



Relative Effects of Uniconazole-p and other growth regulators on Micropropagation ratio of yam (*Dioscorea spp.*) plantlets at different growth phases

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Abstract

Scarcity of planting materials, pest and diseases are major constraints to yam production. The tissue culture techniques, meristem culture combined with heat therapy has been used to produce high-yielding virus-tested plantlets of root crops, which are later multiplied through micropropagation. This procedure, however has low regeneration and multiplication rates in yam. We investigated effects of different plant growth regulators on rate of growth of yam plantlets when applied at different growth phases. Three concentrations each of uniconazole-p (UP), gibberellic acid (GA3), jasmonic acid (JA) and naphthalene acetic (NAA) acids were applied at single node and primary nodal complex (PNC) formation phases in a split-plot design using two genotypes each of white and water yam. Number of nodes per plantlet was recorded after 16 weeks. Results showed that number of new nodes varied significantly with genotype by growth phase interaction and the main effect of PGRs. After 16 weeks, the control, 1.7 μ M UP and 0.03 μ M JA showed highest means of 7, 9 and 8 nodes per plantlet respectively compared to 2-3 nodes in the GA treatments. We demonstrated that 4900, 8100 and 6400 plantlets could be obtained in one year using 100 nodes by sub-culturing them four times. Addition of PGRs at PNC doubled the number of nodes per plantlet. However the effect of stage of treatment varied with genotypes. The GA and its inhibitor, UP influenced the multiplication rate of yam.

Keywords: *Dioscorea*, micropropagation, Uniconazole-p, primary nodal complex, growth regulators.

Introduction

The yams (*Dioscorea spp*) are starchy root staples and a primary source of income in West Africa, from where 94% of global yam production emanates. Nigeria alone produces 68% (FAO, 2013) of global yam production, equivalent to 37 million tons. However, yam production is constrained by its relatively slow rate of propagation (less than 1:10 compared to 1:300 in some cereals) which is vegetative and encourages build-up of diseases especially

as certified or quality declared seeds are scarce and seed system is informal. Seed therefore account for up to 63% of production costs (Agbaje et al., 2005; Ironkwe 2005). Use of unclean planting materials, pre-infected by viruses, anthracnose and nematodes either singly or in combination, up to 25% yield reduction (Degras, 1993). Consequently, increase in yam production was reported to be due to increased land area under cultivation rather than productivity per unit area and global annual

production may reach a plateau, actually decreasing by 11.5% in 2007 (Manyong et al., 1996). The erratic rate of propagation does not facilitate genetic improvement either, as only one generation is produced per year while flowering is irregular in genotypes, making hybridization difficult.

Among yam diseases, viruses are most challenging, as no cure is available yet. Meristem culture combined with heat therapy has been successfully used to produce virus-tested plantlets, which are used in rapid multiplication of superior clones (Mantell et al., 1980; Ng, 1984, 1992; Sengupta. et al., 1984; Mitchell et al., 1995a and 1995b). This technique ensures that the viral inoculum is not passed on to subsequent generations. However, the slowness of yam propagation *in vivo* is also witnessed in tissue cultures and a 1:4 multiplication rate (Malaurie et al., 1995; Medero et al., 1999; Chu and Ribeiro, 2002; Borges et al., 2004; Ovono et al., 2007) is reported averagely for yam tissue cultures. Meristem cultures took between 6 and 24 months, while embryo cultures and somatic embryogenesis are yet to be perfected for economically important yam genotypes. This low multiplication rates limit the use of *in vitro* produced, virus-tested plantlets in addition to losses during transplanting. Also, there is limited application of biotechnological techniques like genetic transformation and marker assisted selection to yam relative to crops like cassava, potatoes and cowpea.

