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Full Length Research Paper

Micropropagation of *Dioscorea alata* L. from microtubers induced *in vitro*

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A new method made up of different steps was established for micropropagation of *Dioscorea alata*. First plantlets were regenerated from shoots proliferating on nodal cuttings cultured on half strength Murashige and Skoog salt medium (MS/2) (basal medium) supplemented with 0.5 mg l^{-1} BAP and 1 mg l^{-1} NAA. These plantlets were used to induce microtubers on the basal medium supplemented with 1 to 5 mg l^{-1} 6-benzylaminopurine (BAP), kinetin (Kin) or α-naphthalene acetic acid (NAA) and 10 to 60 g l^{-1} sucrose. BAP at 2 to 3 mg l^{-1} combined with 20 to 30 g l^{-1} sucrose was more effective than Kin and NAA. It gave rise to 92% plantlets producing microtubers and the highest numbers of microtubers per plantlet varied between five and six. Microtubers, when sectioned and cultured on the basal medium supplemented with different BAP/NAA or Kin/NAA ratios, differentiated into shoots that, when isolated and subcultured in the same media, gave rise to rooted plantlets. The highest percentage of microtubers that differentiated into shoots was 98.8%, the highest number of shoots per microtuber was 7.5 and hence the highest number of rooted plantlets regenerated from those shoots was induced with BAP/NAA ratio (3/2 mg l^{-1}) compared to that of BAP/NAA and Kin/NAA ratios. When the plantlets were acclimatized in different substrates, 97% survived in the mixture black soil/sand at equal volume (V/V) and this was the best result for the final step of the micropropagation of *D. alata* in this study. The different steps here described, allowed the regeneration of 45 and 16 plantlets from microtubers in about 251 days using BAP and NAA, respectively, and constituted a new and rapid method for the production of healthy seeds of this species.

Key words: Growth regulators, micropropagation, microtubers, yam.

INTRODUCTION

Yams (*Dioscorea* spp.) belong to the family *Dioscoreaceae*. The genus *Dioscorea* contains over 600 species, which are all monocotyledonous. They are mainly cultivated in South America, Asia and West Africa (Ayensu and Coursey, 1972). *Dioscorea alata* is classified between the sixth most cultivated species in Africa; the others are *Dioscorea rotundata, Dioscorea bulbifera, Dioscorea trifida, Dioscorea cayenensis* and *Dioscorea esculenta.* Yam tubers are important in different domains. Nutritionally, yams are a major source of nourishment to many populations in the world (Craufurd et al., 2006; Hahn, 1995; Yan et al., 2002). Pharmaceutically, some species of *Dioscorea*, particularly

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxy acetic acid; **BAP,** 6-benzylaminopurine; **IBA,** indole -3-butyric acid; **Kin,** Kinetin; **MS,** Murashige and Skoog; **NAA,** α-naphthalene acetic acid.

Dioscorea zingiberensis, produces high concentration of diosgenin, a chemical used for the commercial synthesis of sex hormones and corticosteroids (Yongqin et al., 2003; Yuan et al., 2005). Agriculturally, yams tubers are used as planting material. The conventional multiplication of *Dioscorea* species is by tuber seeds, a tuber fragment that grows and develops into a new tuber. The absence of viable seeds, the long period required for obtaining usable tubers and phytosanitary problems are some of the factors that limit the rapid conventional propagation and economic exploitation of *Dioscorea* species (Balogum et al., 2006; Tschannen et al., 2005). As many species of *Dioscorea*, *D. alata* is dioecious and cultivated forms have the larger diversity compared to the cultivated forms of other species (Mantell et al., 1978). It is then necessary to propagate vegetatively the selected plants of this species.

In fact, since about 30 years ago, *in vitro* propagation of *Dioscorea* species has been increased and performed by using different types of explants such as immature leaves (Kohmura et al., 1995), zygotic embryos (Viana and Mantell, 1989), nodal cuttings (Alizadeh et al., 1998; Yongqin et al., 2003), bulbils (Asokam et al., 1983), roots (Twyford and Mantell, 1996), cells and protoplasts (Tor et al., 1998). However, few studies have been reported on the micropropagation and microtuberisation of *D. alata* (Mantell and Hugo, 1989; John et al., 1993; Jasik and Mantell, 2000; Borges et al., 2005).

In general, the different results obtained show that it is possible to propagate *D. alata in vitro* from different explants such as leave fragments, nodal cuttings, apex and meristems. But none has been reported on the micropropagation of this species from microtubers induced *in vitro*.

The aim of this work was thus to multiply this species *in vitro*, especially to produce microtubers and use them as explants for micropropagation. Many factors such as the presence or the absence of growth regulators, the concentration of sugars and jasmonic acid in the medium, the mineral composition of the medium and the photoperiod were known to influence the tuberization *in vitro* (Mantell and Hugo, 1989; Santos and Salema, 2000; Ovono Ondo et al., 2007). Therefore, the effects of growth regulators (BAP, Kin and NAA; alone or in combination) and sucrose, on microtuberization and micropropagation of *D. alata* were studied.

MATERIALS AND METHODS

Plant material and disinfection

About 30 mature tubers of *D. alata* (Figure 1a) were obtained from local market of Mbam division, Center Region, Cameroon. Each tuber was divided into two fragments: the posterior and the anterior (Figure 1b). Each anterior fragment bearing single apex (Figure 1c) was cleaned and put under running tap water for 24 h. Cleaned fragments were then cultured in polyethylene bags containing a sterile black soil. After 35 days, each apex had grown into a long stem with about 10 to 16 nodes (Figure 1d). Nodal cuttings about 1.5 cm long were isolated from different stems, then cleaned under running tap water for 2 h and disinfected in 1% Tween 80 for 5 min followed by 1% sodium hypochlorite for 35 min and then rinsed four times (10 min each) in sterilized distilled water.

