Plant Cell, Tissue and Organ Culture 41: 229–235, 1995.

Effect of growth regulators concentrations on morphological development of meristem-tips in *Dioscorea cayenensis-D. rotundata* complex and *D. praehensilis*

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Received 19 October 1993; accepted in revised form 2 February 1995

Key words: growth regulators, meristem-tip, morphological development, regeneration, tissue culture, yam

Abstract

The effects of NAA/BA concentrations and GA_3 supply, on the development of meristem-tips excised from microshoots of one *Dioscorea cayenensis-D. rotundata* cultivar and of one *D. praehensilis* genotype, have been examined. Three NAA/BA concentrations and one addition of GA_3 to one medium were compared after 3, 8 and 11 months of culture without subculturing. Culture support was a superimposed agar/agarose (5 v/1v); growth regulators were used in a MS medium with agarose as top layer support. For the two clones, rooting was significantly decreased by the highest NAA/BA concentrations, and budding was independent of treatments. Shoot elongation was more associated with lower NAA/BA concentrations (*Dioscorea cayenensis-D. rotundata*) or independent (*D. praehensilis*). Callusing appeared more linked to higher NAA/BA concentrations (*D. praehensilis*) or independent *Dioscorea cayenensis-D. rotundata*). GA_3 supply did not improve regeneration. The genotypic differences are marked by a better survival and a better regeneration into rooted plantlets for the cultivar.

Abbreviations: BA – 6-benzyladenin, GA_3 – gibberellic acid; IBA –indolbutyric acid, NAA – naphthaleneacetic acid

Introduction

The first successful regeneration from shoot apex culture were achieved by Robbins (1922), Ball (1946), Wetmore & Morel (1949). Following these early works, meristem-tip culture has been used extensively in plants clonal propagation, such as horticultural plants. But it was with the work of Morel & Martin (1952) that meristem and shoot-tip culture came into their own by virtue of their capacity to produce virus-free plants. Long-term storage of meristem and shoot-tips through cryopreservation techniques was first achieved by Seibert (1976) with *Dianthus caryophyllus* shoot-tips.

To regenerate whole plants from these tissues, a proper balance of cytokinin and auxin is required. In yam, different combinations of growth regulators were used for plantlet formation from meristem and shoot-tip culture:

- NAA (2.69 to 5.38 μM) combined with BA (0.44 to 5 μM), according to Grewal *et al.* (1977) and Mantell *et al.* (1980) with *D. deltoidea* and *D. alata*, respectively;
- NAA (0.054 μM) or IBA (0.049 μM) combined with BA (8.88 μM) plus GA_3 (2.9 μM) with *D. trifida* (Saleil *et al.* 1990)

The first species, *D. deltoidea*, belonging to medicinal yams, is a wild species used for its sapogenin content, and the two others, *D. alata* and *D. trifida*, belonging to cultivated species, are used for their edible tuber tropical crops.

The present paper discusses the influence of NAA/BA concentrations and GA_3 supply on the morphological development of meristem-tips excised from

microshoots of two edible yams, one cultivar of *D. cayenensis* Lamk. - *D. rotundata* Poir. complex cultivar and one genotype of *D. praehensilis* Benth.

Materials and methods

Plant material

Two clones from the yam *in vitro* germplasm collection (Malaurie *et al.* 1993) were used for meristem-tip culture. Their identification numbers were clone PB04 for the 'Grosse Caille' cultivar of *D. cayenensis-D. rotundata* complex, and clone EA08 for the genotype of *D. praehensilis*. Meristem-tips were excised under a stereo microscope (x12, x50) from microplants sub-cultured in test tubes (24 mm diameter; 15 cm long) filled with 10 ml culture medium. The basal medium was a MS modified medium (Murashige & Skoog 1962), with 5% sucrose and 0.2% activated charcoal.

