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Effect of various growth regulators on growth of yam (*Dioscorea trifida* L.) *in vitro* shoot tips

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In this work, we observed the effect of hormonal content of four culture media on the growth of *Dioscorea trifida* shoot tips. Medium S included 0.6 µM 6-benzylaminopurine, 1.07 µM naphthalene acetic acid, and 0.23 µM gibberellic acid (GA_3), medium EBR 0.1 µM 24-Epibrassinolide and 0.23 µM GA_3, medium T 25 µM meta-topolin and 0.23 µM GA_3 and medium ZR 25 µM zeatin riboside and 0.23 µM GA_3. After 2 months in culture, shoot length was highest on medium EBR and ZR, with values of 11.63 and 11.30 mm, respectively, intermediate on medium S (9.70 mm) and lowest on medium T (3.07 mm). Oxidation reached a similar level on medium S, T and ZR (2.17 - 2.40) while it was only 1.63 on medium EBR. Well-developed shoots were obtained on medium EBR with an average of 1.93 leaves and 0.70 roots per shoot. Shoots were less developed on medium S and ZR, with an average of 1.30 leaves and 0.20 roots per shoot on medium S and 1.60 leaves and 0.00 roots per shoot on medium ZR. On medium T, buds showed a tuberized aspect and no leaves or roots were produced. In conclusion, medium EBR proved superior to the three other media tested based on the parameters tested.

Key words: Yam, *Dioscorea trifida*, shoot tip growth, 6-benzylaminopurine, 24-Epibrassinolide, meta-topolin, zeatin riboside.

INTRODUCTION

Yam, a multi-species, polyploid and vegetatively propagated crop, is an economically important staple food for more than 300 million people in low income, food-deficit countries of the tropics (Gedil and Sartie, 2010). Out of the more than 600 yam species identified, 10 are generally cultivated as food crop including *Dioscorea alata, D. rotundata, D. cayenensis, D. bulbifera, D. esculenta, D. opposita-japonica, D. nummularia, D. pentaphylla, D. transversa* and *D. trifida*. *D. trifida* L. (cush-cush yam) is believed to originate from the Guyana region of South America; it is by far the most important of the indigenous American yams.

Because of its vegetative mode of propagation, the field gene bank is the traditional *ex situ* method for conserving yam germplasm (Ng and Ng, 1999). However, such collections remain exposed to attacks by pests and pathogens, both in the field and during tuber storage, and viruses can accumulate progressively in plants, leading to significant yield decreases and ultimately to loss of accessions. Moreover, field gene banks are very costly to maintain properly, requiring large land areas, high input and labor resources (Ng and Ng, 1999).

In the case of yam, *in vitro* culture techniques have been developed for virus eradication by means of

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meristem culture (Mantell et al., 1980), multiplication (Mantell et al., 1978), distribution, and conservation of germplasm using *in vitro* plantlets and microtubers (Ng and Ng, 1999). For yam medium-term storage, *in vitro* slow growth protocols have been developed, which have been applied to several thousand accessions (Dumet et al., 2013). For long-term conservation of vegetatively propagated plants such as yam, cryopreservation (liquid nitrogen, -196°C) is currently is the only safe and cost-effective option (Engelmann, 2004). Indeed, at this temperature, all cell divisions stop and metabolism comes to a halt. Explants can thus be conserved for extended periods (several thousand years) without modification or alteration, sheltered from contamination, in a limited volume and with reduced maintenance.

Cryopreservation protocols have been developed for *D. alata*, *D. cayenensis* and *D. rotundata* shoot tips using different techniques including vitrification (Mandal et al., 2008; Mukherjee et al., 2009; Leunufna and Keller, 2005), encapsulation-dehydration (Mandal et al., 1996; Malaurie et al., 1998; Mandal et al., 2008) and droplet-vitrification (Leunufna and Keller, 2005). A droplet-vitrification protocol jointly established by the International Institute of Tropical Agriculture (IITA, Ibadan, Nigeria) and our laboratory has been applied to a total of 42 *D. cayenensis*, *D. rotundata*, *D. alata*, *D. bulbifera* and *D. mangotiana* accessions, with an average recovery of 29% (Gueye et al., unpublished results). Cryopreservation experiments performed in IRD Montpellier with *D. trifida* shoot tips showed that, even though positive results were obtained with droplet-vitrification, no reproducible protocol was yet available for this species (Engelmann-Sylvestre et al. unpublished results). In all these reports, the authors mentioned that if high survival could be consistently achieved, regeneration of whole plantlets from cryopreserved shoot tips was highly variable, depending on the species and the technique used, and was generally much lower than survival. An additional problem identified during regeneration of cryopreserved yam shoot tips was the occurrence of severe oxidation phenomena, which had a strong negative impact on re-growth.