Regeneration of plantlets

For regeneration of plantlets, disinfected nodal cuttings were cultured in closed test tubes containing each 10 ml of half strength Murashige and Skoog salt medium (1962) (MS/2) supplemented with Morel and Wetmore (1951) vitamins, 20 g l^{-1} sucrose, 0.5 mgl^{-1} 6-benzylaminopurine (BAP), 1 mgl^{-1} α-naphthalene acetic acid (NAA), 3 gl^{-1} activated charcoal and 6 gl^{-1} Difco agar. The pH of the medium was adjusted to 5.8 with NaOH solution (1 N) or HCl solution (0.1 N) before autoclaving at 115°C for 30 min under a pressure of 1.6 ± 0.1 kg cm^{-2}. All cultures were incubated under 80 µmol m^{-2} s^{-1}light provided by cool white fluorescent tube lamps (Mazda) at a photoperiod of 16 h at 26 ± 1°C.150 nodal cuttings were cultured and the experiment was repeated twice. After 21 days, shoots proliferated at the level of the node and, when maintained in the same medium for 15 days, each shoot differentiated into roots and became plantlet. After being isolated from the node, those plantlets constituted the explants for the following experiment.

Induction and production of microtubers

Regenerated plantlets from nodal cuttings were subcultured in 150 x 80 mm glass culture tubes containing semi liquid (3 gl^{-1}Difco agar) MS/2 medium supplemented with Morel and Wetmore vitamins, 20 gl^{-1} sucrose, 5 µM jasmonic acid, 1 to 5 mgl^{-1}BAP or Kin or NAA. The effects of these three growth regulators on the induction and production of microtubers were evaluated. The pH of all media was adjusted to 5.8 before autoclaving. All cultures were incubated under the same conditions as during plantlets regeneration. 15 plantlets were subcultured for each concentration of growth regulator and all experiments were repeated twice. The percentage of plantlets inducing microtubers was evaluated after 30 days. 60 days later, without any secondary subculture, the average number of microtubers produced, the average length and the average fresh weight were evaluated for each treatment. For all experiments, the control was the MS/2 semi liquid medium supplemented with vitamins, sucrose and jasmonic acid. The effect of sucrose on the induction and production of microtubers was also evaluated during the same period. MS/2 semi liquid medium was supplemented with 20, 30, 40, 50 and 60 gl^{-1} sucrose together with 2 or 3 mgl^{-1} BAP or Kin or NAA. The control was without sucrose.

Proliferation of shoots from microtubers and micropropagation

Each microtuber produced was divided into two fragments: the posterior and the anterior. All anterior fragments bearing a small apex were then used as explants. They were cultured in closed test tubes each containing 10 ml of MS/2 medium supplemented with Morel and Wetmore vitamins and 20 gl^{-1} sucrose, 6 gl^{-1}Difco agar (basal medium), 0.5 to 3.5 mgl^{-1} BAP and 2 mgl^{-1}NAA (BAP/ANA ratio) or 0.5 to 3.5 mgl^{-1} Kin and 2 mgl^{-1} NAA (Kin/NAA ratio). The effects of the different ratios on the proliferation of shoots were evaluated. The culture conditions were the same as during the production of microtubers. 12 microtubers fragments were used for each ratio and all experiments were repeated twice. The control was growth regulator-free. The percentage of explants inducing shoots and the average number of shoots per explant were evaluated after 60 days of culture. When isolated from microtubers,

