INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19F–030/2019/22–4–693–696
DOI: 10.17957/IJAB/15.1117
http://www.fspublishers.org

Full Length Article

In Vitro Plant Regeneration and Hairy Roots Induction in *Dioscorea alata* (Cultivar Wenshanyao)

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Abstract

Wenshanyao is a special cultivar of yam (*Dioscorea alata* L.) cultured in Zhejiang Province in China with both culinary and medicinal values. In this study, the stem segments with leaf axils of yam cv. Wenshanyao were used as explants to induce callus in MS medium with 2 mg/L of 2,4-D. The induction rate reached up to 95%. The callus was cultured in MS+6-BA 2 mg/L+IAA 0.5 mg/L medium and differentiated into clustered buds with a differentiation rate of 86.7%. Buds were cultured in MS+1.0 mg/L IAA medium and regenerated whole plants, in which each root system had 1–4 or more roots. *Agrobacterium rhizogenes* K599 (with recombinant plasmid pRI101-AN-gfp) was used to infect the seedling stems of Wenshanyao and to obtain hairy roots, which were identified as transgenic hairy roots by PCR and fluorescence microscopy. The tissue-culture regenerated plants obtained in this study provided foundation for the genetic improvement of Wenshanyao varieties. The technology for obtaining transgenic hairy roots provided technical support for the industrialized production of medicinal ingredient of Wenshanyao using hairy root cultures. © 2019 Friends Science Publishers

Keywords: *Dioscorea alata*; Tissue culture; Regeneration; Transgenic hairy roots

Introduction

Dioscorea plants are distributed in tropical and temperate regions of the world. Derived from the genus *Dioscorea*, yam is an important group of economic plants. Yam has a history of more than 2,000 years of cultivation and application in China, with both culinary and medicinal values. Currently, there are mainly four Dioscorea plants cultivated in China, including *Dioscorea alata*, *D. opposita*, *D. persimilis* and *D. fordii*. Among *D. alata* cultivars, cv. Wenshanyao is a special yam in Zhejiang Province in China with a long history of cultivation. It has the effects of strengthening the spleen and stomach, as well as benefiting the lung and kidney (Zheng *et al*., 2004; Zheng, 2005; He *et al*., 2006; Wu *et al.*, 2010).

The traditional breeding method of Wenshanyao is vegetative reproduction, which not only consumes a large number of medicinal parts, but also causes variety degeneration and yield reduction due to long-term vegetative reproduction (Wu *et al.*, 2015). In the production of Wenshanyao, it becomes an urgent challenge to improve quality and increase yield. The tissue culture based rapid reproduction technology can lay the foundation for genetic improvement and breeding of new varieties (Canter *et al.*, 2005; Wang *et al.*, 2017). To tackle this challenge, *in vitro* propagation of Wenshanyao needs to be developed not only to maintain the excellent characteristics of Wenshanyao but also to mitigate the influence of external conditions, leading to the reproduction of a large number of seedlings in a short period of time.

On the other hand, when *Agrobacterium rhizogenes* infects plants, the T-DNA in the Ri plasmid carried by *A. rhizogenes* can be inserted and integrated into the genome of the plant cell, resulting in the generation of hairy root at the wound site of the infected plant cell (Giri and Narasu, 2000; Xiang *et al.*, 2016). Hairy roots can grow vigorously on a hormone-free medium and can be used as a bioreactor to produce plant secondary metabolites. Moreover, they can further regenerate whole plants (Banerjee *et al.*, 2012). In this study, we accomplished the regeneration of Wenshanyao plant *via* tissue culture, and the induced reproduction of hairy roots.

Materials and Methods

Materials

Wenshanyao tubers were kindly provided by Wenzhou Tianhe Biotechnology Co., Ltd. (Zhejiang, China). The tubers were planted in a glass greenhouse to obtain

To cite this paper: Jin, T., C. Zhang, K. Wang, R. Li, Y. Li, C. He and T. Xiang, 2019. *In Vitro* plant regeneration and hairy roots induction in *Dioscorea alata* (cultivar wenshanyao). *Intl. J. Agric. Biol*., 22: 693–696

seedlings. The leaves, stem segments, and stem segments with leaf axils were taken as the explants.

