Pak. J. Bot., 47(2): 665-668, 2015.

A SIMPLE METHOD FOR MICROTUBER PRODUCTION IN *DIOSCOREA OPPOSITA* USING SINGLE NODAL SEGMENTS

MINGJUN LI[1, 2, 4, *], JUNHUA LI[1, 2, 4, *], YIPENG WANG[1], WEN LIU[1], XIAOBO GUO[1], SHUJIE LI[1], LINLIN HAN[1], ZHIHUI SONG[1], XITING ZHAO[1, 2, 4] AND QINGXIANG YANG[1, 3]

[1]College of Life Sciences, Henan Normal University, Xinxiang 453007, China
[2]Engineering Technology Research Center of Nursing and Utilization of Genuine Chinese Crude Drugs, University of Henan Province, Xinxiang 453007, China
[3]Key Laboratory for Microorganisms and Functional Molecules, University of Henan Province, Xinxiang 453007, China
[4]Engineering Laboratory of Green Medicinal Material Biotechnology, Henan Province, Xinxiang 453007, China
[*]Corresponding author e-mail: Mingjun Li, 041013@htu.cn; Junhua Li, lijh0909@gmail.com; Tel.: +86 0373 3328189; Fax: +86 0373 3328189

Abstract

Dioscorea opposita Thunb. (Chinese yam) is an important tuber crop in East Asia because of its dual benefits edible and medicinal properties. Microtubers may provide a feasible alternative to *in-vitro*-grown plantlets as a means of micropropagation and a way to exchange healthy planting material. In this study, we have developed a simplified culture method for *In vitro* production of microtubers from *D. opposita* cv. 'Tiegun'. In this method, microtubers formed in 98% of the internodes of single nodal segments after four weeks of dark-incubation when cultured in MS medium supplemented with 60 g sucrose l^{-1} with shaking. Anatomical observations strongly supported the process of tuberization. We also found that 66% of the microtubers produced *In vitro* sprouted two months after transfer to vermiculite. The protocol presented here provides a simple model for studying the physiological, biochemical, and molecular mechanisms of tuberization in *D. opposita*, and shows good potential for large-scale production of microtubers as well.

Key words: *Dioscorea opposita*; Liquid medium; Microtuber; Shaking culture; Single nodal segments.

Introduction

Yams (*Dioscorea* spp.) are an important tuberous crop in many countries, especially in West Africa, East and South Asia, and the Caribbean. Tiegun yam (*D. opposita* cv. 'Tiegun') is a well known but rare type of *D. opposita* that has a long history of domestication in special areas such as Wenxian, Wuzhi, etc. along the Yellow River in China. Tiegun yam has been used both as a source of food and also as a top grade traditional medicine. Viral disease infection is the main cause of cultivar degradation in *D. opposita*, and significantly decreases the yield (Li *et al*., 2003). Successful studies were conducted to produce viral free plants (Ahmed *et al*., 2011; Hussain *et al*., 2011). Previously, we have performed studies on techniques for detoxification and rapid propagation of *D. opposita* (Li, 2004). However, *In vitro* virus-free plantlets are not convenient for handling, storage, or transport, and poor field establishment of *In vitro*-grown plantlets has restricted the implementation of *D. opposita* tissue culture techniques. Therefore, a feasible alternative to *In vitro*-grown plantlets is required for sustainable propagation of *D. opposita* as done elsewhere for such purposes (Abbassi *et al*., 2011; Khan *et al*., 2014).

Microtubers are *In vitro*-derived small storage organs that are superior to shoots for mass propagation because they are easily acclimated, stored, and transported. Microtubers also have considerable advantages in the commercial production of virus-free germplasm.

There have been several successful efforts in microtuber production in other yam species, such as in two clones of *D. alata* cultured in a temporary immersion system (Jova *et al.*, 2005), and in *D. opposita* by using a drum type bioreactor (Akita & Ohta, 2002). The induction of microtubers in Tiegun yam is challenging, possibly due to the large genetic difference between Tiegun yam and other yam cultivars (our unpublished data) and a poor growth ability of Tiegun yam (Wang *et al.*, 2005). Recently, we have developed a two-step culture process for production of microtubers in *D. opposita*, by which large numbers of microtubers (2-3 microtubers per plantlet) can be achieved (Li *et al.*, 2014). Although this protocol shows great potential for industrial production of microtubers, it is not suitable for studying the mechanisms of microtuber formation due to the uncertainty of the time and position of the microtuber formation in the yam plantlets. Therefore, it is important to establish a more accurate model in which the time and positions of microtuber formation can be controlled. In this study, a simple method was developed for microtuber formation which can be used to investigate the mechanisms underlying this process in *D. opposita*.

