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In vitro propagation of yam via nodal segment culture

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Yam is common non-traditional expensive foods in Egypt that commercially propagates by tubers. To overcome long dormancy problem and obtain free disease tubers, *in vitro* culture techniques are commonly applied. Therefore, a sufficient protocol for *in vitro* plantlet regeneration of *Dioscorea rotundata* using nodal segment culture has been used. Different concentrations of BA alone or in combination with 0.5 mg/l of NAA have been tested to alter proliferation and regeneration of nodal cutting excised from *D. rotundata* micro-shoots during 2016 and 2017 seasons. Our results indicate that supplying MS medium with 2mg/l of BA +0.5mg/l of NAA presented the highest survival ratio (92.3 %)if compared to other shoot induction treatments. Moreover, better number of leaves and nodes were obtained with1.5 mg/l BA+ 0.5 mg/l NAA treatment. On contrast, maximum leaves area and number of roots were obtained with MS basal medium. For root induction, *in vitro* shoots resulted from BA (1.5 mg/l) and 0.5mg/l NAA treatment were excised and cultured on MS basal medium supplemented individually with IAA, IBA and NAA at 0.5 mg/l. Our results reveal that BA (1.5 mg/l) + (0.5 mg/l) NAA alters *D. rotundata* shoot induction if compared to other treatments. However, culturing shoots of *D. rotundata* on 0.5 mg/l NAA found to increase root formation for commercial acclimatization processes.

Keywords: Yam, growth regulators, nodal segment, regeneration, *in vitro* culture

INTRODUCTION

*Dioscorea rotundata,*belongs to the *Dioscorea* genus that includes 600 species, is known as yam (Huber, 1998).In semi-arid regions, yam has two major challenges limiting its sufficient production, long dormancy (Ile et al. 2006) and lack uniform of germinated seeds(Donnelly et al. 2003). Traditionally, yam propagates by tubers (Aighewi et al. 2015). Thus, pests and pathogens would contaminate tubers across propagation procedure (Lebot, 2009). Recently, *in vitro* culture technique proved to overcome problems of dormancy, virus and fungal diseases (Das et al. 2013), which provide yam market with enough tubers for sustainable production and adequate yield for human consumption as well. Many researchers successfully propagated yam *in vitro* using nodal segment as explants (Jova et al. 2011 and Asha et al. 2016). Furthermore, plant growth regulators have positive effect on initiation of aseptic culture (Adeniyi et al. 2008). In this respect, Obssi et al (2015) recorded the highest shoot induction of *D. japonica* with medium contains mixture of 0.2µM BA + 0.1µM NAA. Notably, increasing BA concentration up to 0.44 µM resulted in higher number of nodes, shoots and fresh weight of Japanese yam (Kadota and Niimi 2004). In connection, Mahesh et al. (2010) observed better shoot formation of *D. wighti* with all tested growth regulators of BA, Kinetin and 2iP. For rapid *in vitro* propagation, Chen et al. (2003) developed a protocol for *D.zingiberensis* using stem as explants. The authors reported that 4.4µM BA+1.1µMNAA accelerated shoots production from nodal segments within 20 days, while4.9µM IBA found to promote callus and root initiation.

Poornima and Ravishankar (2007) used nodal segments to propagate *D. oppositifolia* and *D. pentaphylla*. The authors observed multiple shooting on MS medium supplied with 8.8 µM BA and 0.3% activated charcoal, while rooting was detected in MS medium supplemented with 2.67 µM IBA. To regenerate plantlet of *D. oppositifolia,* Behera et al. (2009) cultured nodal segment on MS medium supplemented with BA and NAA. The best shoot proliferation was observed in MS + 2mg/l kinetin + 1.0mg/l BA + 0.5mg/l NAA + 100 mg/L ascorbic acid, whereas half strength MS supplemented with 2.0mg/l NAA found to improve root formation. The D-571 medium, supplied with 1.5% manitol +1mg/l BA + 2g/l activated charcoal, showed 100% regeneration of *D. rotudata* explants. This study provides a suitable protocol for *in vitro* plantlet regeneration of *D. rotundata* using nodal segment culture. This work provides large-scale free-disease plantlets to meet purpose of yam conservation and domestication under Egyptian conditions.

MATERIALS AND METHODS

Plant materials

Mature *Dioscorea rotundata* tubers were obtained from the Federal Ministry of Agriculture and Rural Development (FMARD), Nigeria. Each tuber was divided into five fragments and cleaned under running tap water (Fig.1 A). Fragments were cultured in plastic pots contain sterile peatmoss. One month later, apex was differentiated into stem includes 10-16 nodes. Node cuttings were cut from stems for sterilization. Briefly, cuttings washed in tap water and liquid soap, immersed in 70% ethanol for 1 min and surface sterilized in 5% of NaOCl contains two drops of Tween 20 for 15-20 minutes, with continuous agitation (Fig1.B). Growing medium was adjusted to 5.8 pH and autoclaved at 121°C for 20 min. Cultures were incubated at 25°C and 3000 Lux 16/8 h light/dark for two months.

