agar powder. Again the study has provided an insight to the fact that crude extracts from sprouted *Z. mays* can substitute for 2,4-D and BAP combinations as good substrate supplements while NH₄NO₃ and KH₂PO₄ can substitute for the relatively

expensive MS salts and allied conventional media.

Significance statement

The study discovered that the combination of ammonium nitrate (NH₄NO₃) and potassium bi phosphate (KH₂PO₄) is as good as MS basal salt. It can serve as its substitute at aliquot measures or molar concentrations. These local salts are readily available and less expensive. Garden soil is a good gelling agent and can substitute for agar in tissue culture protocol. Soil is everywhere; it is highly available and affordable relative to agar. The *Zea mays* seedling extracts can substitute for highly expensive phytohormones, BAP and 2,4–D in *in vitro* propagation event. *Z. mays* grains are available in all local markets. They are highly affordable and can easily sprout within two days on a moist filter paper, in a modest environment. Researchers and plant breeders irrespective of their expertise and financial capabilities can conveniently use this protocol to improve on agro–horticultural businesses.

Conflict of interest

There is no conflict of interest.

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