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Somatic embryogenesis and plantlet regeneration from juvenile leaf explants of two cassava cultivars (TME 419 & TMS 98/0505).

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Abstract

In vitro regenerated juvenile plantlets from two cassava cultivars (TME 419 and TMS 98/0505) leaf lobes were used to induce organized embryogenic structures (OES) on DKW2 50P medium for 3 weeks under dimmed light conditions at 26±2 ^{0}C. Maximum stable frequencies of OES for the two cultivars were obtained with 5mg/L picloram. Friable embryogenic calluses (FEC) were further produced on GD2 50P medium supplemented with 500 µM tyrosine. The percentage plantlets recovered on hormone free MS2 agar medium of the two cassava cultivars were 85 and 80% respectively with more than 90% post acclimatization survivals.

Key words: Acclimation; friable embryogenic callus; organized embryogenic structures; regeneration; TME 419; TMS 98/0505; tyrosine.
Abbreviations: DKW-DKW basal salt mixture; FEC-Friable embryogenic callus; GD-Gresshoff & Doy; OES-Organized embryogenic structures; TME-Tropical *Manihot esculenta*; TMS-Tropical *manihot* specie

Introduction

Cassava (*Manihot esculenta* Crantz) is a major food crop in Nigeria and to over 500 million people in Africa, Asia, and Latin-America. It is often referred to as the world's most reliable food security crop because harvesting of some cultivars can vary between 6 months and two years. Besides its use as food, cassava is a multipurpose crop by nature; and can be processed into a variety of food forms, feed for livestock and raw material for a wide array of value-added products (FAO, 2013). Grown mostly in the Southeast, Southwest, Southsouth and the middle belt region of Nigeria, the crop is cultivated mainly by low-income farmers and often intercropped with maize, melons, and legumes. Its ability to tolerate adverse environmental conditions makes the warm tropical climate of Nigeria quite unique for its cultivation. Currently, the estimated average yield per hectare in Nigeria is 10.6 tonnes per ha compared to the targeted 15.0 tonnes per ha. The wide variety of food products that are made from cassava roots, its high yield compared to other food crops, the crop's low labour input requirements, ability to produce good yield on degraded soils, drought tolerance, and its resistance to pests and diseases by some cultivars are some of the added reasons why cassava cultivation is expanding. Recently, increasing urbanization has prompted entrepreneurial farmers to expand production close to major cities and towns in order to capitalize on the concentration of prospective consumers. Arguably, the two unique advantages of cassava over most crops are its flexible harvesting time which makes it excellent famine food crop and its ability to be stored in the ground for over 36 months (Ubalua, 2015). Despite all these positive attributes, cassava is low in protein, succumbs easily to postharvest deterioration and some cultivars are susceptible to insect pests and virus diseases. Addressing these limitations through biotechnological approaches has been identified and will hopefully compliment efforts in traditional breeding. Plantlet regeneration from somatic embryos is a prerequisite targeted at improving crop quality (Zhang et al., 2004). Cassava plantlets have been successfully regenerated from callus grown from buds, *in vitro* shoot apexes and stem cuttings. Somatic embryogenesis and plantlet regeneration in cassava could generate improved genotypes with desirable traits. The ability to regenerate *in vitro* and the potential for rapid mass production makes somatic embryogenesis particularly appealing. Apart from these advantages somatic embryogenesis provides continuous source of target tissues for plantlet regeneration and transformation studies, facilitates development of synthetic seeds, and rapid multiplication and distribution of disease-free elite cassava cultivars. Furthermore, new cassava plantlets through somatic embryogenesis by contrast are photosynthetically active and often yield better simply because they are healthier and are more vigorous. Vasil (1982) and Atanasov (1985) reported on the variable nature of somatic embryogenic potential from one species to another and between cultivars of the same species. Rossin and Rey, (2011) emphasized that for successful embryogenesis in cassava, that the source and type of the initial explants is an important factor and that the efficiency

of the technique is dependent on the genotypes of the cultivars as not all the cultivars are prone to embryo formation, regeneration or transformation. Similarly, Fregene and Puonti-Kaerlas, (2002) reported that meristems are the explant of choice for regeneration as they represent "growth centers" of plants. TME 419 and TMS 98/0505 are elite cassava cultivars highly valued for their high yielding, high dry matter and high starch content potentials. In this study we sought to ascertain the embryogenic responses of these two cassava cultivars using their juvenile leaves as explants. A positive response opens up a window of opportunity to genetically improve on some of their agronomic deficiencies.