In the leaf axils of old yam nodes, there are two axillary buds and one shoot primordium, which also later develop into an axillary bud. A primary nodal complex (PNC), preceded by a meristematic PNC-initial is developed at the base of the first-formed axillary bud. This PNC-initial has capacity for multiple bud production, roots and a tuberous storage organ (Wickham et al., 1982). The PNC-initial is the organ of renewed growth and the only true organ of vegetative propagation in *Dioscorea* species (Wickham et al., 1981; 1982). In yam, axillary shoot proliferation, leaf expansion and rooting were reported (Bimbaun et al., 2002) to occur simultaneously, the leaves expanding to several centimeters. However, such size is not needed in the stage meant for plantlet multiplication. In order to focus on one aspect of growth at a time, and considering that yam has both dormant and active growth phases, we hypothesized that application of different growth regulators at different growth phases will re-direct

yam morphogenesis *in vitro*. The objective of this study was to investigate effects of different plant growth regulators on rate of growth of yam plantlets when applied at different growth phases

Materials and Methods

Two genotypes each of *D. rotundata* (TDr 608 and TDr 93-23) and *D. alata* (TDa 297 and TDa 92-2) collected from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria were used. The genotypes had been established *in vitro* at the tissue culture laboratory of IITA. Single node cuttings from the plantlets were sub-cultured into liquid, MS (Murashige and Skoog, 1962) medium, containing 5% sucrose (Ng, 1988) and different regimes of one of jasmonic acid (JA, Sigma Chemicals), gibberellic acid (GA, BDH laboratories), naphthalene acetic acid (NAA, Sigma Chemicals) and uniconazole-p (UP, Agros Chemicals, Japan). Each regime was applied at the onset of culturing (single node (SN) phase) and at primary nodal complex (PNC) formation phase (10 weeks after culturing). The concentrations used were 3, 0.3 and 0.03 μM JA; 3, 0.3 and 0.15 μM GA; 2.7, 0.54 and 0.27 μM NAA and 34, 3.4 and 1.7 μM UP. At each phase, application of sterile distilled water served as the control.

For application at SN phase, GA and NAA were autoclaved along with other medium constituents at 121°C and 103.4 kPa for 15 minutes while JA and UP were sterilized using membrane filters of size 0.2 μm and added into the autoclaved, cooled medium in the laminar flow hood. Each medium was dispensed in 10 ml quantities into vials containing a filter paper bridge. There were 60 vials for each PGR regime, 15 of which were used per genotype. All cultures were incubated at $25 \pm 2^\circ\text{C}$, 4,000 lux of light and 12 h photoperiod. For application at the PNC phase, nodes were first cultured in PGR-free medium. At about 10 weeks after culture initiation when there was obvious PNC formation (Plate 1), 10x of the desired final concentration of each of the thirteen PGR regimes were prepared, filter-sterilized, and one ml was added into each culture.

The experimental design was factorial in split-plot arrangement (Snedecor and Cochran, 1967), with growth phase as main plot and the PGR regimes as subplot. Data were taken on numbers of nodes and roots per plantlet at 4 months after culture initiation. The number of days

required to add one new node was calculated by dividing 120 by the number of nodes obtained four months after culture initiation and this was denoted as the rate of micropropagation. Analysis of variance was done using the GLM procedure of the Statistical Analysis Systems (SAS, 2000) and means were separated at $p=0.05$.

Results

Number of nodes and roots produced

The main effect of PGR, interaction between growth phase and genotype were significant for most of the traits (Table 1). Growth phase by PGR regime

Table 1. Mean square values for micropropagation parameters of yam plantlets to which 13 PGR regimes were applied at 2 growth phases 16 weeks after culture initiation.