Meristem-tip culture conditions

Excised meristem-tips ($0.3 < t \leq 0.5$ mm) were cultured in test tubes (24 mm diameter, 15 cm long) filled with 25 ml nutrient medium, without subcultures during the eleven months experiment. Culture support was two superimposed agar / agarose (5 v/1v) layers. Agarose (Sigma, type VII) was poured under laminar air flow, after agar solidification. Test tubes, closed by a polycarbonate cap, were sealed with an extensible transparent film.

The basal medium was a MS modified medium for the top layer; for the bottom layer, the Knop's modified medium, proposed by Malaurie *et al.* (1993), was used without activated charcoal. Bottom layer support was provided by the addition of 0.8% agar (Sigma, agar-agar), and top layer support by 0.4% agarose. The three culture media M1, M2 and M3 [respective NAA and BA concentrations of M1: 2.69 / 0.44 μM, M2: 5.38 / 0.89 μM and M3: 8.06 / 1.33 μM), and a fourth, M4, consisting of M2 supplemented with 0.1 μM GA_3, were used for the top layer. The pH of all the media was adjusted to 5.8 before addition of the gelling agents. Culture media were autoclaved at 116°C for 30 min. Meristem-tip cultures were maintained at 28°C ± 2°C, with a 16-h per 24 h photoperiod. Light flux of about 120 μmol m^{-2} s^{-1} was provided by equal numbers of Grolux and Coolwhite fluorescent tubes.

Parameters and data analysis

Three, eight and eleven months after meristem-tip inoculation, the following parameters were evaluated: callusing, rooting, shoot elongation and bud formation. Evaluation was qualitative: presence/abscence. Any meristem-tip which in the process of development became completely necrosed, was discarded after each observation; it was maintained when necrosis was weak. For the contingency analysis, the frequencies were compared with the Pearson's χ^2. The significance level was adjusted according to Ryan (1960) to allow multiple comparisons two by two.

Results

Variability in response to BA and NAA

D. cayenensis-D. rotundata complex, (PB04) clone
Meristem-tips cultured without growth regulators, referred to as controls, allowed only 33% survival three months after excision; they reached just a swelling stage and afterwards did not survive. No significant differences in survival rate were observed between treatments. Mean survival of treated ones was 87%, 77% and 59% after 3, 8 and 11 months of culture (Table 1).

No significant between-treatment differences in morphological development were noted after 3 months of culture. Five months later, the middle concentration of NAA and BA (M2) gave the best rooting (81%) and shoot production (73%), significantly different compared to the highest concentration (M3) with 45% and 50%, respectively. However, the results with M1 and M2, as well as with M1 and M3, were not significantly different. Eleven months after meristem-tip excision, M1 and M2 gave the best results (87 to 96%) in rooting and shoot elongation. As previously, rooting was significantly higher compared to M3 (67%). No significant difference in bud production was observed between treatments (Table 1).

In summary, from 3 to 11 months of culture, the high percentage of regeneration into shoots and roots favoured by the lower NAA/BA concentrations could explain, by correlative inhibition, the slow regeneration into buds. The time-course of regeneration in response to total NAA/BA treatment showed these two tendencies after 3 months of culture: a high rate for shoot and root production, a slow rate for bud and callus production.

Table 1. Effect of NAA and BA concentration increases, in a constant NAA/BA ratio, on meristem-tip growth of the *D. cayenensis-D. rotundata* complex (clone PB04) and *D. praehensilis* (clone EA08) 3, 8 and 11 months after meristem excision.

