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Improving Yam Micropropagation

Series 2

Development of micropropagation system for yam *(Dioscorea spp.)* using somatic embryogenesis

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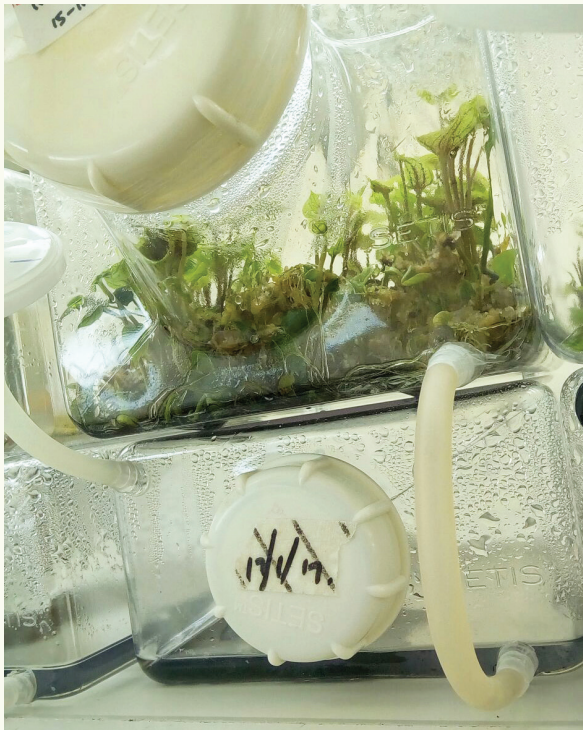

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Cover photo: Somatic embryo formation and plantlet regeneration from callus induced from young axillary buds of the improved white yam variety (Kpamyo: TDr 95/19177) in SETIS Type Temporary Immersion Bioreactor System.

Abstract

Inadequate availability of disease-free planting materials remains a major constraint to yam production. The tissue culture technique has been used to regenerate disease-free plantlets from pre-formed, heat-treated meristems followed by micropropagation. This procedure, however, has a low multiplication ratio with an average of 1: 4 every eight weeks. Embryo production from somatic cells (somatic embryogenesis, SE) is a system in which each somatic cell can regenerate a complete plantlet. However, previous reports show low SE induction frequencies and significant variations in success rates among different genotypes while hardly any report exist for improved varieties that farmers desire, especially in Nigeria. Studies were carried out to evaluate the effects of different plant growth regulators (PGRs) on induction of somatic embryogenesis of the following genotypes: one improved *Dioscorea alata* (TDa 291) and three improved (TDr 95/19177, TDr 89/2665, TDr 95/18544) and one landrace (Obioturugo) of *Dioscorea rotundata.* Leaf, stem, and axillary bud explants were cultured in MS basal medium containing fifteen treatment combinations of 2,4-Dichlorophenoxyacetic acid (2,4-D), Naphthaleneacetic acid (NAA), Benzylaminopurine (BAP), Picloram, and Uniconazole-P (UP). The genotype TDr 95/19177 was tested for SE in Temorary Immersion Bioreactor System (TIBS). The incidence of induction of callus formation and plantlet regeneration from the three explants were recorded. Embryogenic callus induction was highest (87%) from axillary buds cultured on modified MS + 2 mg/l of 2,4-D + 1 mg/l of NAA while 1 mg/l of BAP + 9. 9 mg/l of UP had the highest percentage plantlet regeneration of 50% in TDr 95/18544 and an average of 37% across genotypes at a mean of 5 plantlets per explant. The genotype TDr 95/19177 was successfully regenerated via indirect somatic embryogenesis in the SETIS Type Temporary Immersion Bioreactor System.

Purpose of study

The general aim of the study is to develop a through-put system for (scaling up) yam propagation in temporary immersion bioreactors systems (TIBS) using indirect somatic embryogenesis. The specific objective was to investigate the effects of different growth regulators at varying concentrations, on callus formation, somatic embryo induction, and plantlet regeneration among different explants and genotype regimes in agar-solidified medium.