Source of variation	Degree of freedom	Nodes per plantlet	Days to add new node ($\times 10^2$)	Roots per plantlet ($\times 10^2$)	Days to add new root
Genotype	3	35.55 ^{ns}	21.2 ^{ns}	45.1 ^{ns}	84.3 ^{ns}
Growth phase	1	125 ^{ns}	203.5*	28.4*	832.7*
Genotype*Phase	3	49.8*	9.5 ^{ns}	2.72*	52.1*
PGR	11	15.4*	87.2**	2.02*	67.7*
PGR*Phase	11	4.8 ^{ns}	66.2*	0.63 ^{ns}	54.7*
Error	66	3.16	757.6	0.40	8.7
Total	95				

Table 2. Mean numbers of nodes and days to add a new node in yam plantlets at different growth regulator regimes.

Growth regulator regime	Days to add a new node	Number in of nodes 120 days
Control	25.6 ^{cd}	5.5 ^b
3 μM GA	137.7 ^a	2.7 ^e
0.3 μM GA	68.6 ^b	3.2 ^{de}
0.15 μM GA	48.3 ^{bc}	3.6 ^{cde}
3 μM JA	37 ^{cd}	4.4 ^{bcd}
0.3 μM JA	35.3 ^{cd}	5 ^{bc}
0.03 μM JA	22.5 ^{cd}	6.1 ^b
2.7 μM NAA	25.8 ^{cd}	5.4 ^b
0.54 μM NAA	26.4 ^{cd}	5.4 ^b
0.27 μM NAA	34 ^{cd}	4.6 ^{bcd}
3.4 μM UP	Died	Died
3.4 μM UP	25.8 ^{cd}	5.3 ^{bc}
1.7 μM UP	18.8 ^d	7.9 ^a
Standard error	13.76	0.89

Means in each column followed by the same letters are not significantly different at $p=0.05$.

GA1:3.0 μM ; GA2:0.3 μM ; GA3:0.15 μM ; JA1:3 μM ; JA2:0.3 μM ; JA3:0.03 μM ; NAA1:2.7 μM ; NAA2:0.54 μM ; NAA3:0.27 μM ; UP1:34 μM ; UP2:3.4 μM ; 1.7 μM UP3:1.7 μM ; Control:hormone-free. SN: Single node stage; PNC: Primary nodal complex formation stage; For each genotype, values in each column followed by the same letter are not significantly different. SE: Standard error; Probability Level = 5%.

interaction were significant for number of days to add a new node or root.

Table 2 shows mean actual numbers of nodes and roots produced in 120 days. Seven, eight and nine nodes were recorded for control, 0.03 μM JA and 1.7 μM UP respectively when added at PNC formation. This implies that from a yam plant having 100 nodes, it is possible to obtain 4,900, 6,400 and 8,100 plantlets in 1 year within a four month propagation cycle in contrast to 1,600 plantlets per year in conventional micropropagation procedures where PGRs are added at culture initiation. The highest number of roots was also recorded in 1.7 μM UP. Up to 24 nodes were recorded in UP-treated explants in one genotype each of *D. rotundata* and *D. alata* after 28 weeks of culture (Table 2). Highest mean of 29.38 and 12.25 nodes for 3.4 μM UP applied at single node phase were obtained in TDr 608 and TDr 93-23 respectively, while application of 1.7 μM UP to *D. alata* genotypes had 23.71 and 27.00 at PNC and single node phases respectively. A maximum of 14 new nodes were recorded in the control.

Growth retardants like UP and flurprimidol, which are inhibitors of gibberellin biosynthesis, have been used to reduce or prevent abnormal leaf development and enhance the development of compact bud aggregates (Graebe, 1987, Rademacher, 1991, Ziv. 1990). UP actually reduced leaf extension but increased axillary branching as the leaves were smaller and the internode was shorter. Uniconazole-p is an inhibitor of protein synthesis, which

promoted sprouting in *Dioscorea* (Okagami, 1978; Park et al., 2003; Balogun, 2005). It also has a dwarfing effect on a number of plants by inhibiting stem elongation (Izumi et al., 1984). This indicates that the mode of action of uniconazole-p is related to endogenous gibberellin content. Matsumoto et al. (2010) also reported that in *Dioscorea*, UP enhanced shoot formation. In addition, Oyelami (2011) also reported that highest regeneration from meristems was recorded in GA-free medium.