Materials and Methods

The *D. opposita* cv. 'Tiegun' (which originated from Wen County, Henan Province) was used for the establishment of an *In vitro* microtuber production protocol. Microbe-free plants were grown in plant tissue culture jars (240 ml) with plastic caps containing 50 ml Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) supplemented with 0.005 mg L^{-1} thidiazuron and 30 g L^{-1} sucrose. The pH of the culture medium was adjusted to 5.6-5.8 prior autoclaving for 20

min at 121°C. Cultures were maintained at 60 $\mu E \cdot s^{-1} \cdot m^{-2}$ at 29 ± 1°C with a 14 h light/10 h dark photoperiod. Subcultures were performed every 30 d, during which single nodal segments with petioles, but without leaves, were excised from plantlets and transferred to 240 ml jars (12-15 nodal segments per jar), and were sub cultured onto fresh medium.

For microtuber production, single nodal segments (2 cm in length) were excised from healthy plants and transferred to 240 ml jars containing 50 ml MS basal medium supplemented with 60 g L^{-1} sucrose. Each jar was inoculated with 12 single nodal segments. Cultures were incubated in the dark either under stationary conditions, or in a rotary shaking incubator (ZWY-2102, Zhicheng, Shanghai, China) at 29 ± 1°C, 120 rpm. Each treatment consisted of twelve jars, with three replications per treatment. Microtubers larger than 3 mm in diameter were defined as valid microtubers.

Histological sections were prepared according to a method described previously (Iranbakhsh *et al.*, 2007). The sections were stained with 1% safranine for 1 hour and 1% fast green for 30 seconds at room temperature. Excess dye was rinsed off with ethanol. Photographs were taken on Zeiss Axioskop 40 microscope (Carl Zeiss, Jena, Germany) for Fig. 3a-3e, and an Olympus SZ61 stereomicroscope (Olympus, Tokyo, Japan) for Fig. 3f and 3g.

Results and Discussion

Under our culture conditions, etiolated axillary buds sprouted from the nodal segments in week one (Fig. 1b), and further developed into etiolated shoots (1-2 per nodal segment) in the following two weeks (Fig. 1c). In the fourth week, obvious tuber tissue was observed emerging from the primary internodes (Fig. 1d). Six weeks after inoculation, the microtubers reached an average diameter of 0.61 cm (Figs. 1f and Fig. 2). In all of the tests, ~98% of the explants formed microtubers. Table 1 shows that tuberization was clearly stimulated by rotary shaking in comparison to the static cultures, and this effect was seen in both in the light and in the dark.

To confirm the formation of microtubers under our induction conditions, we examined the anatomical structure of the leaf-axil region at several time points during tuber induction (Fig. 3). The results showed that microtubers formed from the auxiliary buds under the induction conditions (Fig. 3a-3f). Starch grains accumulated in the tuber matrix as indicated by the red structures after staining with safranine (Fig. 3g). The positions from which the microtubers formed and the structures of the microtubers observed in this study were consistent with results previously reported by Iranbakhsh *et al*. (2007). After transfer from culture jars to vermiculite, 66% of the microtubers sprouted and developed into young plantlets in two months at room temperature (approximately 25°C).

Here we present a simple method for microtuber production in *D. opposita* using single nodal segments. In several previous reports, shoots were used as starting material for microtuber induction (Akita & Ohta, 2002; Jova *et al.*, 2005; Li *et al.*, 2014), and tuberization occurred in an unpredictable portion of internodes, on both aerial and submerged parts of plantlets. In the present study, microtubers were induced only at the internodes of single nodal segments (Fig. 3a-3f). Compared to our previously reported two-step method, this protocol was much simpler, and greatly reduced the incubation time (from 3 months to 4 weeks). At least 10 separate experiments confirmed that the time of microtuber formation was quite reliable and reproducible, i.e., obvious microtubers definitely appeared by day 28. Therefore, this model was very suitable for our underlying research on the molecular mechanisms behind the process of microtuber formation related to differences in gene expression and transcriptome profile.