Length of culture	Treatment	Number observed	Survival (%)	Percentage survivors exhibiting :				Number observed	Survival (%)	Percentage survivors exhibiting :			
				Callusing	Rooting	Shoot elongation	Budding			Callusing	Rooting	Shoot elongation	Budding
		PB04 clone (*D. cayenensis-D. rotundata* complex)						EA08 clone (*D. praehensilis*)					
3 months			[1),2)]									[1)]	
	control	15	33	0	0	0	0	15	0	0	0	0	0
	M1	59	88	32	19	9[a]	14	32	81[a]	41	47[a]	3	16
	M2	117	85	37	26	18[a]	29	68	40[b]	34	18[b]	7	25
	M3	53	87	21	9	4[a]	19	34	62[c]	38	15[b]	3	29
	X^2		< 3	5.356	5.840	7.032	4.190		35.393	0.489	12.336	< 3	5.035
	df = 2			NS	NS	*	NS		***	NS	**		NS
8 months													
	M1	49	80	41	76[ab]	65[ab]	27	15	40	40	80[a]	33	60
	M2	97	80	43	81[a]	73[a]	42	25	35	64	52[ab]	40	44
	M3	42	72	43	45[b]	50[b]	31	20	44	50	25[b]	20	55
	X^2		2.434	0.084	19.427	7.036	4.033		1.699	2.304	10.440	2.080	1.095
	df = 2		NS	NS	***	*	NS		NS	NS	**	NS	NS
11 months						[1)]							
	M1	37	63	27	87[ab]	89	32	11	31	27[b]	91[a]	45	64
	M2	75	64	39	93[a]	96	51	18	22	78[a]	61[ab]	56	50
	M3	27	51	41	67[b]	78	44	12	26	42[ab]	33[b]	42	67
	X^2		4.338	2.941	11.965	< 3	7.420		2.096	7.985	7.996	0.622	0.985
	df = 2		NS	NS	**		NS		NS	*	*	NS	NS

Values followed by different letters are significantly different for $p = 0.05$, at least, after 2-by-2 comparison with the Ryan test. [1] Insufficient theoretical sample size (<3)
[2] df = 3. ***, **, * respectively significantly different for the 0.001, 0.01 and 0.05 levels, after comparison with Pearson's χ^2 test. NS, not significant.

Table 2. Effect of GA_3 supply on meristem-tip growth of the *D. cayenensis-D. rotundata* complex (clone PB04) and the *D. praehensilis* clone EA08 3, 8 and 11 months after meristem excision.

Length of culture	Treat-ment	Number observed	Survival (%)	Percentage survivors exhibiting :				Number observed	Survival (%)	Percentage survivors exhibiting :			
				Callusing	Rooting	Shoot elongation	Budding			Callusing	Rooting	Shoot elongation	Budding
		PB04 clone (*D. cayenensis-D. rotundata* complex)						EA08 clone (*D. praehensilis*)					
3 months			[1),2)]									[1)]	[1)]
	control	15	33	0	0	0	0	15	0	0	0	0	0
	M2	117	85	37	26	18	29	68	40	34	18	7	25
	M4	53	85	11	4	9	8	25	68	32	20	0	8
	X^2		< 3	11.499	11.415	2.041	9.726		15.781	0.027	0.068	< 3	< 3
	df = 1			***	***	NS	**		***	NS	NS		
8 months													
	M2	97	80	43	81	73	42	25	35	64	52	40	44
	M4	42	77	29	52	62	24	11	29	36	27	0	18
	X^2		0.267	2.676	12.462	1.772	4.299		0.827	2.363	1.892	6.092	2.07
	df = 1		NS	NS	***	NS	*		NS	NS	NS	*	NS
11 months						[1)]				[1)]	[1)]	[1)]	[1)]
	M2	75	64	39	93	96	51	18	22	78	61	56	50
	M4	30	53	23	67	93	27	6	3	17	0	0	17
	X^2		2.492	2.236	12.444	< 3	5.014		16.503	< 3	< 3	< 3	< 3
	df = 1		NS	NS	***		*		***				

Values followed by different letters are significantly different for $p = 0.05$, at least, after 2-by-2 comparison with the Ryan test. [1] Insufficient theoretical sample size (<3)
[2] df = 2. ***, **, * respectively significantly different for the 0.001, 0.01 and 0.05 levels, after comparison with Pearson's χ^2 test. NS, not significant.

D. praehensilis (EA08) clone
Survival percentage differed significantly between treatments at 3 months; then no difference was observed in subsequent development. Mean survival of treated was 61%, 40% and 26% after 3, 8 and 11 months of culture. Control, without growth regulators, did not survive after short term culture (Table 1).