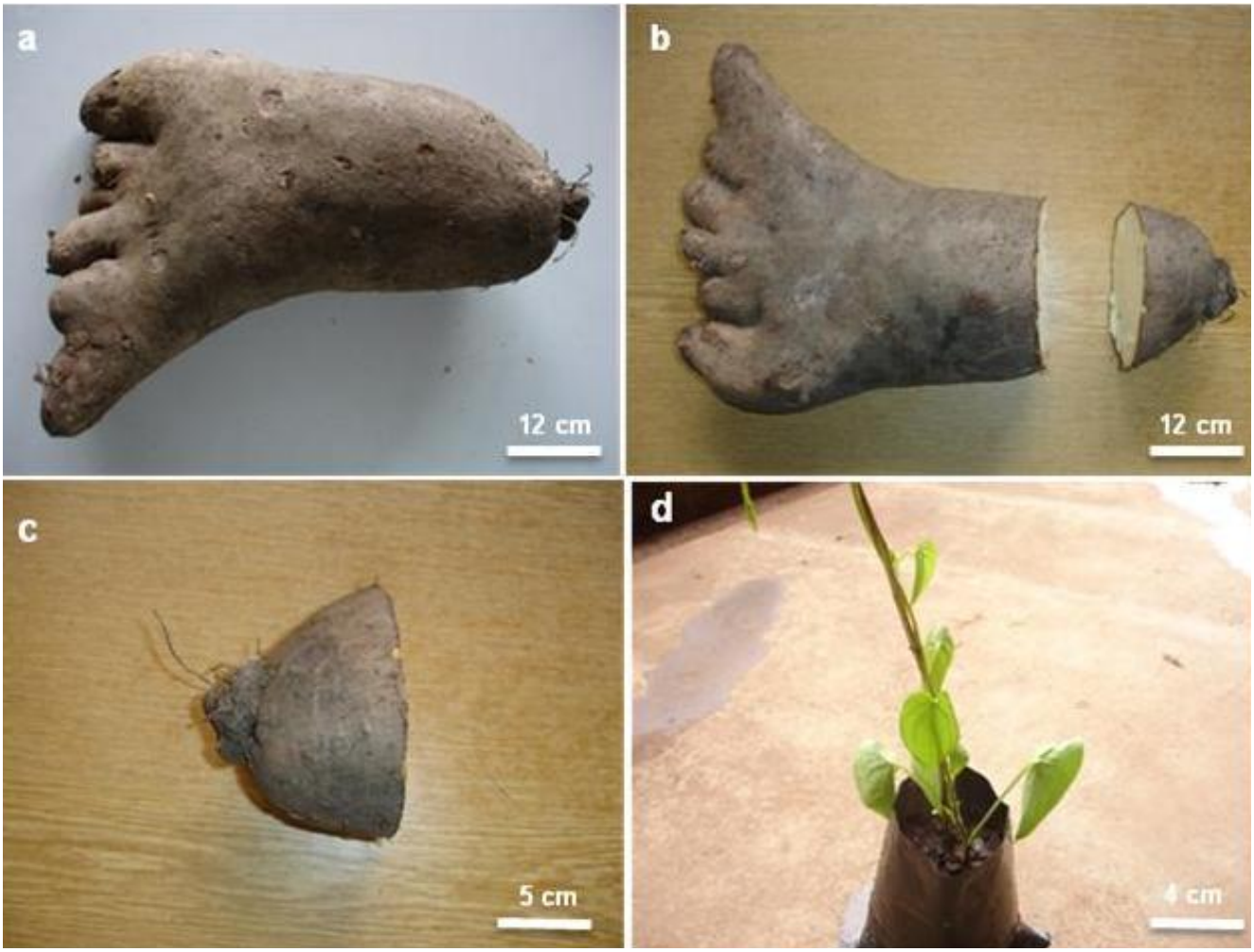

Figure 1. Material of *Discorea alata* used for the production of nodal cuttings. **(a)** Mature tuber of *D. alata*. **(b)** Mature tuber divided into anterior (right) and posterior fragment (left). **(c)** Anterior fragment bearing single apex (sa) cultured to obtain stem with nodes. **(d)** Nodal plant derived from the growth of anterior fragment after 35 days of culture in polyethylene bag.

shoots were then subcultured in the same proliferation media supplemented with 3 gl^{-1} activated charcoal for the regeneration of plantlets. 12 isolated shoots were used for each ratio and all experiments were repeated twice. The percentage of regeneration was evaluated and the growth of shoots into plantlets was measured by counting the average number of nodes, leaves and roots after 30 days of subculture.

Effect of substrate on the acclimatization of plantlets

The acclimatization of plantlets was tested in polyethylene bags containing the following sterilized substrates; vermiculite, black soil, mixture black soil/vermiculite at equal volume (v/v), sand, mixture sand/vermiculite at equal volume (v/v), mixture black soil/sand at equal volume (v/v). 33 plantlets were cultured per substrate and the experiment was repeated twice. All cultures were incubated under a temperature of 26 ± 1°C, 72 to 76% of relative humidity and a photoperiod of 16 under a light period of 80 µmol m^{-2} s^{-1}during 56 days. During this period, the relative humidity was progressively reduced as plantlets were watered firstly with sterilized tap water during 20 days and then with tap water during 36 days before being transplanted to the field. The percentage of survival plantlets was evaluated in each substrate.

Data analysis

All experiments were set up in a completely randomized design. Differences between means were scored with Duncan's multiplication range test. The analysis of samples from each treatment was statistically evaluated by analysis of variance (ANOVA, $p \leq 0.05$) and the interactive effect of two phytoregulators was assessed by a two-way ANOVA. The program used was SPSS (version 17 for Windows).

RESULTS

Regeneration of plantlets from nodal cuttings culture

When cultured on MS/2 supplemented with 0.5 mg l^{-1} BAP and 1 mg l^{-1}NAA, the lateral buds appeared on the