Explant Disinfection

The explants were immersed in a diluted detergent solution for 5 min, rinsed with tap water, sterilized with five-time diluted sodium hypochlorite (saturated solution) for 20 min, rinsed 3 times with sterile water and finally blotted away surface water using sterile filter paper. The leaves were cut into small pieces of approximately 1 cm square and the stems with or without leaf axils were cut to a length of 0.5–1 cm.

Explant Culture

The MS (Murashige and Skoog, 1962) basic medium with different ratios of 2, 4-D (2, 4-dichlorophenoxyacetic acid) and NAA (1-naphthlcetic acid) was used as culture medium for explant-induced callus. The MS basic medium with different ratios of 6-BA (6-benzylaminopurine) and IAA (indole-3-acetic acid) was used as culture medium for buds. The MS basic medium with different ratio of IAA and NAA was used as culture medium for roots (Table 1). The explants were inoculated on the induction medium after disinfection at a density of 3–5 explants per bottle with shading. The plant callus was obtained, and then transferred and cultured with lights in a differentiation medium after removing the browning part of the callus. The obtained buds were transferred to a rooting medium to regenerate roots and obtain whole plants. For all the cultures, the temperature was 24 ± 2°C.

***Agrobacterium rhizogenes* K599 Activation**

Recombinant *A. rhizogenes* K599 (stored at -80°C, with endogenous plasmid pRi2659 and exogenous plasmid pRI101-AN-GFP) (Du *et al.*, 2015) was cultured with LB + 50 mg/L Km (kanamycin) + 50 mg/L Str (streptomycin) medium at 28°C. A single colony was cultured overnight in LB + 50 mg/L Km + 50 mg/L Str medium at 28°C with a rotation rate of 200 r/min. When the *OD* value of Agrobacterium liquid was approximately 1.0, 1 mL of the bacterial solution was centrifuged with a 1.5 mL centrifuge tube. The supernatant was removed, followed by adding 25 mg/L of AS (acetosyringone) liquid culture medium to suspend the cells, which were centrifuged again. The supernatant was removed again, and the culture medium was diluted with MS liquid medium to an *OD* value of about 0.1, which was used as an infecting solution.

Infection of Sterile Seedling *in Vivo*, Hairy Root Induction and Reproduction

After 12–15 days of growth, vigorously grown seedlings were picked for infected materials. A sterile needle was used to draw multiple wounds in the seedling stems. 2–5 μL of inoculum was added to every wound and then the infested seedlings were further cultured at 24 ± 2°C with shading. When the hairy roots induced at wounds were about 2–3 cm in length, the hairy roots were cut and transferred to MS + 500 mg/L Cef (cefotaxime sodium) + 50 mg/L Km medium. In every 20–30 days the hairy roots were subcultured. After 3–4 times subcultures, Agrobacterium was completely killed, and the hairy roots were transfer to MS basic medium to propagate.

Molecular Identification of Hairy Roots and Observation by Fluorescence Microscopy

The hairy roots and common roots DNA were extracted using a Plant Genomic DNA Extraction Kit (Sangon Biotech, Shanghai, China). Primers 5'-GCCAGCATTTTTGGTGAACT-3' and 5'-CTGGCCCATCGTTCTAAAAA-3' were designed according to the *rolB* sequence of *A. rhizogenes* K599 Ri plasmid pRi2659 T-DNA (GenBank accession number is EF433766), and primers 5'-GTCAGTGGAGAGGGTGAAGG-3' and 5'-AAAGGGCAGATTGTGTGGAC-3' were designed based on *gfp* gene sequence (GenBank accession number is U17997). For transgenic hairy roots, a band of 700 bp or so can be amplified with the *rolB* gene primers and a band of about 500 bp with the *gfp* gene primers, which are the same as the sizes of the amplified bacterium. The primers were synthesized by the Sangon Biotech. A PCR amplification reaction was prepared according to the method developed by Du *et al.* (2015). Amplification was performed using a Bio/Rad 9700 type PCR instrument. The PCR reaction procedure was: pre-denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 90 sec, and extension at 72°C for 10 min after all the cycles. The amplified products were electrophoresed on a 1.2% agarose gel for 0.5–1 h (5 V/cm), stained with ethidium bromide, and visualized and photographed using a Bio/Rad gel imaging system. In addition, the induced hairy roots were fluorescently detected under blue excitation light (filter FITC) using a Zeiss fluorescence microscope and photographed with a digital imaging system equipped with the Zeiss fluorescence microscope.