After 3 and 8 months of culture, only rooting data allowed statistical comparison of treatments on morphological developement. The lowest NAA and BA concentrations (M1) significantly increased the percentage of meristem-tips producing roots, with 47% at 3 months and 80% at 8 months, versus 15% and 25% for M3 (Table 1).

After 11 months of culture, the qualitative data allowed statistical comparison for two parameters: rooting and callusing. For rooting, the significant difference observed previously remained between M1 and M3, with M1 giving the best result (91% versus 33%). For callusing, M2 (78%) had the greatest and significantly different effect compared to medium M1 (27%). No significant difference was observed in the bud and shoot production frequencies ranging from 42 to 67% (Table 1).

In summary, changes in regeneration show that M1 treatment is best for rooting throughout culture. In response to all NAA/BA treatments, 50 to 60% of the surviving meristems produced roots, buds, shoots and callus after 11 months of culture.

Variability in response to GA_3 supply

D. cayenensis-D. rotundata complex, (PB04) clone
The addition of 0.1 μM GA_3 (in treatment M4) to the medium referred to as M2, did not influence significantly the survival rate. At 3 months of culture, GA_3-free medium gave the best and significantly different results for callus, root and bud production. After 8 months of culture, as seen before, a significant positive effect on rooting and bud production was observed in the absence of GA_3. Eleven months after meristem-tip excision, the significant inhibitory effect of GA_3 persisted in rooting and bud production. Opposite to it, a very high percentage (93 to 96%) of shoot elongation was observed for the two media (Table 2).

D. praehensilis (EA08) clone
Addition of GA_3 to the medium stimulated (first 3 months) and then inhibited meristem-tip survival. After three months of culture, no significant differences were observed between the morphological development of meristems cultured with or without GA_3. After 8 months of meristem culture, no shoots were produced on GA_3-medium. After 11 months, no statistical comparison was possible (Table 2).

Genotypic effect

The EA08 clone was significantly more sensitive to meristem-tip excision and culture; its survival rates was 20% after 11 months of culture against 56% for the PB04 clone. In the *D. cayenensis-D. rotundata* complex, clone PB04, 68 and 83% of meristem-tips surviving at 8 and 11 months of culture produced roots, versus 47 and 53% for *D. praehensilis* clone EA08. Moreover, this clone PB04 belonging to the complex exhibited significantly the best shoot production at 3, 8 and 11 months, with 11, 65, 91% success compared to 4, 27 and 43% for the EA08 clone (Table 3).

Discussion

The effect of the three NAA/BA treatments on morphological development showed that:
- a) for the two clones: a1) budding was independent of the treatments; a2) rooting was strongly decreased by the highest NAA/BA concentration;
- b) for one clone, the PB04: b1) callusing was independent of treatments; b2) shoot elongation was more associated with lower NAA/BA concentrations;
- c) for another clone, the EA08: c1) callusing was more linked to higher NAA/BA concentrations; c2) shoot elongation was independent of treatments.

The effect of GA_3 supply seen at all morphological inspections was unfavourable, and depending of the genotype was expressed as:
- decreased survival and decreased production of shoots for EA08,
- a decrease in root, bud and sometimes callus production for PB04.

More marked and significant differences were observed between the 2 clones in response to all treatments used. The PB04 clone was distinguished by better survival and by better rooting and development of buds into shoots, which led to improved regeneration by production of rooted leafy shoots.