The number of nodes obtained in a 4-month period (Figure 1) in the *D. alata* genotypes when PGR were added at PNC formation doubled what was obtained with application at the onset of culturing. In *D. rotundata* however, the differences between the two stages were only significant for TDr 93-23. Also, the two *D. alata* genotypes formed new roots at the same rate while in *D. rotundata*, TDr 608 added a new root every 4 days, significantly higher than 8 day-interval recorded in TDr 93-23. It will therefore be necessary to standardize protocols for each *D. rotundata* accessions while a more general protocol may be applicable to more of the *D. alata* accessions.

The morphogenetic expression of the organ of renewed growth in yam (the primary nodal complex: PNC) is under hormonal control (Wickham et al., 1982). It is important to consider the duration of the growth cycle of the two species when deciding the optimum stage of application of PGR. The genotypes have different crop growth durations, suggesting that at a particular point in time, genotypes were at different stages of physiological maturity. This is a first report of the effect of PGRs applied at different growth phases on yam plantlets although similar observations have been reported in vivo (Onjo

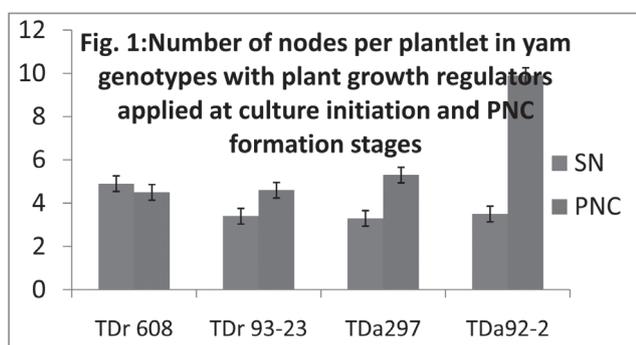


Figure 1. Number of nodes per plantlet in yam genotypes with plant growth regulators applied at culture initiation and PNC formation stages.

and Hayashi, 2001). Thus, micropropagation rate can be optimized if specific PGR regimes are applied at a growth phase optimum for a particular genotype.

Days required for formation of a new node and root

The number of days required to add one new node and root varied significantly with the PGR regime (Table 1). Control, 1.7 μM UP, and 0.03 μM JA added a new node within the shortest periods of 19, 17 and 16 days respectively, significantly faster than all concentrations of GA in which new nodes were formed 48-137 days after culturing. 1.7 μM UP (5 days) was the fastest to root while GA was the latest (13 days). The lower the concentrations of GA and UP, the faster it took to add a new node or root. At the highest concentration of 34 μM UP, all the plantlets died. It therefore becomes necessary to determine an optimum balance of GA/UP concentration for yam rapid micropropagation. The control added a new node and root, 25 and seven days after culture initiation respectively.

There were significant interactions between stage of application and PGR regime for rate of micropropagation. In all PGR regimes except UP, 2.7 μM NAA and 0.54 μM NAA in which the differences were not significant, application at PNC formation induced addition of new nodes and roots earlier than application at culture initiation (Figure 2).

On the average, it took the plantlets a mean of 56 and 12 days respectively to add a new node and root when PGRs were applied at start of culture while the duration was halved when PGRs were applied at the PNC formation