An appropriate bioreactor is required to reduce the cost of mass propagation (Li, 2004). The method presented here used a common rotary shaking incubator, which is inexpensive and versatile in comparison with forced aeration that is usually included in bioreactor systems. Our result indicates that tuberization of *D. opposita* can be induced without forced aeration by modifying the bioreactor. The technique developed here should be easily adapted to other yam cultivars and tuberous crops, such as potato (*Solanum tuberosum*).

Acknowledgments

We are grateful for Dr. David Zaitlin and Dr. Muqiang Gao from University of Kentucky, USA for comments on the manuscript. This research was financially supported by the National Natural Science Foundation of China (NSFC 81274019), the Program for Innovative Research Teams (in Science and Technology) of the University of Henan Province (IRTSTHN, 13IRTSTHN009), the Innovation Scientists and Technicians Troop Construction Projects of Henan Province (114200510013), and the Young Scientist Fund of Henan Normal University (2013QK11).

Table 1. Comparison of culture conditions for tuberization in *Dioscorea opposita* cv. 'Tiegun'.

Culture conditions	14 d		28 d		42 d	
	Tuber formation (%)	Shoot formation (%)	Tuber formation (%)	Shoot formation (%)	Tuber formation (%)	Shoot formation (%)
Rotary shaking culture in the dark	0.0 a	100.0 a	98.1 a	100.0 a	98.1 a	100.0 a
Static culture in the dark	0.0 a	100.0 a	5.3 b	100.0 a	5.3 b	100.0 a
Static culture in light	0.0 a	100.0 a	47.4 c	100.0 a	57.2 c	100.0 a

Different lower-case letters in each column represent significant differences by Fisher's least significant difference (LSD) multiple comparisons ($p<0.05$), using Statistical Product and Service Solutions (SPSS) software (version 13.0) (Li *et al.*, 2013)