Background

Up to 93% of global yam production emanates from West Africa (FAO, 2013) where it is a primary source of income and staple food. However, its production is limited by scarcity of clean seed yam. Several propagation methods have been developed to find a solution to the constraint of planting materials in yam production (Orkwor et al. 1998; Aighewi et al. 2015). *In vitro* multiplication of *Dioscorea* species has been performed using explants since 1980s (Shu et al. 2005) and this has been achieved mainly through organogenesis (Balogun and Gueye 2013). However, organogenesis was improved in TIBS (Adelberg and Simpson 2002; Balogun et al. 2014) relative to semi-solid tissue culture due to improved culture aeration from intermittent nutrient immersion, labor, and cost efficiency. Somatic embryogenesis is a more efficient system because all somatic cells are potentially able to produce bipolar embryos and regenerate into a complete plantlet (Mousavizadeh 2009), such that as many as there are in a plant is the number of seed yam it can produce. The white yam variety 9811-090 from Latin America, and the accession TDr 2436 were reported regenerated through somatic embryogenesis (Padron et al. 2011; Manoharan et al. 2016). Quain et al. (2011) while studying transgenic potential of *Dioscorea rotundata*

using agrobacterium-mediated genetic transformation, reported successful transformation of the local Ghanaian white yam variety "Pona" but regeneration was via organogenesis. However, a system for somatic embryogenesis, especially via an intervening callus phase, has not been reported for improved, released varieties of white yam in West Africa, and especially using TIBS.

In contrast, somatic embryos are bipolar structures and have been reported in *D. floribunda, D. composita, D. alata, and D. bulbifera* (Ammirato 1976; 1982). Recently, (Suarez et al. 2011) somatic embryos were induced from leaf tissues of *D. rotundata* in medium containing 2,4-D and incubated in darkness. However, low induction frequencies (< 30%) were recorded and protocols will have to be optimized. In *D. alata* and *D. opposita*, embryogenic cell masses were induced from root explants in liquid MS supplemented with 2,4-D and cultured in light (Twyford and Mantell 1996; Nagasawa and Finer 1989). Germination of somatic embryos of *D. alata* increased in the presence of GA3 (Deng and Cornu 1992; Twyford and Mantell 1996). Plantlet recovery from somatic embryos of *D. rotundata* was enhanced at 4.5% sucrose but not affected by benzylaminopurine (Okezie et al. 1994; Suarez et al. 2011).

Materials and Methods

The stem and leaf explants (approximately 0.5 cm × 0.5 cm) of TDr 89/02665, Obioturugo, TDr 95/18544, and TDr 95/19177; and the axillary buds of TDr 95/18544, and TDa 291 were introduced into Murashige and Skoog (1962) medium modified with different concentrations of plant growth regulators (PGRs) (Table 1) using petri plates. The media had been autoclaved at 121 ^{o}C and 15 Psi for 15 minutes before being dispensed into petri plates. Cultures were incubated in darkness at 25 + 2 ^{o}C for a period of 3 weeks in a completely randomized design (CRD) with 3 replicates and incidence of callus formation was recorded. The calli formed were transferred to the same set of media and incubated at 16 h photoperiod and 25 ± 2 ^{o}C. Incidence of somatic embryo formation and plantlet regeneration was recorded. Explants found to be most responsive for the different genotypes were introduced into the optimum callus induction media for more callus production and %callus formation was recorded. For somatic embryogenesis in TIBS), 2 weeks old nodal cuttings of TDr 95/19177 containing axillary buds were introduced into the SETIS type TIBS in MS modified with 1mg/L NAA for a period of 3 weeks for callus induction. The calli were introduced into PGR-free MS medium for callus maturation and formation of somatic embryos (SE) for 2 weeks, and finally to a medium containing 1 mg/l of BAP + 9.9 mg/l of UP for plantlet regeneration. Percentage callus induction and number of regenerated plantlets were recorded. Data was analyzed using ANOVA and means were separated using the Duncan Multiple Range Test (DMRT) at 5% level of probability.

Results and Discussion

Table 2 shows that callus induction was highest on modified MS + 2 mg/l of 2,4-D + 1 mg/l of NAA (87.50 ± 14.43%) as was reported by Marilyn and Jocelyn (2008), but not significantly different from MS + 5 mg/l of Picloram at 62.50 ± 43.30% and this was from plantlet buds (Tables 1 and 2) (Thao et al. 2003) while both were significantly higher than other regimes. Medium containing 4 mg/l of 2,4-D induced callus from both leaf and stem explants of TDr 95/19177 and TDr 95/18544. On MS + 2 mg/l of 2,4-D + 1 mg/l of NAA medium, up to 40% of the explant formed callus (Table 2).

Table 1. Different plant growth regulator regimes used in the study.