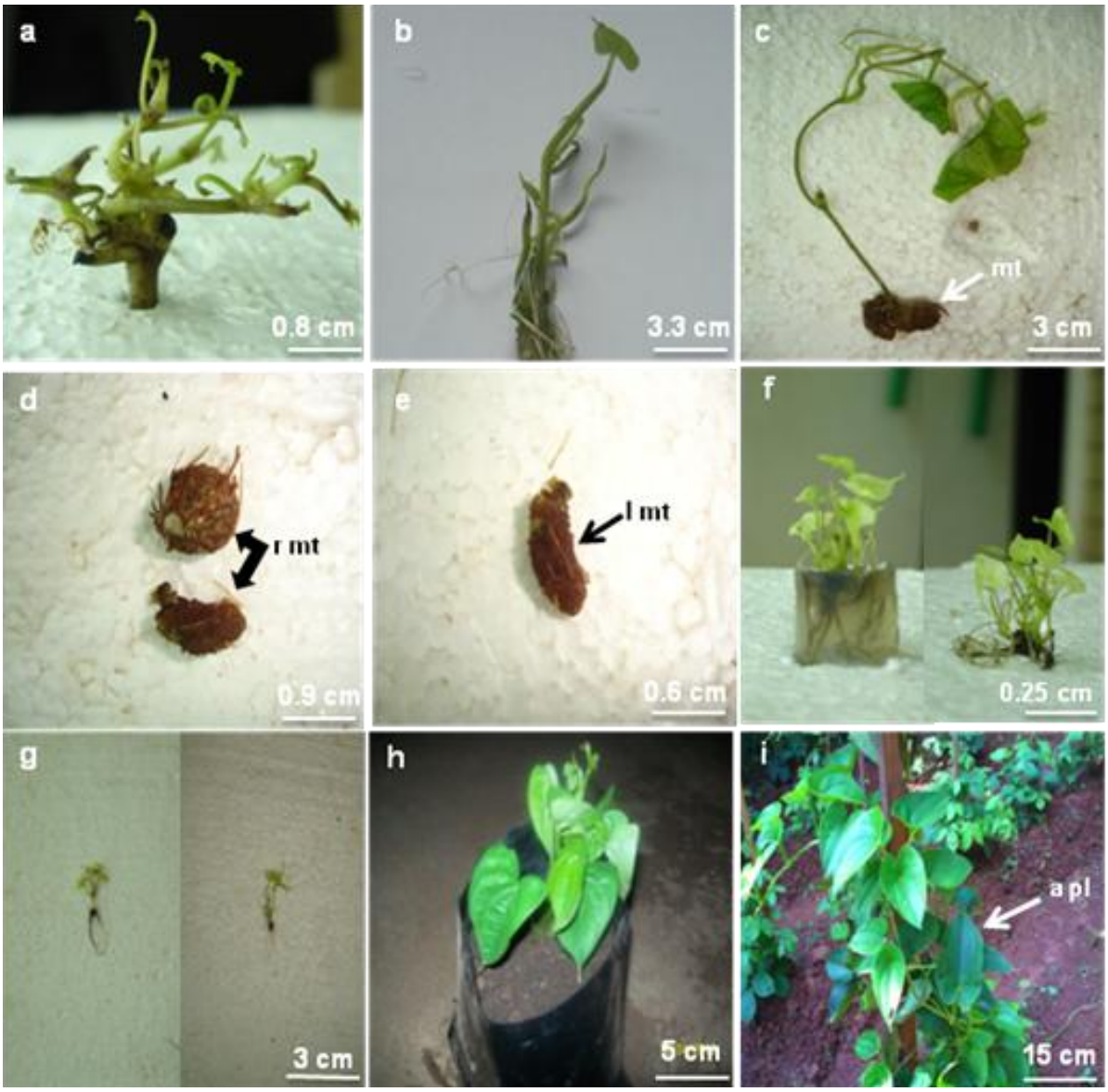

Figure 2. Microtuberization and micropropagation of *Dioscorea alata*. **(a)** Shoot proliferated on nodal cuttings cultured during 21days on MS/2 medium supplemented with 0.5 mg l^{-1} BAP and 1 mg l^{-1} NAA. **(b)** Rooted plantlet regenerated from nodal shoot cultured during 15 days in the same medium. **(c)** Plantlet with microtubers (mt) induced on MS/2 medium supplemented with 1 mg l^{-1} BAP after 30 days of culture. **(d)** Isolated rounded microtubers (r mt) from plantlet cultured in the presence of 5 mg l^{-1} BAP or 2 to 5 mg l^{-1} Kin or 1 to 5 mg l^{-1} NAA and 20 to 40 g l^{-1} sucrose. **(e)** Isolated long microtuber (l mt) from plantlet cultured in the presence of 3 mg l^{-1} BAP and 20 to 30 g l^{-1}sucrose. **(f)** Multiple shoot proliferated in microtuber culture during 60days on MS/2 medium supplemented with 3/2 mg l^{-1} BAP/NAA (left) and 1.5/2 mg l^{-1} KIN/NAA (right). **(g)** Rooted plantlet regenerated from microtuber shoot subcultured during 30 days in the same media. **(h)** Acclimatized plant in mixture sand/ black soil (v/v) after 56 days of culture. **(i)** Adult plant (a pl) in the field 90 days after transfer.

nodal cuttings after 7 days; almost all grew into shoots with an average length of 5.25 ± 0.52 cm within 21 days (Figure 2a). When maintained in the same medium, 67.14% of shoots developed into plantlets with an average number of leaves per shoot of 7.34 ± 1.64 and an average number of roots per shoot of 4.11 ± 0.41 within 15 days (Figure 2b).

Effect of BAP, NAA and Kin on the production and growth of microtubers

Plantlets regenerated from nodal cuttings were cultured in the presence of 1 to 5 mgl^{-1} BAP, Kin or NAA for the production of microtubers (Figure 2c). After 30 days of culture, the higher percentages of plantlets producing

Table 1. Effect of BAP, Kin and NAA on the production (30 days) and growth (60 days) of microtubers of *D. alata.*

Phytohormone	Concentration ($mg.l^{-1}$)	Number of plantlets cultured	% of plantlets producing microtubers	Production of microtuber		
				Number of microtubers per plantlet	Average length of microtuber (mm)	Average fresh weight of microtubers per plantlet (mg)
BAP	0	30	0	0	0	0
	1	30	72.2^{a}	2^{c}	12^{b}	221.3^{c}
	2	30	33.2^{d}	4^{b}	13^{b}	298.1^{b}
	3	30	68.4^{b}	6^{a}	23^{a}	641.1^{a}
	4	30	39.6^{c}	2^{c}	12^{b}	220.8^{c}
	5	30	23.1^{e}	2^{c}	11^{b}	207.1^{d}
ANA	0	30	0	0	0	0
	1	30	0	0	0	0
	2	30	12.6^{c}	4^{a}	12^{d}	207.5^{b}
	3	30	40.9^{a}	3^{b}	17^{a}	328.3^{a}
	4	30	34.2^{b}	2^{c}	15^{b}	209.7^{b}
	5	30	07.1^{d}	1^{d}	12^{b}	128.2^{c}
Kin	0	30	0	0	0	0
	1	30	12.1^{d}	2^{b}	14^{b}	303.1^{b}
	2	30	17.9^{c}	2^{b}	12^{b}	296.2^{c}
	3	30	31.2^{b}	2^{b}	14^{b}	299.1^{c}
	4	30	46.7^{a}	3^{a}	17^{a}	406.7^{a}
	5	30	09.6^{e}	1^{c}	16^{a}	133.2^{d}

Duncan's multiple range test was used to evaluate the difference in the percentage of plantlet production of microtubers, number of microtubers per plantlet, average length of microtuber and average fresh weight of microtuber per plantlet, respectively; data sharing the same letter in the same column were not significantly different at 5% level.