Modifying the hormonal content of culture media can have a dramatic impact on re-growth of plant material (George, 1993). Among the numerous plant growth regulators (PGRs) identified, cytokinins, including natural ones such as zeatin (Z) or zeatin riboside (ZR), and synthetic cytokinin analogues such as 6-benzylaminopurine (BAP) have been broadly used for decades for their stimulatory effect on cell division, adventitious shoot formation and axillary shoot proli-feration (George, 1993). Topolins, another category of naturally occurring aromatic cytokinins, and especially the meta-topolin and its derivatives, have been employed for culture initiation, protocol optimization and for counteracting various *in vitro* induced physiological disorders in many species (Amoo et al., 2011; Aremu et al., 2012). Topolins have been reported to increase shoot multiplication, maintain histogenic stability, improve rooting efficiency and subsequently reduce production costs (Bogaert et al., 2006). Of more recent use in *in vitro* culture are brassinosteroids (BRs), a class of plant steroid hormones, which possess significant growth-controlling activity, and are involved in the promotion of cell elongation, cell division, differentiation, disease resistance, stress tolerance, and senescence throughout the plant life cycle (Bajguz and Hayat, 2009). BRs have been reported to help modulating the plant antioxidant defense system and thus scavenging the free radicals and help the plant protecting itself from oxidative stress (Verma et al., 2012). BRs have also been found to have an activity *in vitro.* They were reported to increase the rate of cell division and colony formation of Chinese cabbage mesophyll protoplasts (Nakajima et al., 1996) and *Petunia hybrida* protoplasts (Oh and Clouse, 1998). BRs proved also to be essential for the differentiation of isolated *Zinnia* mesophyll cells into tracheary elements (Iwasaki and Shibaoka, 1991) and in the morphogenesis of *Arabidopsis* (Li et al., 1996).

In this study, we compared the effect of four culture media on re-growth of *D. trifida* shoot tips: 1) the recovery medium used in the droplet-vitrification protocol developed in IRD, which contained BAP, naphtalene acetic acid (NAA) and gibberellic acid (GA_3); 2) a medium containing topolin and GA_3; 3) a medium containing Z and GA_3; and 4) a medium containing 24-epibrassinolide and GA_3. Our objective was to study if these changes in PGR content in the recovery medium were effective in stimulating shoot tip re-growth and in reducing oxidation.

MATERIALS AND METHODS

This study was performed using *in vitro* shoot cultures of *D. trifida* accession N° 278, provided by the Institut National de la Recherche Agronomique (INRA) Guadeloupe, French West Indies. Mother-plants were sub-cultured every 3 to 5 months on medium containing Murashige and Skoog (MS, 1962) basal salts and vitamins, 3% sucrose, 0.2% activated charcoal and 0.7% agar. The pH was adjusted to 5.8 ± 0.1 and the medium was autoclaved for 20 min at 120°C. Cultures were maintained at 27 ± 1°C under a 12 h light/12 h dark photoperiod and a light intensity of 50 µmol m^{-2} s^{-1}.

Single node cultures were transferred to yam multiplication medium (YMM) consisting of MS basal salts and vitamins (Murashige and Skoog, 1962), 0.476 µM KIN, 0.164 µM L-cysteine, 3% sucrose and 0.7% agar. After 3 weeks, shoot tips (1-2 mm in length) were excised under the binocular microscope and transferred on four different culture media for re-growth.