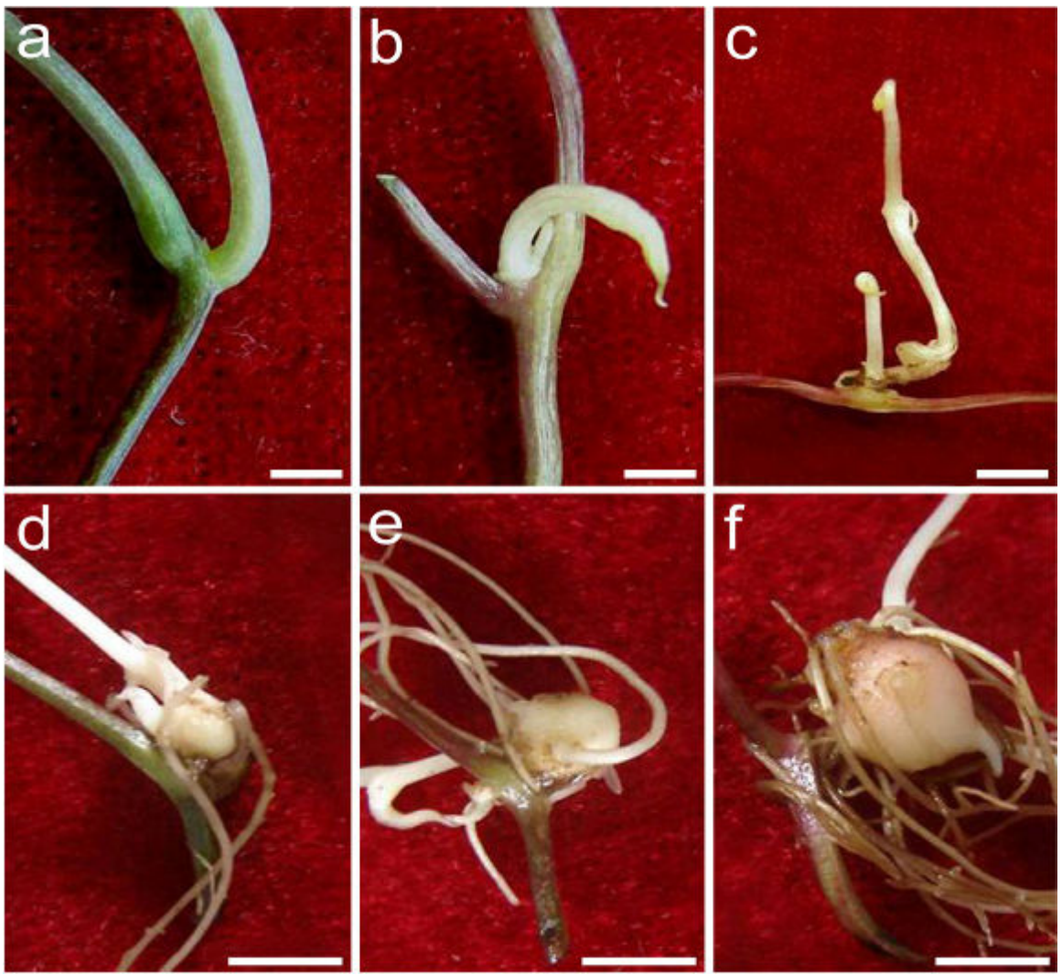

Fig. 1. Generation of microtubers in single nodal segment cultures of *D. Opposita* cv. 'Tiegun'.
a, initial nodal segment before inoculation; b, an etiolated axillary bud sprouted from the nodal segment after one week culture; c, axillary bud developed into etiolated shoots after three weeks culture; d, young spherical microtuber from the primary internode and adventitious roots from tuber tissue after four weeks; e, microtuber after five weeks culture; f, the microtubers with multiple adventitious roots after six weeks culture. Bars = 0.25 cm in a-b, 0.5 cm in c-f.

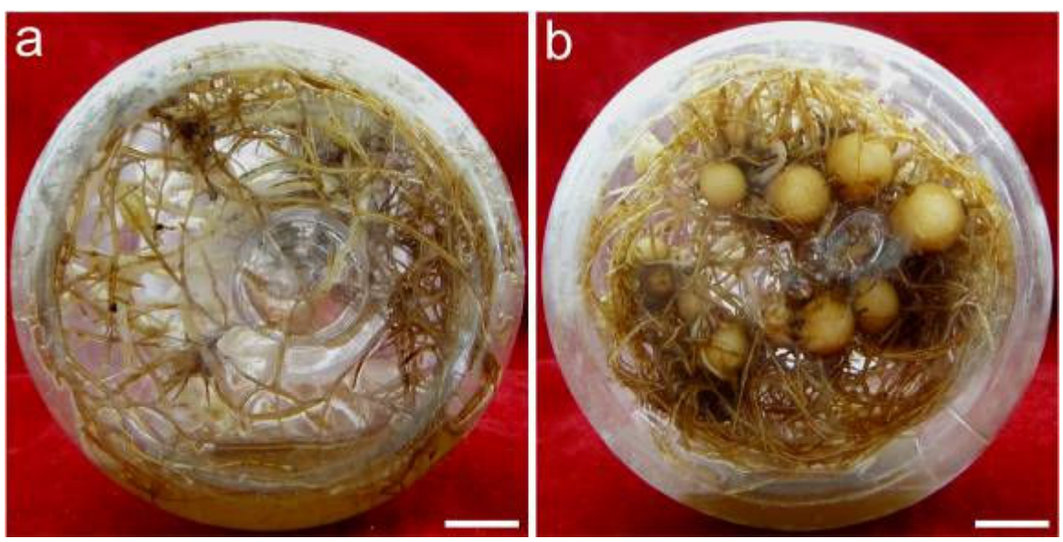

Fig. 2. Representative culture jars incubated in the dark under conditions for microtuber induction.
a, static culture. b, rotary shaking culture. The cultures were maintained for 42 d. Bars = 1 cm.