microtubers were 72.17, 40.19, and 46.17% with 1 mgl^{-1} BAP, 3 mgl^{-1} Kin and 4 mgl^{-1} NAA, respectively (Table 1). Comparatively, BAP is more effective than Kin and NAA. Indeed, the percentages of production obtained with BAP at all concentrations were higher than that of Kin and NAA. With 5 mgl^{-1}, low percentages of 23.6, 07.08, and 09.06% were obtained, respectively, with BAP, Kin and NAA (Table1). The number of microtubers per plantlet after 60 days of culture varied according to the growth regulators and the highest numbers of 6, 4 and 3, were obtained, respectively, with 3 mgl^{-1} BAP, 2 mgl^{-1} Kin and 4 mgl^{-1} NAA (Table 1). With the other concentrations, this number was 1 or 2. Based on the length of microtubers, there were two sizes: for some of them, the length varied from 16 to 23 mm (long microtubers) and for the others from 11 to 14 mm (rounded microtubers) (Figure 2d, 2e). The best concentration for the production of long microtubers of *D. alata* was 3 mg l^{-1} BAP (23 mm) while 5 mgl^{-1} of the same growth regulator was the best for the production of rounded microtubers (11 mm) (Table 1). With Kin and NAA, both types were obtained almost with all concentrations tested and they were found on the same plantlet. Concerning the fresh weight of microtubers per plantlet, the variation was from 128.2 mg with 5 $mgl^{-1}$of Kin to 641.2 mg with 3 mgl^{-1} of BAP (Table1).

Effect of sucrose on the production and growth of microtubers

When the basal medium was supplemented with 10 to 60 gl^{-1} of sucrose and 2 to 3 mgl^{-1} of BAP, Kin or NAA, the plantlets also produced microtubers after 30 days. The highest percentages (92%) were obtained with 20 or 30 gl^{-1} in the presence of BAP, 88% with 40 gl^{-1}in the presence of Kin and 53% with 30 gl^{-1} in the presence of NAA (Table 2). On the other hand, 60 gl^{-1} of sucrose gave rise to low percentages of plantlets producing microtubers which were 17, 11 and 9% for BAP, Kin and NAA, respectively (Table 2). The highest number of microtubers per plantlet was 5 with 20 gl^{-1}, 3 with 20 and 40 gl^{-1} and 3 with 30gl^{-1} in the presence of BAP, Kin and NAA, respectively. The other concentrations of sucrose gave 1 or 2 microtubers per plantlet. Based on the length, there were two kinds of microtubers; the long type with

Table 2. Effect of sucrose on the production (30 days) and growth (60 days) of microtubers of *D. alata* in the presence of 2 to 3 mg.l^{-1} of BAP, Kin or NAA.

Growth regulator (mg l^{-1})	Sucrose (g.l^{-1})	% of plantlets producing microtubers	Average number of microtubers per plantlet	Average length of microtubers (mm)	Average fresh weight of microtuber per plantlet (mg)
BAP (2 or 3)	0	0	0	0	0
	10	26.7^{d}	2^{c}	08^{d}	195.4^{c}
	20	92.1^{a}	5^{a}	21^{b}	1005.8^{a}
	30	92.3^{a}	4^{b}	26^{a}	998.4^{b}
	40	71.8^{b}	2^{c}	12^{c}	192.9^{c}
	50	38.6^{c}	1^{d}	11^{c}	86.8^{d}
	60	17.9^{e}	1^{d}	12^{c}	85.4^{d}
Kin (2 or 3)	0	0	0	0	0
	10	23.4^{d}	1^{c}	10^{c}	97.2^{e}
	20	77.8^{b}	3^{a}	23^{a}	642.7^{b}
	30	82.4^{a}	2^{b}	19^{b}	202.3^{d}
	40	88.2^{a}	3^{a}	19^{b}	728.6^{a}
	50	48.9^{c}	2^{b}	11^{c}	225.5^{c}
	60	11.2^{e}	1^{c}	11^{c}	85.2^{e}
NAA (2 or 3)	0	0	0	0	0
	10	17.4^{d}	2^{b}	13^{b}	220.5^{b}
	20	49.2^{b}	2^{b}	22^{a}	164.7^{c}
	30	53.3^{a}	3^{a}	23^{a}	641.2^{c}
	40	22.7^{c}	1^{c}	11^{c}	59.7^{d}
	50	12.8^{e}	1^{c}	11^{b}	56.3^{d}
	60	09.4^{f}	2^{b}	07^{c}	162.4^{c}

Duncan's multiple range test was used to evaluate the difference in the percentage of plantlet production of microtubers, number of microtubers per plantlet, average length of microtuber and average fresh weight of microtuber per plantlet respectively; data sharing the same letter in the same column were not significantly different at 5% level.

the length of 19 to 26 mm obtained with 20 or 30 gl^{-1} sucrose for all growth regulators (Figure 2e) and the rounded type with the length of 07 to 12 mm obtained with 10, 40, 50 or 60 gl^{-1}sucrose (Table 2). The fresh weight and number of microtubers per plantlet varied according to the concentration of sucrose. In fact it varied from 56.3 mg with 50 gl^{-1}sucrose in the presence of NAA to 1005.8 mg with 20 gl^{-1} in the presence of BAP (Table 2). Generally, looking at Tables 1 and 2, it was noticed that the sucrose is more useful for the production of long microtubers (26 mm) than growth regulators (23 mm).

Effect of BAP/NAA and Kin/NAA ratio on the proliferation of shoots and regeneration of plantlets from microtubers

When cultured in the presence of BAP/NAA or Kin/NAA ratio, the fragments of microtubers produced shoots after 60 days (Figure 2f). With BAP/NAA, the highest percentage of microtubers differentiating shoots (98.8%) were obtained with 3/2 ratio, followed by 2.5/2 (81.3%) and 2/2 (76.1%) (Table 3). A significant number of shoots per microtuber (5.3 to 7.5) was obtained with these same ratios as when treated with any of the other combinations (Table 3). With Kin/NAA, the highest percentage of microtubers differentiating shoots (66.6%) was obtained with the ratio 1.5/2. A significant number of shoots per microtuber (4.6 to 4.9) was obtained with 1/2; 1.5/2 and 2/2 ratios (Table 3).