The first medium was the standard yam meristem medium (medium S) used for shoot tip regeneration after cryopreservation, which consisted of MS mineral salts and vitamins (Murashige and Skoog, 1962), 0.164 µM L-cysteine, 0.22 mM adenine hemisulfate, 0.6 µM BAP, 1.07 µM NAA, 0.23 µM GA_3 (filter-sterilized), 3% sucrose and 0.7% agar. In the other three media, BAP and NAA were replaced by 0.1 µM 24-Epibrassinolide (Sigma-Aldrich ref. E1641, filter-sterilized, medium EBR); 25 µM meta-topolin (Duchefa Biochemie ref. T0941, filter-sterilized, medium T); or 25 µM zeatin riboside (trans-isomer, Duchefa Biochemie ref. Z0937, filter-sterilized, medium ZR).

Table 1. Effect of culture medium on shoot length and oxidation level after 1 month and on shoot length, oxidation level, number of leaves and roots produced per shoot at 2 months.

Medium	1 month		2 months			
	Shoot (mm)	Oxidation	Shoot (mm)	Oxidation	Leaves/ shoot	Roots/ shoot
S	2.93^{b}	1.00^{a}	9.70^{b}	2.40^{a}	1.30^{c}	0.20^{b}
EBR	5.50^{a}	0.17^{c}	11.63^{a}	1.63^{b}	1.93^{a}	0.70^{a}
T	1.30^{c}	0.90^{ab}	3.07^{c}	2.17^{a}	0.00^{d}	0.00^{b}
ZR	4.77^{a}	0.67^{b}	11.30^{ab}	2.30^{a}	1.60^{b}	0.00^{b}

S: 0.6 µM BAP + 1.07 µM NAA + 0.23 µM GA_3; EBR: 0.1 µM24-epibrassinolide + 0.23 µM GA_3; T: 25 µM meta-topolin + 0.23 µM GA_3; ZR 25 µM zeatin riboside + 0.23 µM GA_3. In columns, figures followed by different letters are significantly different ($p < 0.05$).

Shoot tips were kept in the dark for 1 week, and then transferred to the culture conditions employed for mother-plants.

Observations performed and statistical analysis of results

The experiment was performed once, with three replicates of 10 shoot tips per experimental condition. After 1 and 2 months of culture on medium S, EBR, T or ZR, the size of shoot tips (mm) was measured and the oxidation level was evaluated using a scale from 0 (no oxidation) to 3 (very high oxidation). One-way ANOVA was performed to compare the growth of shoot tips and oxidation levels on the four media tested. Means were statistically differentiated using Duncan test at a significance level of $p \leq 0.05$.

RESULTS

After one month in culture, growth of shoot tips was highest on medium EBR and ZR, with shoot tips lengths of 5.50 and 4.77 mm, respectively, intermediate on medium S (2.93 mm) and lowest on medium T (1.30 mm) (Table 1). Oxidation was highest on medium S and T, with values of 1.00 and 0.90, respectively, intermediate on medium ZR (0.67) and lowest on medium EBR (0.17).

After 2 months in culture, shoot length was highest on medium EBR and ZR, with values of 11.63 and 11.30 mm, respectively, intermediate on medium S (9.70 mm) and lowest on medium T (3.07 mm). Oxidation reached a similar level on medium S, T and ZR (2.17 - 2.40) while it was only 1.63 on medium EBR. Well-developed shoots were obtained on medium EBR with an average of 1.93 leaves and 0.70 roots per shoot (Figure 1A, Table 1). Shoots were less developed on medium S and ZR, with an average of 1.30 leaves and 0.20 roots per shoot on medium S (Figure 1B, Table 1) and 1.60 leaves and 0.00 roots per shoot on medium ZR (Figure 1C, Table 1). On medium T, buds showed a tuberized aspect and no leaves or roots were produced (Figure 1D, Table 1).

DISCUSSION

These experiments showed that the hormonal content of the shoot tip culture medium had a strong impact on their growth pattern. Medium EBR, which included 24-epibrassinolide, led to the production of well-developed shoots with leaves and roots and strongly limited oxidation. This result illustrated the role of BRs in stimulating the plant antioxidant system (Verma et al., 2012) and showed for the first time the positive effect of 24-epibrassinolide on shoot tip morphogenesis. Media S and ZR produced slightly shorter shoots with a lower number of leaves per shoot and very few (medium S) or no roots (medium ZR) and a higher oxidation level. The lower results obtained with two media may be due either to the non-optimal concentrations of the PGRs they contained and/or to their lower effect on shoot tip morphogenesis and reduction of oxidation, compared to medium EBR. Finally, medium T had a detrimental effect on the development of shoot tips, as it induced the production of tuberized shoots and led to an oxidation level comparable to that noted with media S and ZR. Despite their positive effect on shoot regeneration which has been observed with many species (Aremu et al., 2012), Bogaert et al. (2006) noted the unfavorable effect of topolin on shoot regeneration and multiplication of *Rosa* hybrid cultures. The induction of tuberization in shoot tips by their exposure to meta-topolin may be of interest for medium-term storage and international exchange of yam germplasm in the form of microtubers.