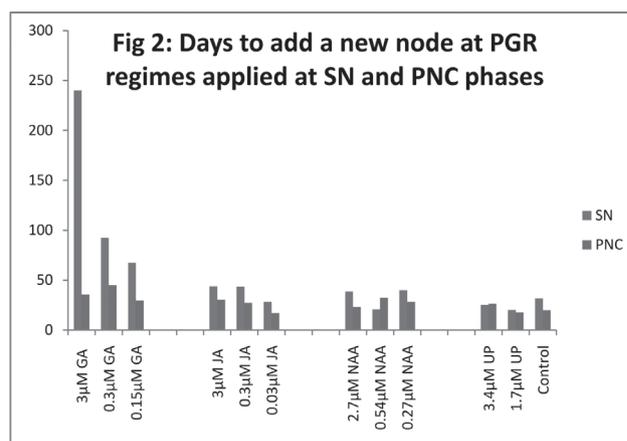


Figure 2. Days to add a new node at PGR regimes applied at SN and PNC phases

stage. This difference was highly significant for 3 μM GA and 0.3 μM GA in which new nodes were added 240 and 92 days after culturing relative to 35 and 44 days respectively when the same PGR regimes was added at PNC formation. The same trend was observed for root formation, as the rate of root formation at least doubled when the GA was applied at PNC formation.

The regimes in which the highest numbers of nodes were recorded for each genotype (Table 3) after 28 weeks of culturing were UP3 at PNC formation (23 nodes) and

UP3 at onset of culture (27 nodes) in TDa 297 and TDa 92-2 respectively. In the *D. rotundata* genotypes, it was UP2 applied at the onset of culture (29 nodes) and UP2 applied at the onset of culture or JA1 applied at PNC formation (12 nodes).

Gibberellin inhibitors were reported to induce multiple shoots from nodal explants in *D. Oppositifolia* and *D. Pentaphylla* (Poornima and Ravishankar., 2007) and numerous axillary nodes in *D. alata* and *D. rotundata* (Bimbaun et al., 2002; Balogun, 2005). The enhancing

Table 3: Numbers of nodes per plantlet of 4 genotypes each of yam at different PGR regimes applied at single node and PNC formation stages, 28 weeks after culturing.

Growth phase	PGRs	Genotypes				Means
		TDa 297	TDa 92-2	TDr 608	TDr 93-23	
SN	Control	10.00 ^{ab}	10.50 ^{bc}	7.78 ^{cd}	8.00 ^{abcd}	9.07 ^c
	GA1	0.21 ^d	1.86 ^{bc}	0.27 ^e	2.42 ^{ef}	1.12 ^e
	GA2	6.50 ^{bcd}	5.09 ^{bc}	9.36 ^{bcd}	5.07 ^{cde}	6.51 ^{cd}
	GA3	2.60 ^{cd}	7.09 ^{bc}	9.69 ^{bcd}	5.33 ^{cde}	6.18 ^{cd}
	JA1	4.83 ^{bcd}	8.12 ^{bc}	4.31 ^{de}	6.08 ^{bcd}	5.84 ^d
	JA2	7.60 ^{bc}	10.45 ^{bc}	10.79 ^{bc}	9.00 ^{abcd}	9.46 ^c
	JA3	6.23 ^{bcd}	11.58 ^b	9.29 ^{bcd}	10.47 ^{ab}	9.39 ^c
	NAA1	6.22 ^{bcd}	7.75 ^{bc}	8.14 ^{bcd}	4.83 ^{de}	6.74 ^{cd}
	NAA2	6.89 ^{bc}	6.71 ^{bc}	9.22 ^{bcd}	8.75 ^{abcd}	7.89 ^{cd}
	NAA3	6.00 ^{bcd}	5.17 ^{bc}	9.56 ^{bcd}	5.50 ^{cde}	6.56 ^{cd}
	UP1	0.00 ^d	0.00 ^c	0.00 ^e	0.00 ^f	0.00 ^e
	UP2	14.50 ^a	24.60 ^a	29.38 ^a	12.25 ^a	20.18 ^a
	UP3	15.99 ^a	27.00 ^a	13.50 ^b	9.88 ^{abc}	16.57 ^b
	Mean	6.70 ^C	9.70 ^B	9.60 ^B	6.70 ^C	8.10 ^A
	S.E	0.63	1.05	0.70	0.47	0.36
PNC	Control	7.12 ^c	14.25 ^{ab}	5.00 ^{bc}	9.25 ^{ab}	8.91 ^c
	GA1	3.50 ^{cd}	6.00 ^{cd}	2.78 ^c	3.14 ^{cd}	3.86 ^{de}
	GA2	3.50 ^{cd}	11.50 ^{abc}	1.67 ^c	6.00 ^{bcd}	5.67 ^{cd}
	GA3	4.62 ^{cd}	13.25 ^{abc}	5.00 ^{bc}	5.57 ^{bcd}	7.11 ^{cd}
	JA1	7.83 ^c	11.80 ^{abc}	7.86 ^{abc}	12.00 ^a	9.87 ^{bc}
	JA2	3.80 ^{cd}	16.83 ^a	6.43 ^{bc}	5.71 ^{bcd}	8.19 ^c
	JA3	4.00 ^{cd}	15.20 ^{ab}	6.56 ^{bc}	7.71 ^{abc}	8.37 ^{cd}
	NAA1	5.60 ^{cd}	17.86 ^a	6.88 ^{bc}	7.43 ^{abc}	9.44 ^{bc}
	NAA2	9.62 ^c	13.00 ^{abc}	4.11 ^{bc}	5.29 ^{bcd}	8.01 ^{cd}
	NAA3	4.00 ^{cd}	11.40 ^{abc}	6.67 ^{bc}	8.25 ^{abc}	7.58 ^{cd}
	UP1	1.00 ^d	3.00 ^d	1.29 ^c	0.89 ^d	1.54 ^e
	UP2	18.38 ^b	19.17 ^a	9.86 ^{ab}	7.71 ^{abc}	13.78 ^a
	UP3	23.71 ^a	7.83 ^{bcd}	13.50 ^a	5.33 ^{bcd}	12.60 ^{ab}
	Mean	7.40 ^C	12.40 ^A	6.00 ^C	6.50 ^C	8.10 ^A
	S.E	0.83	0.83	0.60	0.51	0.37