When subcultured in the same media, shoots regenerated plantlets at 100% after 30 days (Figure 2g). With BAP/NAA, the highest number of nodes per plantlet (6.3) was obtained with 2.5/2 and 3/2. A significant number of leaves of 7.1 were obtained in the ratio of 3/2 and a root number between 6.1 and 6.6 with the ratio of 1/2; 1.5/2 and 2/2 (Table 4). With Kin/NAA, the ratios 1.5/2 and 2.5/2 gave higher numbers of nodes (5.3 to 5.6 per plantlet). A significant higher number of leaves (5.8 to 6.2) was obtained with 1.5/2; 2/2 and 2.5/2 and of roots (5.5 to 5.8) with 1/2; 1.5/2 and 2/2 compared to the other combinations (Table 4).

Table 3. Effect of BAP/NAA and Kin/NAA ratios on the proliferation of shoots on microtuber of *D. alata* after 60 days of culture.

Growth regulator ratio (mg.l^{-1})	% of microtubers differentiating shoot	Average number of shoots per microtuber
BAP/ANA		
0.5/2	21.2^{d}	3.8 ± 0.6^{d}
1/2	21.4^{d}	3.6 ± 0.5^{d}
1.5/2	39.6^{c}	4.3 ± 0.2^{c}
2/2	76.1^{b}	4.4 ± 0.3^{c}
2.5/2	81.3^{b}	5.3 ± 0.4^{b}
3/2	98.8^{a}	7.5 ± 0.3^{a}
3.5/2	41.1^{c}	2.3 ± 0.6^{e}
Kin/ANA		
0.5/2	16.4^{d}	3.3 ± 0.4^{b}
1/2	31.7^{c}	4.9 ± 0.2^{a}
1.5/2	66.6^{a}	4.6 ± 0.5^{a}
2/2	35.4^{c}	4.4 ± 0.6^{a}
2.5/2	42.2^{b}	2.4 ± 0.6^{c}
3/2	31.8^{c}	3.1 ± 0.4^{b}
3.5/2	19.6^{d}	2.1 ± 0.3^{c}

Standard error; only the shoots longer than 7 mm were counted. Duncan's multiple range test was used to evaluate the percentage of explants differentiating shoot and average number of shoots per microtuber respectively; data sharing the same letter in the same column were not significantly different at 5% level.

Effect of substrate on the acclimatization of plantlets

When transferred on different substrates, the plantlets developed from microtuber shoots gave rise to vigorous plants after 56 days. After this day, each plant had an average number of leaves of 12.8 ± 2.6 and average number of nodes of 11.9 ± 1.7 (Figure 2h). The percentage of survival varied with the type of substrate. A significant percentage of 97% was obtained with the mixture black soil / sand (v/v), followed by a mixture of black soil/vermiculite (v/v) (71%) and a mixture of sand/vermiculite (v/v) (67%) (Figure 3). The black soil and sand gave average percentage of survival of 48 to 52%) and vermiculite gave the lowest percentage of 6% (Figure 3). When transferred to the appropriate land (black soil containing sand), all survival plants grew and developed well and gave rise to adult plants after 90 days (Figure 2i).

DISCUSSION

D. alata as the other species of yam can be successfully propagated by *in vitro* method using different kinds of explants such as nodal cuttings (Lauzer et al., 1992; Alizadeh et al., 1998), adventitious buds (Yongqin et al., 2003), protoplasts (Tor et al.,1998), leaves (Kohmura et al., 1995), zygotic embryos (Viana and Mantell, 1989). The results of this work showed that in the presence of 0.5 mg l^{-1} BAP and 1 mg l^{-1} NAA, the nodal cuttings of *D. alata* from preexisting axillary buds were differentiated into shoots which, when subcultured in the same media, gave rise directly to rooted plantlets at 67.14% without any tuberization within 15 days. This direct regeneration was also reported on the same species by Mantell et al. (1978) from the same material but with a lower percentage (52%), or by other authors on other species of *Dioscorea* such as *Dioscorea floribunda* (Borthakur and Sing, 2002) and *D. zingiberensis* (Yan et al., 2002). But the number of shoots and plantlets regenerated in different cases are function of the species, the nature and the concentration of growth regulators used.

In fact, in this work, BAP and NAA were supplemented in the same medium at a single concentration while in other species they were used separately and sometimes at various concentrations (Malaurie et al., 1995b). The microtuberization in *Dioscorea* species have been already reported (Ng Syc, 1998; Ondo Ovono et al., 2007; Passam, 1995). This microtuberization is generally induced directly on the explants cultured and is influenced by factors such as jasmonic acid (Bazabakana et al., 2003; Jasik and Mantell, 2000), growth regulators and light (John et al., 1993), photoperiod, mineral medium and sucrose (Chu and Ribeiro, 2002; Mantell and Hugo, 1989; Tsafack et al., 2009). These factors are generally studied in combination. In the present study, the effects of growth regulators and sucrose on the microtuberization of *D. alata* were studied separately.

Table 4. Effect of BAP/NAA and Kin/NAA ratio on the growth of shoots from microtubers of *D. alata* after 60 days of culture.