Results and Discussion

Juvenile *in vitro*-raised cassava leaf explants excised from *in vitro* cassava plantlets of the two cassava cultivars (TME 419 & TMS 98/0505) were used to determine the effect of picloram (auxin) concentration on callus induction.

Callus Induction

After 3 weeks of incubation on DKW2 50P, the explants became swollen and gradually turned dark green and finally yellowish (Fig.1a). However those on DKW2 picloram–free medium remained unchanged, reflecting the importance of picloram in inducing embryogenic callus in the two cassava cultivars. But moderate variations were observed among the two cultivars tested although TME 419 produced the best result. We observed that as high as 80% of the TME 419 explants formed somatic embryos while 70% of the TMS 98/0505 explants formed somatic embryos (Fig. 1b). Table 1 shows that in all of the concentrations of the picloram examined, the two cassava cultivars were able to produce organized embryogenic callus. For TME 419, the percentage response of the explants in the medium at 1 mg/L picloram concentration was significantly lower when compared to 5 and 9 mg/L (Table 1). A similar trend was observed with TMS 98/0505 at 1 mg/L picloram concentration. But callus induction was enhanced when the concentration of picloram was increased. Thus, when picloram concentration was increased from 3 to 5 mg/L, organized embryogenic callus induction appreciated from 40-70% and 20-50% for TME 419 and TMS 98/0505 respectively (Table 1). Beyond 5 mg/L picloram, gradual inhibitions of callus production were observed. This equally demonstrates that for callus induction to occur in TME 419 & TMS 98/0505, an auxin (picloram) is a necessity. Szabados et al., (1987) achieved maximum embryo formation between 8-16 mg/L 2,4-D in different cassava cultivars. On the other hand, Vidal et al., (2015) compared cassava explants response to somatic embryo formation in culture media containing auxin 2,4-D and picloram with picloram favouring maximum formation of calli with embryos than in medium containing 2,4-D. Comparatively we obtained the highest embryo induction in culture medium containing 5 mg/L of auxin (picloram). Although Hankoua et al., (2006) reported on efficient production of somatic embryos in a number of African cassava cultivars in medium containing 12 mg/L picloram, we achieved our highest frequency of calli with embryos with 5 mg/L picloram suggesting that picloram concentration requirement may vary among cultivars. We therefore presumed that such variation in response by different cassava cultivars may be genotype-specific, and to some extent on explants source. Other regulators like dicamba and copper sulphate has also been reported to be amenable in inducing OES production (Duncan, 1997; Kiong et al., 2008; Mousavizadeh et al., 2010).

Induction of somatic embryos

Embryo maturation and somatic embryo induction (Fig. 1B) were examined on GD2 50P+500 µM tyrosine medium (Taylor et al., 2001; Ubalua, 2015). To determine the effect of medium composition on the induction of somatic embryos, the induced calluses were further sub-cultured and incubated on GD2 50P+500 µM tyrosine and on GD2 50P medium without tyrosine. Productions of somatic embryos were assessed at the end of 4 weeks of incubation (Table 2). Though the embryogenic calluses formed somatic embryos regardless of the concentration of tyrosine in the medium, proliferation of the somatic embryos were promoted by continuous subculture. However, induction was not observed in the medium without tyrosine implying that tyrosine is necessary for embryo formation. But beyond the optimum performance of the tyrosine (at 500 µM), depreciation in the embryogenic potential of the cultures were observed probably as a result of embryogenic cell disruptions (Bhojwani and Razdar, (1996).

Plantlet regeneration

Subsequently the somatic embryos were transferred to regeneration medium (MS2 0.5µM NAA) (Taylor et al., 2001) for plantlet regeneration. Following incubation in a growth chamber under light and at 28^0C, development of embryos with green cotyledons, swollen hypocotyls and meristematic regions were observed after 21 days (Fig. 1c & d). However, in this *in vitro* regeneration studies, two major drawbacks namely, low conversion/recovery rates and a number of morphologic abnormalities in the form of cornet-shaped cotyledon, fused and trumpet-shaped cotyledons were observed (Fig. 2a, b & c).