In this study, lower NAA/BA concentrations had a better effect on shoot production for the clone from

Table 3. Genotypic effect on morphogenetic orientation 3, 8 and 11 months after meristem excision.[1]

Length of culture after meristem excision	Clones	Number observed	Survival (%)	Percentage survivors exhibiting :			
				Callusing	Rooting	Shoot elongation	Budding
3 months	EA08	174	52	32	21	4	14
	PB04	297	83	27	16	11	19
	X^2		51.585	1.674	1.535	7.093	1.994
	df = 1		***	NS	NS	**	NS
8 months	EA08	71	35	51	47	27	47
	PB04	230	74	40	68	65	34
	X^2		69.401	2.543	11.058	32.587	3.954
	df = 1		***	NS	***	***	*
11 months	EA08	47	20	49	53	43	53
	PB04	169	56	34	83	91	41
	X^2		58.422	5.568	8.848	32.132	3.760
	df = 1		***	*	**	***	NS

***, **, * respectively significantly different for the 0.001, 0.01 and 0.05 levels, after comparison with χ^2 Pearson test. NS, not significant. [1] Data have been collected on the five treatments (control, M1, M2, M3, M4)

the *D. cayenensis-D. rotundata* complex. The distinct NAA/BA requirements of the two 'species' *D. praehensilis* and the *D. cayenensis-D. rotundata* complex may be due to differences in their endogenous auxin and cytokinin levels. This difference in response depending on growth regulator concentration could partly explain the differences that we observed between the 2 'species'. It may also explain why some authors reported regeneration from meristem-tips with only one growth regulator, e.g. BA with strawberry (Boxus *et al.* 1977) or flax (Lane 1979a), and NAA with pea (Kartha 1981).

In our study, we observed that GA_3 supply did not improve morphological development, as observed by Lane (1979a) for the rooting of flax meristem-tips. With *Pyrus communis* L. meristem-tips, Lane (1979b) observed that shoot multiplication was not modified if medium contained NAA plus GA_3 or BA alone. Moreover, GA_3 supply have some stimulation effect, such as in rooting of potato meristem-tips (Pennazio & Redolfi 1973), or in plantlet production of *D. alata* at high concentration (Ng & Hahn 1985). In studies of *D. trifida*, Saleil *et al.* (1990) reversed the NAA/BA balance (NAA: 0.054 μM; BA: 8.88 μM), but added GA_3 (2.9 μM) during apical outgrowth. This medium differed greatly from our GA_3-containing medium (M4).

Microplant regeneration from excised meristem-tips has been reported in one, two or three phases. In an one-phase system, Kartha (1981) noted only shoot formation in meristem cultures of cassava on BA and GA_3. Complete microplants developed in a second phase, in the presence of BA and NAA. This author achieved 50% production of rooted leafy shoots, in different clones of cassava, in an one-phase system with the three growth regulators BA, GA_3 and NAA (0.5, 0.1 and 1 μM, respectively). Some authors (Kartha 1981; Kartha *et al.* 1977; Lane 1979a; Dale 1977) have reported one-phase production of microplants from meristem-tips in which the three phases were contracted: outgrowth, lengthening and multiplication of leafy bud and rooting. But, according to Boxus *et al.* (1977) if the second medium is not absolutely necessary, its use increases the chances of success. Other authors obtained microplants after two phases shoot production and rooting (Kartha 1981; Lane 1979b; Mantell *et al.* 1980; Kartha *et al.* 1981) and after three phases, outgrowth, shoot production and rooting, Saleil *et al.* (1990) obtained yam microplants of *D. trifida*. In our study, the use of two layers, one with

MS medium and growth regulators in contact with the excised meristem-tips, the other without growth regulators but with basal medium allowing rooting and shooting (Malaurie *et al.* 1993), could be understood as a two-phase system in an one-container system. The top layer allowed the outgrowth and lengthening of the meristem-tips, the bottom layer allowing the shoot elongation and root production without any transfert. This method of plantlets production from meristem-tips, could be a good way of micropropagation without any transfert and so with minimal manipulation and risk of contamination.

Acknowledgements

This work was carried out in the Laboratory of Biotechnology at ORSTOM, and later at IIRSDA (Institut International de Recherche Scientifique pour le Développement en Afrique), Adiopodoumé research station, near Abidjan, in Côte d'Ivoire, with EEC support [STD2, TS2A- 0116- CI. (CD)]. The authors would like to thank Dr. Ph. Lashermes for his advice and comments.

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