Growth regulator ratio (mg.l^{-1})	Average number of nodes per plantlet	Average number of leaves per plantlet	Average number of roots per plantlet
BAP/ANA			
0.5/2	3.1^{c}	3.8^{c}	4.7^{b}
1/2	2.3^{d}	3.3^{c}	6.1^{a}
1.5/2	4.4^{b}	5.2^{b}	5.3^{b}
2/2	4.6^{b}	5.2^{b}	6.6^{a}
2.5/2	6.2^{a}	5.4^{b}	6.4^{a}
3/2	6.3^{a}	7.1^{a}	4.6^{b}
3.5/2	2.5^{d}	3.4^{c}	4.5^{b}
Kin/ANA			
0.5/2	2.0^{d}	2.3^{e}	3.4^{c}
1/2	3.1^{c}	4.0^{c}	5.5^{a}
1.5/2	5.9^{a}	6.2^{a}	5.8^{a}
2/2	4.8^{b}	5.8^{b}	5.7^{a}
2.5/2	5.2^{a}	5.9^{b}	4.7^{b}
3/2	2.1^{d}	2.2^{e}	3.8^{c}
3.5/2	2.6^{c}	3.0^{d}	3.5^{c}

χ^2 and Duncan's multiple range test was used to evaluate the average number of nodes per plantlet, average number of leaves per plantlet and average number of roots per plantlet; data sharing the same letter in the same column were not significantly different at 5% level.

The results obtained show that under these conditions, the microtuberization is easily obtained with plantlets produced *in vitro* from nodal cuttings. Similar results were reported by Mantell and Hugo (1989) on the same species and *D. bulbifera* but under the control of different factors. The percentage of plantlets producing microtubers, the number of micro tubers per plantlet, their length and their fresh weight varied according to the concentration of BAP, Kin and NAA used or according to the concentration of sucrose in the medium.

This variation is comparable to that obtained by Alizadeh et al. (1998) in *D. composita* and by Ondo Ovono et al. (2007) in *D. cayenensis* and *D. rotundata*. Between the three growth regulators used in this study, BAP at 3 mg l^{-1} seems to be more effective for the production of microtubers (68.41% of plantlets produced microtubers, 6 microtubers per plantlet with an average weight of 641.2 mg) compared to Kin at the same concentration (40.19% plantlets produced microtubers, 4 microtubers per plantlet, with an average weight of 328.3 mg) and NAA at 4 mg l^{-1} (46.17% of plantlets producing microtubers, 3 microtubers per plantlet and an average fresh weight of 406.7 mg). Similar results were reported in different species of yam (Forsyth and Van Staden, 1984; Kadota and Niimi, 2004). But the work of Ammirato (1982) and Scarcelli et al. (2006) on different species of yam, showed that the tuberization of those species did not depend only on the experiment or culture conditions but also on the genotype of species. This can partly explain why in this work the microtuberization was obtained only after producing plantlet instead to be obtained directly from the explants cultured as in other species such as *D. floribunda* (Sengupta et al., 1984), *D. bulbifera* (Forsyth and Van Staden, 1984), *D. rotundata* (Ng Syc, 1988), *Pterostylis sanguinea* (Debeljak et al., 2002). When the basic medium is supplemented with 10 to 60 g l^{-1} sucrose and 2 to 3 mg l^{-1}BAP, Kin or NAA, the highest percentages of plantlets forming microtubers (53.3 to 92.3%) were obtained with 20 to 40 g l^{-1} in the presence of these growth regulators.

These results are in contrast with those obtained by Ondo Ovono et al. (2007) on *D. cayenensis-D. rotundata* complex which showed that an increase of sucrose from 3 to 5% in the presence of Kin had no effect on tuberization. It had also been shown that, on MS medium containing 20 g l^{-1} sucrose, *D. composita* microtubers were not induced, whereas they were on media containing 80 or 100 g l^{-1} sucrose (Alizadeh et al., 1998; Nyochembeng and Stephen, 1998). In this study, the highest number of microtubers per plantlet (3 to 5), the greatest microtuber size (19 to 26 mm) and higher fresh weight of microtubers (641.2 to 1005.8 mg) were obtained when 20 to 40 g l^{-1} sucrose were used. These results are different to those obtained by Jasik and Mantel (2000) in *D. cayenensis* where higher results were obtained with 40 g l^{-1} sucrose. The proliferation of shoots from microtubers and micropropagation from those shoot are obtained when the basal medium is supplemented