Development of the embryos into plantlets

Further transfer of the embryos to an elongation medium (MS2 2µM BAP) (Taylor et al., 2001) for 4 weeks and on a hormone-free (MS2 agar) medium for another 4 weeks led to the recovery of 85 and 80% of TME 419 & TMS 98/0505 plantlets respectively with normal roots and shoots (Fig. 1e). The plantlets were again transferred to potting medium and maintained under conditions of high humidity and low light intensity (Fig. 3). When transferred to soil, over 90% of the regenerated plantlets survived. Interestingly, the regenerated plantlets were normal and morphologically similar to the mother plants confirming the assertion by Ammirato (1983) that plants regenerated through somatic embryogenesis are of single cell origin and are of true-to-type.

Materials and Methods

Plant material

The *in vitro* plantlets (TME 419 & TMS 98/0505) were obtained from the National Root Crops Research Institute

Table 1. Effect of auxin (picloram) concentrations on callus induction (OES) from young leaf explants of TME 419 & TMS 98/0505

Picloram (mg/L)	TME 419 Percentage response	TMS 98/0505 Percentage response
0	0	0
1	20	10
3	40	20
5	70	50
7	50	30
9	50	20
11	30	20
13	30	20
15	10	10

Values represents mean for 3 replications for each treatment.

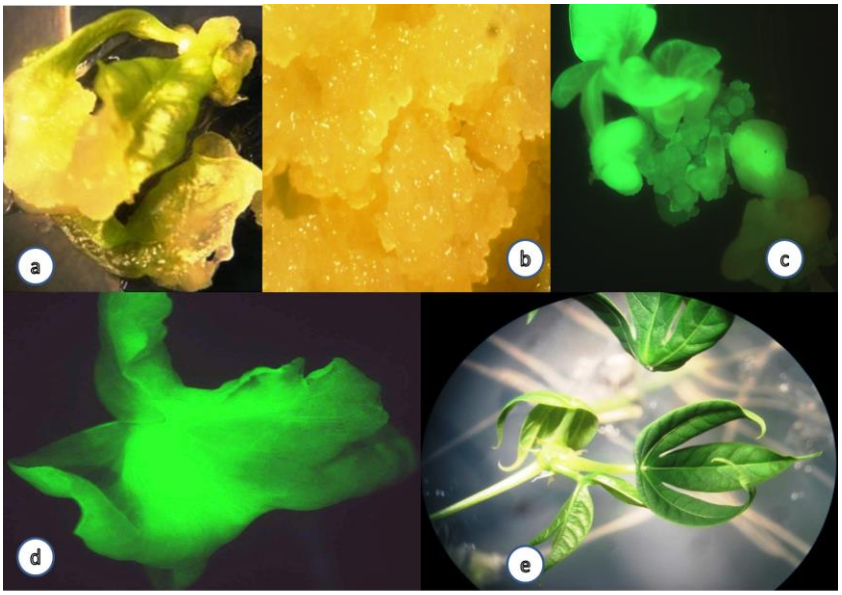

Fig. 1: Somatic embryogenesis and plantlet regeneration in cassava (*Manihot esculenta*).
(a) Developing callus. (b) Somatic embryos. (C) Torpedo stage. (d) Developing cotyledon.
(e) Regenerated plantlet in culture.

Table 2. Emergence and development of TME 419 & TMS 98/0505 plantlets in medium supplemented with tyrosine.

Tyrosine (µM)	TME 419	TMS 98/0505
	% somatic embryos with regenerants	
0	0	0
300	55	45
350	65	55
400	70	65
450	80	75
500	85	80
550	80	75
600	75	70

Values represents mean for 3 replications for each treatment.