Fig. 1. Tubers of *D. rotundata* (left) and sterilized nodal segments (right).

Shoot and root proliferation

Nodal segment was cultured in MS (Murashige and Skoog, 1962) supplemented with different concentrations of a-naphthalene acetic acid (NAA) and benzylaminopurine (BA). Activated charcoal (AC) 2 g/l was added to prevent browning. For shoot induction, single application of BA was applied to MS medium at 0.5 and 1mg/l. In addition, 0.5mg/l NAA combined with 0.5, 1, 1.5 and 2mg/l BA were tested. Free hormone MS medium was used as control. Survival ratio, shoot length, number of leaves and roots, root length per explant and fresh weight of plantlet were recorded at 60 days after shoot initiation. For root induction, nodal cuttings of *in vitro D. rotundata* were cultured on MS supplemented with single concentration (0.5 mg/l) of IAA, IBA and NAA. One month after sub-culturing, rooting ratio, root length and number per explants were recorded.

Acclimatization

Single rooted plantlets were transferred to sterile poly pot (2.5 cm) filled with peat moss+ vermiculite (1:1). Pots were placed in growth room at 28°C with 70-80% relative humidity. After 3 weeks, shoots were shifted to larger pots (8 cm) contain mixture of soil and vermiculite (1:1). For acclimatization, pots were kept at plastic house for 3 weeks. Plants were irrigated with ¼ MS solution 3 days intervals.

Statistical Analysis

Factorial complete randomized design with three replications was used for data analysis. Means were compared by least significant difference (L.S.D) according to Snedecor and Cochran (1994).

RESULTS

Analysis of morphology of micro propagated shoots

This study was carried to establish an optimal regeneration protocol of *D. rotundata* via nodal segment culture using different concentrations of BA and NAA at lab of department of vegetable crops, Faculty of Agriculture, Cairo University. Different growing medium were formed to asses survival ratio, plant height, number of leaves, total leaf area, number of nodes and roots as well as root length of *D. rotundata*. As shown in Table 1, the highest survival ratios 89.8 and 92.3 % were obtained with 1.5 and 2m/l BA +0.5mg/l NAA, respectively. Regarding plant height, 1mg/l BA + 0.5mg/l NAA produced taller plantlets with longer internodes than other concentrations. Maximum number of leaves and nodes were recorded with 1.5m/l BA +0.5mg/l NAA. On contrast, control revealed higher leaf area and number of roots if compared to other treatments. In addition, minimum single concentration of BA (0.5 mg/l) presented the longest roots.

Table 1.Effect of different BA concentrations on shoot proliferation of *D. rotundata*

Concentration	Survival %	Plant height (cm)	Number of leaves	Leaves area	Number of nodes	Number of roots	Root length (cm)
Control	35.0 d	8.2 b	6.8 d	14.1 a	4.7 d	5.0 a	0.7 b
0.5mg/l BA	66.3c	4.5 de	8.1 bc	11.4 b	4.9 d	4.0 b	2.2 a
1mg/l BA	73.4 b	5.4 cd	8.8 b	6.6 c	8.0 b	0.0 c	0.0 c
0.5mg/l BA+0.5 mg/l NAA	62.2 c	8.1 b	6.4 b	7.5 c	8.3 b	0.0 c	0.0 c
1mg/l BA+0.5 mg/l NAA	73.6 b	13.7 a	8.5 b	5.2 d	6.3 c	0.0 c	0.0 c
1.5mg/l BA+0.5 mg/l NAA	89.8 a	6.1 b	11.2 a	3.9 e	10.0 a	0.0 c	0.0 c
2mg/l BA+0.5 mg/l NAA	92.27 a	3.50 e	9.04 b	3.50 e	8.30 b	0.00 c	0.00 c
L.S.D	6.46	1.33	0.84	1.07	1.45	0.91	0.54

*Values marked with the same letter (s) are statistically similar using Revised L.S.D test at P = 0.0

Table 2. Effect of NAA, IAA and IBA on root proliferation of *D. rotundata* resulted from 1.5mg/l BA+0.5 mg/l NAA treatment

Concentration (mg/l)	Rooting %	Root length (cm)	Number of roots
Control	83.3 b	2.9 b	3.6 b
0.5 IBA	80.8 b	4.3 b	3.9 b
0.5 IAA	93.3 a	3.0 b	2.5 b
0.5 NAA	90.2 a	8.9 a	8.5 a
L.S.D	3.21	1.57	2.10

*Values marked with the same letter (s) are statistically similar using Revised L.S.D test at P = 0.05

Fig.2. Regenerated *D. rotundata* plantlets resulted from single node cutting under acclimatization conditions (left - middle), and 30 days old shoot grown under filed conditions (right)

Root initiation

For rooting experiment, well-developed shoots, resulted from 1.5mg/l BA+0.5 mg/l NAA were excised from the shoot clumps and transferred to MS media+2 g/l (AC) containing 0.5 mg/l of IBA, IAA or NAA, individually. Rooting percentage presented the highest significant increase 93.3 and 90.2 % with IAA and NAA, respectively, when compared to control. Moreover, number of roots and root length showed significant increase with NAA compared to other treatments (Table. 2).