S/N	Plant Growth Regulator Regime	Callus induction (%)	Embryo formation	Plantlet regeneration (%)
1	0 mgl^{-1} 2,4-D + 0 mgl^{-1} BAP	0.00	Torpedo	0.00
2	0 mgl^{-1} 2,4-D + 1 mgl^{-1} BAP	20.70	NA	9.38
3	1 mgl^{-1} 2,4-D + 0 mgl^{-1} BAP	23.40	NA	0.00
4	1mgl^{-1} 2,4-D + 1 mgl^{-1} BAP	0.00	NA	0.00
5	1 mgl^{-1} 2,4-D + 1 mgl^{-1} BAP	11.70	NA	0.00
6	2 mgl^{-1} 2,4-D + 1 mgl^{-1} BAP	0.00	NA	0.00
7	0 mgl^{-1} 2,4-D + 0 mgl^{-1} BAP + 1 mgl^{-1} NAA	9.00	NA	0.00
8	0 mgl^{-1} 2,4_D + 1 mgl^{-1} BAP + 1 mgl^{-1} NAA	9.00	NA	0.00
9	1 mgl^{-1} 2,4-D + 0 mgl^{-1} BAP + 1 mgl^{-1} NAA	0.00	NA	0.00
10	1 mgl^{-1} 2,4-D + 1 mgl^{-1} BAP + 1 mgl^{-1} NAA	0.00	NA	0.00
11	2 mgl^{-1} 2,4-D + 0 mgl^{-1} BAP + 1 mgl^{-1} NAA	71.88	NA	0.00
12	2 mgl^{-1} 2,4-D + 1 mgl^{-1} BAP + 1 mgl^{-1} NAA	18.00	NA	0.00
13	1 mgl^{-1} BAP + 9.9 mgl^{-1} UP	0.00	NA	56.25
14	4 mgl^{-1} 2,4-D	38.55	NA	0.00
15	5 mgl^{-1} Picloram	62.5	NA	0.00

2,4-D: 2,4-Dichlorophenoxyacetic acid; NAA: Naphthalene acetic acid; BAP: Benzylaminopurine; UP: Uniconazole-P;

Table 2. Medium regimes at which highest callus induction from specific explants of different yam genotypes were obtained.

		Callus Induction	
Genotype	Explant	Medium	%
TDr 89/02665	Stem	2 mg/l 2,4-D + 1 mg/l NAA	52.8
Obioturugo	Stem	2 mg/l 2,4-D + 1 mg/l NAA	42.0
TDr 95/19177	Stem	4 mg/l 2,4-D	50.0
TDr 95/19177	Buds	2 mg/l 2,4-D + 1 mg/l NAA	87.5
TDr 95/19177	Leaf	4 mg/l of 2,4-D	50.0
TDr 95/18544	Stem	4 mg/l of 2,4-D	25.0
TDr 95/18544	Leaf	4 mg/l of 2,4-D	25.0
TDr 95/18544	Buds	5 mg/l of Picloram	62.5
TDa 291	Buds	MS	9.4

2,4-Dichlorophenoxyacetic acid (2,4-D)., α-Naphthaleneacetic acid (NAA)., 6-Benzylaminopurine (BAP).

Table 3. In vitro regeneration of plantlets from somatic embryogenic calli of white and water yams using different regimes of PGRs.

Genotypes	Treatments	D2SReg	PSReg
TDa 291	MS + 1 mg/l of BAP	0.0	0.0
TDa 291	MS + 1 mg/l of BAP + 9.9 mg/l of UP	121.0	25.0
TDa 291	MS	0.0	0.0
TDr 95/18544	MS + 1 mg/l of BAP	0.0	0.0
TDr 95/18544	MS + 1 mg/l of BAP + 9.9 mg/l of UP	108.0	50
TDr 95/18544	MS	0.0	0.0
TDr 95/19177	MS + 1 mg/l of BAP	85.0	25
TDr 95/19177	MS + 1 mg/l of BAP + 9.9 mg/l of UP	0.0	0.0
TDr 95/19177	MS	0.0	0.0
SE		2.67	4.16

Plant Growth Regulators (PGR), 6-Benzylaminopurine (BAP), Uniconazole-P (UP). Standard error (SE), Days to Shoot Regeneration (D2SReg), Percentage Shoot Regeneration (PSReg).

Table 4. Days to regeneration and average number of plantlets regenerated per callus in yam.

Genotypes	Treatments (mg/l)	TotalD2R of callus	Minimum NOR	Maximum NOR	Average NOR
TDa 291	1 BAP	104	0	0	0
TDa 291	1 BAP + 9.9 UP	93	4	6	5
TDr 95/18544	1 BAP	–	0	0	0
TDr 95/18544	1 BAP + 9.9 UP	111	0	16	8
TDr 95/19177	1 BAP	–	0	0	0
TDr 95/19177	1 BAP + 9.9 UP	123	2	2	2
S.E		1.24			2.18

D2R = SE: Days to plantlet regeneration, NOR = Number of plantlets per callus, SE = Standard error.