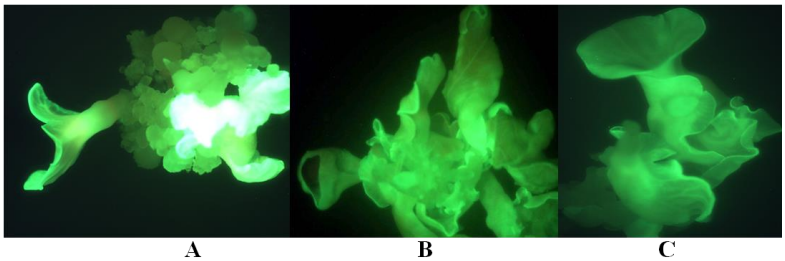

Fig. 2: Somatic embryos developing into abnormal cotyledons. All are expressing GFP fluorescent protein under fluorescent microscopy.
A-Cornet-shaped cotyledon; B-Fused-shaped cotyledon; & C-Trumpet-shaped cotyledon

Fig. 3: Potted plantlets 8 weeks post acclimatization

(NRCRI), Umudike, Umuahia, Nigeria. They were regenerated and multiplied for a period of 2 months at Donald Danforth Plant Science Center, 975 N, Watson Road, St. Louis, MO. 63132, USA.

Medium composition

Prior to autoclaving at 121^{0}C for 15 minutes, the medium pH was adjusted to 6.12, and supplemented with 8g/L of agar and 20g/L of sucrose. Before dispensing the medium in sterile petri dishes, filter sterilized picloram was added when the medium temperature dropped to 42^{0}C.

Treatments

The juvenile leaves of the *in vitro*-raised TME 419 and TMS 98/0505 were excised and cultured on DKW2 50P for 3 weeks (Taylor et al., 2001). Ten young leaves of each of the cassava cultivars were aseptically inoculated onto each petri dish containing 25 ml of sterilized DKW2 50P in a lamina air flow cabinet to provide sterile environment. Same was done on petri dishes containing only 25 ml of solidified DKW2 as control. After 3 weeks of incubation at 26±2^{0}C and under dimmed light condition, the cultures were assessed for the presence of callus (organized embryogenic structures (OES). The developing yellowish structures were meshed with a sterile spatula on a sterile mesh. For the induction of somatic embryos, the meshed calluses (OES) were placed in small dots on freshly prepared GD2 50P+500 µM tyrosine plates and incubated in a closed paper box (Taylor et al., 1996). The embryos were recycled every 3 weeks for a period of 9 weeks for maximum embryo proliferation.

The emerging somatic embryos with green cotyledons, swollen hypocotyls and meristematic regions were harvested and transferred to regeneration medium (MS2 0.5µM NAA) and elongation medium (MS2 2µM BAP) (Taylor et al., 2001) for a period of 4 weeks each. The putative embryogenic tissues were further cultured on a hormone-free medium (MS2 agar) supplemented with 20g/L sucrose for plantlet regeneration.

Traits measured

The effect of picloram (auxin) concentration on callus induction was determined on the two cassava cultivars after 2 and 3 weeks of incubation. The percentage response of the explants in the medium at 1-15 mg/L picloram concentration was determined. Induction and maturation of the produced somatic embryos on GD2 50P+500 µm tyrosine were also determined and assessed at the end of 4 weeks of incubation. Regeneration of plantlets from the somatic embryos of the two cassava cultivars were examined on (MS2 0.5 µm NAA) (Taylor et al., 2001). The regenerated plantlets were further examined on elongation (MS2 2µm BAP) medium and on MS2 agar for 4 weeks each for growth and development respectively. The plantlets were acclimatized in pots containing jiffy peat under high humidity and low light intensity for a period of 4 weeks before transfer to soil.

Experimental design

Completely randomized design was used for the experiments. Treatments were repeated 3 times and data recorded 3 and 4 weeks after the beginning of the treatments.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) and multiple comparison-least significant difference (LSD) of Gen Stat (DE3) ver. 7.2.

Conclusion

Our protocol revealed the importance of picloram and tyrosine for inducing calluses and somatic embryos in TME 419 & TMS 98/0505 respectively. We also observed that at 5.0 mg/L picloram concentration, callus induction was maximum while 500µM tyrosine was optimum for somatic embryo production. Besides providing a continuous source of target tissues for genetic manipulations, somatic embryos facilitate development of synthetic seeds, rapid multiplication and distribution of disease-free elite cassava cultivars.

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