GA1:3.0 μM ; GA 2:0.3 μM ; GA3:0.15 μM ; JA1:3 μM ; JA2:0.3 μM ; JA3:0.03 μM ; NAA1:2.7 μM ; NAA2:0.54 μM ; NAA3:0.27 μM ; UP1:34 μM ; UP2:3.4 μM ; 1.7 μM UP3:1.7 μM ; Control:hormone-free. SN: Single node stage; PNC: Primary nodal complex formation stage; For each genotype, values in each column followed by the same letter are not significantly different. Bold, mean values followed by the same upper case letter are not significantly different. For each phase, bold, mean values followed by the same lower case letter are not significantly different. Italicised mean values followed by the same letter are not significantly different. SE: Standard error; Probability Level = 5%.

effect of UP on yam vegetative growth is evident since the rate of growth is higher than the control even with application at PNC formation stage. Only limitation of UP was a short internode length which made sub-culturing more difficult. This work will necessitate future studies to optimise concentrations of both UP and GA added together in same culture medium for highest nodes and comfortable internode length. Enhanced vegetative growth with addition of PGR at PNC formation indicate the role of culture aeration since the vials were opened when PGRs were added at PNC formation.

Conclusion

Considering the problems associated with adding the PGRs at later stages in conventional tissue cultures and the associated risk of contamination and culture losses, only the use of automated systems like Temporary Immersion Bioreactors (TIB) (Cabrera et al., 2005; Watt, 2012; Yan et al., 2011) will optimize yam micropropagation rates. In TIBs, medium change during culture is possible without disrupting the cultures. Since meristem culture for virus elimination is most important, it will be worthwhile to investigate the effects of sequential application of PGRs on meristem culture, somatic embryogenesis and plant regeneration. In addition, toxicity tests using residue analysis and animal sensitivity studies should be conducted to determine the safety level of uniconazole-p.

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