Acclimatization and field establishment

Rooted plantlets were transferred to the greenhouse within 3-4 weeks. Plants reached 8 - 10 cm height within 6 weeks (Fig. 2). The acclimatized plants were established in field condition without any morphological variation

DISCUSSION

Success of tissue cultural much depends on the selected growth regulators in culture medium. Nevertheless, survival and multiplication ratio are a key factors that determine best concentration of chosen growth regulators. In particular, root and shoot initiation, callus formation and differentiation are closely regulated by the relative concentration of Auxins and Cytokinin in the growth medium (OndoOvono et al. 2009). In this work, different concentrations of BA found to affect regeneration and development of *D. rotundata*(Tab.1). Lower single application of BA (0.5 or 1mg/l) alone or in combination with 0.5 1mg/l NAA to MS medium stimulated *D. rotundata* survival ratio if compared to MS free medium. Likewise, 0.5 mg/l of BAP reported to increase number of leaves of two *Dioscorea* varieties, *Kounondakou* and *Gnon-boya*. (Ahanhanzo et al. 2010). However, the capability of BA to break bud dormancy and stimulate shoot multiplication has been reported in micro propagated *D. batatas* (Koda and Kikuta, 1991), *D.composita* (Alizadeh et al. 1998), *D. flo-ribunda* (Borthakur and Singh, 2002) and *D.opositifolia* (Behera et al. 2009). Moreover, mixing 0.5mg/l of NAA with higher BA concentrations (1.5 and 2 mg/l) increased survival ratio up to 89.9 and 92.3 %, respectively. Whereas, a comparatively lower response was recoded when BA was added alone in the medium. In connection, Ramierez-Magon et al. (2001) reported that combinations of BAP and IAA increased shoot multiplication of *Spathiphyllum floribundam* from 1.8 to 11.6 shoots per explant. Furthermore, combination in the range of 0.5-2.0 mg/l BA and 0.5-2.0 mg/l NAA has been reported to improve micro-tuber induction of *D. nipponica* Makino (Chen et al. 2007). In this respect, it can be concluded that interaction of BA and NAA

plays important role for *in vitro* propagation of nodal explant for multiple shoot induction. Therefore, Chen et al. (2003) reported that supplementing MS medium with 1.0 mg/l NAA and 0.5-1.0 mg/l BA was the best concentration for multiple shoot bud induction in *D. opposita*. Similarly, Adeniyi et al. (2008) indicates that addition of NAA in the culture medium improves the response of *D. rotundata*in terms of shoot growth. The author found that shoot regeneration was increased up to 75% with 0.1 µM NAA + 0.20 µM BAP. Data in Tab. 1 shows an increase in number of leaves and nodes up to 11.2 and 10.0, respectively, per plantlet at combination of1.5 and 2.0mg/l BA+0.5mg/l NAA, respectively, if compared to lower BA concentrations. Meanwhile, 1mg/l BA+0.5mg/l NAA was more efficient to produce taller plants than 1.5 or 2.0 mg/l BA concentrations (13.7, 6.1 and 3.5 cm), respectively. This results are in harmony with Yan et al. (2011) who reported significant higher shoot length of *D. fordii* in MS basal medium supplemented with 1.0 mg/ l BAP, 0.1 mg /l NAA, 30 g/ l sucrose and 1.5 g /l AC (activated charcoal). On contrast, both free MS medium and the minimum BA concentration (0.5 mg/l) reveal better leaf area 14.1 and 11.4 cm, respectively. Meanwhile, adding0.5mg/l NAA to different BA concentrations didn't affect root length or number. While, single applications of BA (0.5mg/l) altered root morphology than MS control. Regenerating yam shoots with higher number of roots is an important factor that affects acclimatization stage. To address which growth regulator is preferred for rooting under our condition. Plantlets resulted from 1.5 mg/l BA +0.5mg/l NAA were treated with three different individual hormones at 0.5 mg/l. Results in Tab. 2 show that addition of NAA was the best growth regulator for root formation. Behera et al. (2008) got better rooting of *in vitro Dhinjilicatu* raised shoot-lets with ½ strength MS basal medium + 2mg/l NAA+ 2g/l AC. For rooting, Poornima and Ravishankar (2007) reported the most efficient rooting of *D. oppositifolia* and *D. pentaphylla* at MS medium supplied with 2.67µM NAA after 30 days.

CONCLUSION

This protocol recommends using 1.5 mg/l BA of BA to break *D. rotundata* dormancy and stimulate shoot induction, obtains maximum node and subsequent cut nodal segment. To improve root formation for acclimatization, shoots were cultured on media supplemented with 0.5 mg/l NAA.

CONFLICT OF INTEREST

The present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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