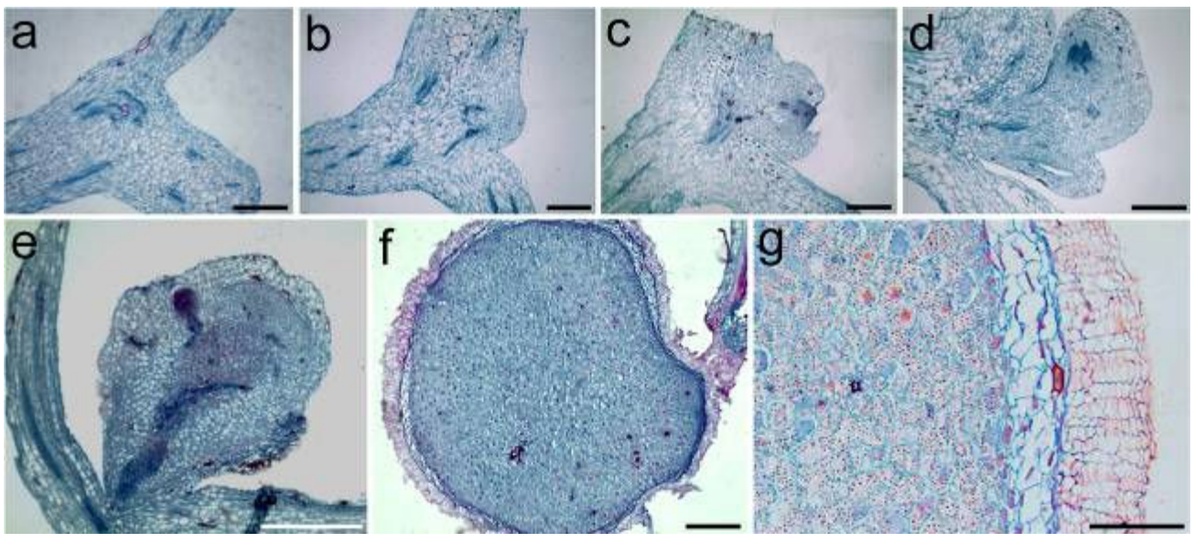

Fig. 3. Microscopic observations of nodal sections during microtuber induction and formation.
Tissue sections were prepared from nodal segments at 10 d (a), 15 d (b), 20 d (c), 25 d (d and e), and 42 d (f and g) after culture initiation and incubation with rotary shaking in the dark. Bars = 10 mm in A-F, 5 mm in g.

References

Abbasi, B.H., A. Rashid, M.A. Khan, M. Ali, Z.K. Shinwari, N. Ahmad and T. Mahmood. 2011. *In vitro* plant regeneration in *Sinapis alba* and evaluation of its radical scavenging activity. *Pak. J. Bot*., 43(Special Issue): 21-27.

Ahmad, N., H. Bin Guo, B.H. Fazal, H. Abbasi1, Chun-Zhao Liu, T. Mahmood and Z. K. Shinwari. 2011. Feasible plant regeneration in black pepper from petiole explants. *J. Medicinal Plants Res.*, 5(18): 4590-4595.

Akita, M. and Y. Ohta. 2002. A simple bioreactor system for production of storage organs of Chinese Yam (*Dioscorea opposita* Thunb.). *Plant Biotechnol.,* 19(5): 353-356.

Hussain, A., S. Naz, H. Nazir and Z.K. Shinwari. 2011. Tissue culture of black pepper (*Piper nigrum* L.) in Pakistan. *Pak. J. Bot*., 43(2): 1069-1078.

Iranbakhsh, A., M. Ebadi and G.B. Khaniki. 2007. The ontogenetic trends of microtuber formation in potato (*Solanum tuberosum* L.). *Pak. J. Biol. Sci.,* 10(6): 843-851.

Jova, M.C., R.G. Kosky, M.B. Pérez, A.S. Pino, V.M. Vega, J.L. Torres, A.R. Cabrera, M.G. García. and J.L.C. de Ventura. 2005. Production of yam microtubers using a temporary immersion system. *Plant Cell Tiss. Org. Cult.,* 83(1): 103-107.

Khan, M.A., B.H. Abbasi and Z.K. Shinwari. 2014. Thidiazuron enhanced regeneration and silymarin content in *Silybum marianum* L. *Pak. J. Bot*., 46(1):185-190.

Li, J., Y. Han, Q. Zhao, C. Li, Q. Xie, K. Chong and Y. Xu. 2013. The E3 ligase AtRDUF1 positively regulates salt stress responses in *Arabidopsis thaliana*. *PLoS One,* 8(8): e71078.

Li, M. 2004. *Tissue culture and application of Dioscorea opposita* Thunb. Science Press, Beijing.

Li, M., J. Li, W. Liu, L. Liu, J. Lu, J. Niu, X. Liu and Q. Yang. 2014. A protocol for *In vitro* production of microtubers in Chinese yam (*Dioscorea opposita*). *Biosci., Biotechnol., Biochem.,* 78(6): 1005-1009.

Li, M., F. Zhang, M. Chen and X. Yu. 2003. Study on virus disease of *Dioscorea opposita*. *Chinese Traditional and Herbal Drugs,* 34(22): U003-U005.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.,* 15(3): 473-497.

Wang, F., H. Liu, C. Lu, Z. Bai, T. Wang and B. Zhang. 2005. Comparison of the agronomical traits and nutrient quality among five yam germplasms. *J. Henan Agric. Sci.,* 3: 58-60.

(Received for publication 13 July 2013