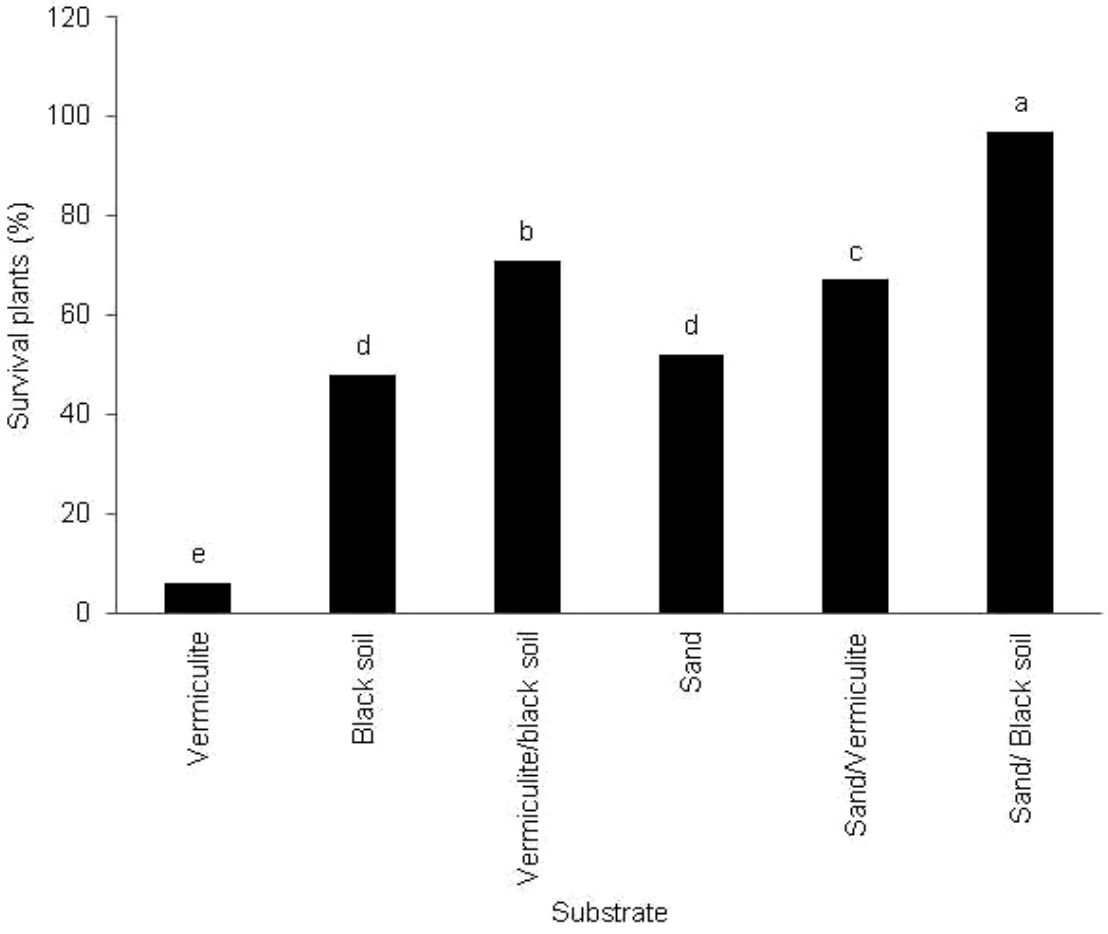

Figure 3. Effect of substrate on the acclimatization of plantlets regenerated from shoot proliferated on microtubers of *D, alata* after 56 days. Duncan's multiple range test was used to evaluate the difference between the percentage of survival plants in different substrates; histogram with the same letter were not significantly different at 5% level.

with different combinations or ratios of BAP/NAA or Kin/NAA. The effects of growth regulators combinations on the organogenesis *in vitro* has been reported in some species such as *D. zingiberensis* (Yongqin et al., 2003; Yuan Shu et al., 2005), *Dioscorea spp* (Ngo Ngwe, 2009) and *Colocassia esculenta* (Yam et al., 1990; 1991).

The results obtained show that the highest percentage of microtubers differentiating shoot (98.8%) was obtained with 3/2 mg l^{-1} BAP/NAA and the highest number of shoot per plantlet (5.5 to 5.7) is obtained with 2.5 to 3/2 mg l^{-1} BAP/NAA. With Kin/NAA, the highest percentage (66.6%) was obtained with 1.5/2 mg l^{-1} and the highest number of shoot per plantlet (4.4 to 4.9) was obtained with 0.5 to 2/2 mg l^{-1}. Auxin/cytokinin ratio is an effective inducer of shoot in *D. alata*. BAP/NAA is more effective than Kin/NAA. The fact that auxin and cytokinin must be combined to induce the proliferation of shoot in *D. alata* confirmed the complementary and synergistic action of these growth regulators as has been shown in several species (Twyford and Mantell, 1996; Jackson, 1999; Yam et al., 1991; Fotso, 2005). The growth and development of shoots to complete plantlets of *D. alata* can also be under the control of BAP/NAA or Kin/NAA complementary and synergistic action.

In fact, when subcultured in the same media, shoot from microtubers regenerate plantlets at 100%. This percentage is comparable to that obtained by Yongqinet al. (2003) in *D. zingiberensis* where almost all subcultured shoots gave rise to rooted plantlets but in the presence of 4.9 or 9.8 µM of IBA. The highest growth parameters (number of nodes, number of leaves and number of roots per plantlet) were obtained with BAP/NAA ratio compared to Kin/NAA ratio. If we consider the fact that the concentration of NAA was constant for all ratio tested (2 mg l^{-1}), it is possible to deduce that BAP had more effect than Kin on the proliferation of shoot and their growth to plantlet in *D. alata*. This higher effect of BAP had been reported by Yuan et al. (2005) on the callogenesis and somatic embryogenesis of *D. zingiberensis* when combined with NAA or 2,4-D, and

also by Gulati and Jaiwal (1996) on the micropropagation of *Dalbergia sisso* from nodal explants when combined with IBA or NAA. In this study, the result of acclimatization showed that plantlets regenerated survived more in the mixture of black soil and sand (97%). This result contrasts with acclimatization of many species where plantlets survived in vermiculite or in mixture of vermiculite and soil (Yongqin et al., 2003; Yassen et al., 1995). This can be partly explained by the fact that, naturally, *D. alata* grows and develops well in the regions where the soil is mixed with sand (Degras et al., 1977; Ngo Ngwe, 2009).

Conclusion

In this study, it was demonstrated that the micropropagation of *D. alata* using microtubers is possible. The originality is that microtubers used are induced on plantlets regenerated from nodal cuttings instead of being induced directly on the cultured explants, as in many species. This induction is due to the use of BAP or Kin or NAA and sucrose at different concentrations. BAP at 3 mg l^{-1} with sucrose at 20 to 40 g l^{-1} are more effective on microtuberization of *D. alata.* The proliferation of shoot on microtubers and their transformation in plantlets were obtained with BAP/NAA or Kin/NAA ratio. In these conditions, BAP/NAA at 3/2 mg l^{-1} and Kin/NAA at 1.5/2 gave the highest percentage of microtubers producing shoots (98.8 and 66.6%, respectively) and the highest number of shoots per microtuber (7.5 and 4.9, respectively) hence the highest number of plantlet regenerated. When taking into consideration the different steps of this work, it was noticed that when using BAP and Kin, the highest number of 45 and 16 regenerated plantlets respectively was obtained in 251days (8 months) from a single nodal shoot. 97% of these plantlets survived when acclimatized in a mixture of black soil/sand. Hence, the micropropagation of *D. alata* by *in vitro* culture method is more effective and more significant than the conventional method where apex culture produced generally a single tuber or seed. Further studies are needed to evaluate the performance of plants transferred to the fields.

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