Plantlets were regenerated from MS + 1mg/l of BAP in TDr 95/18544 and TDr 95/19177, but on hormone-free MS in TDr 95/18544. Marilyn and Jocelyn (2008) reported a low regeneration rate in hormone-free MS and attributed it to the slow rate of maturation of somatic embryos. The addition of BAP enhanced shoot regeneration in *D. bulbifera* and *D. alata* (Ammirato 2004) while the addition of 1 mg/l of BAP + 9.9 mg/l of UP, increased percentage regeneration to 50% and 25% in TDr 95/18544 and TDa 291, respectively, in this study (Table 3). The two-week-old nodal cuttings containing axillary buds introduced into the TIBS gave 100% callus induction as at 3 weeks of culture. Five weeks after transfer of the callus to the optimum plantlet regeneration medium reported above (1 mg/l of BAP + 9.9 mg/l of UP) at least 50 plantlets were regenerated from one-third of the callus. However, the TIBS later became contaminated and the time to regeneration of the remaining callus could not be recorded.

Somatic embryogenesis is one system that can produce the highest possible numbers, in the shortest possible time. Some of the other propagation systems are constrained in terms of small number of plantlets produced per unit time. Using organogenesis plantlet regeneration system from pre-formed meristems like nodes, the propagation ratio is about 5 new plantlets every 10 weeks such that 25 plantlets will be obtained in 20 weeks, giving a ratio of 1: 25. However, with somatic embryogenesis, regeneration of yam plantlets is higher. Up to sixteen plantlets (Table 4) were obtained from a 1 cm^2 tissue section (Plate 1) via a callus phase after 20 weeks. Assuming 25 sections per plantlet, the multiplication ratio for somatic embryogenesis will be 1: 400 (25 × 16) in 20 weeks in CTC. This is expected to increase significantly in TIBS (Plate 2) when perfected due to increased automation and larger number of plantlets per TIBS. Regenerated plantlets were successfully hardened (Plate 3).

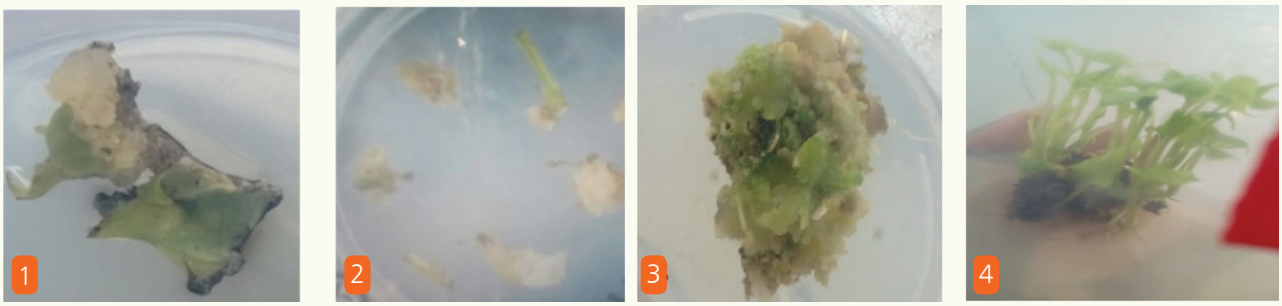

Plate 1. Tissues in semi-soild medium. 1 Callus induced from leaf; 2 Callus induced from stem explants; 3 Somatic embryos of TDr 95/19177; 4 Regenerated plantlets

Plate 2. Left: Somatic embryos and few regenerating plantlets from calli of TDr 95/19177; Middle: Front view of calli with more regenerating plantlets of TDr 95/19177 in Temporary Immersion Bioreactor; Right: Side view of same.

Plate 3. Successfully hardened plantlets of TDr 95/18544 and TDa 291 produced via somatic embryogenesis.

Conclusion and way forward

Five genotypes were tested in this study. Callus was induced in all, but regeneration was achieved in three genotypes, composed of two *D. rotundata* and one *D. alata* varieties. In conventional tissue culture (CTC), MS + 2 mg/l of 2,4-D + 1 mg/l of NAA and MS + 5 mg/l of Picloram were the best combinations for induction and proliferation of callus from the stem explants of yam, while raising the concentration of 2,4-D to 4 mg/l is adequate to induce callus formation in the leaf explants. In TIBs, callus formation was highest in MS + 1 mg/l NAA using 2-week-old nodal cuttings containing young axillary buds. In this experiment, the best condition for the regeneration of plantlets from embryogenic calli in both TIBs and CTC was MS + 1 mg/l of BAP + 9.9 mg/l UP. This protocol will be further validated for other farmer-preferred varieties in TIBS.

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