In conclusion, experiments should now be performed to observe if medium EBR, which provided the best results with control shoot tips, has also a beneficial effect on growth recovery of cryopreserved shoot tips of *D. trifida* accession N° 278. The effect of this medium should also be tested on the regeneration of other *D. trifida* accessions and of other *Dioscorea* species.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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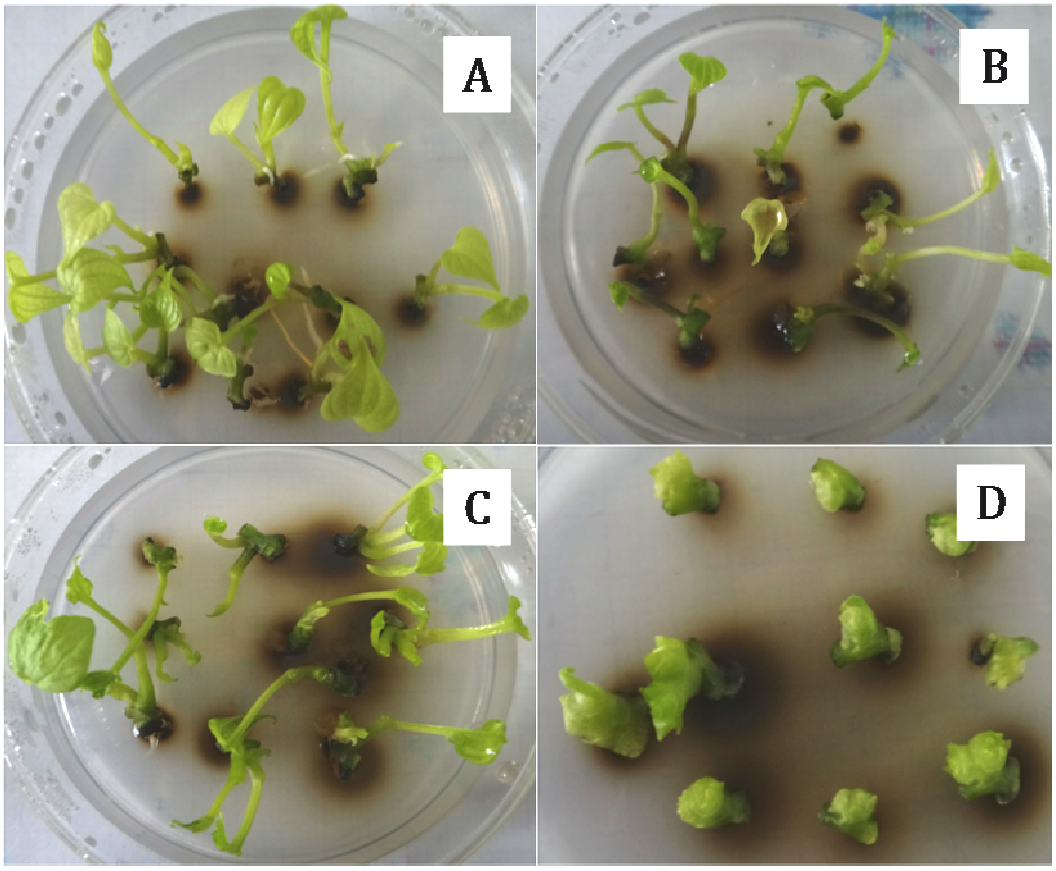

Figure 1. Effect of culture medium on shoot development after 2 months. A: medium EBR (0.1 µM24-epibrassinolide + 0.23 µM GA_3); B: medium S (0.6 µM BAP + 1.07 µM NAA + 0.23 µM GA_3); C: medium ZR (25 µM zeatin riboside + 0.23 µM GA_3); D: medium T (25 µM meta-topolin + 0.23 µM GA_3).

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