Results

Callus Induction, Differentiation and Plantlet Regeneration

The results of different explants culture were different. Callus was induced by leaf in MS medium added 2,4-D but was not induced by leaf in MS medium supplemented with NAA. Callus was induced by both stem and stem with leaf

Table 1: Callus induction results

Explant	2, 4-D (mg/L)	NAA (mg/L)	Callus induction rate
Leaf	0.5	0	0.05(1/20)
	1.0	0	0.1(2/20)
	1.5	0	0.1(2/20)
	2.0	0	0.15(3/20)
	2.5	0	0.25(5/20)
	0	0.5	0
	0	1.0	0
	0	1.5	0
	0	2.0	0
	0	2.5	0
Stem segments	0.5	0	0.1(2/20)
	1.0	0	0.3(6/20)
	1.5	0	0.6(12/20)
	2.0	0	0.7(14/20)
	2.5	0	0.2(4/20)
	0	0.5	0.05(1/20)
	0	1.0	0.15(3/20)
	0	1.5	0.15(3/20)
	0	2.0	0.35(7/20)
	0	2.5	0.3(6/20)
Stem segments with leaf axils	0.5	0	0.25(5/20)
	1.0	0	0.45(9/20)
	1.5	0	0.7(14/20)
	2.0	0	0.95(19/20)
	2.5	0	0.75(15/20)
	0	0.5	0.1(2/20)
	0	1.0	0.2(4/20)
	0	1.5	0.35(7/20)
	0	2.0	0.6(12/20)
	0	2.5	0.65(13/20)

Table 2: Results of callus re-differentiation into buds

6-BA (mg/L)	IAA (mg/L)	Re-differentiation of callus into buds
1.0	0	0.267(4/15)
1.0	0.5	0.533(8/15)
1.0	1.0	0.267(4/15)
2.0	0	0.667(10/15)
2.0	0.5	0.867(13/15）
2.0	1.0	0.2(3/15)

Table 3: Results of callus re-differentiation into roots

IAA (mg/L)	NAA (mg/L)	Re-differentiation of callus into roots
0.5	0	++
1.0	0	++++
1.5	0	++
2.0	0	+
0	0.5	- (Base became callus)
0	1.0	- (Base became callus)
0	1.5	- (Base became callus)
0	2.0	- (Base became callus)

+: 1-2 roots per plant; ++: 1-3 roots per plant; +++: 1-4 or more roots per plant; -: no roots

axils in MS medium supplemented with 2,4-D or NAA. Among them, in the MS medium supplemented with 2.0 mg/L of 2,4-D the induction rate by using stem with axils was the highest, reaching 95% (Table 1). In MS+6-BA 2 mg/L + IAA 0.5 mg/L medium the bud differentiation rate was the highest at 86.7% (Table 2) and all pieces of callus differentiated into clustered buds. Individual bud from cutting of clustered buds can differentiate into roots

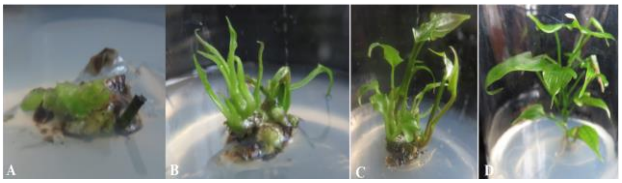

Fig. 1: Regeneration of Wenshanyao whole plants
A: callus formation; B, C: callus differentiated into clustered buds; D: regeneration of whole plants with roots

Fig. 2: Induction of hairy roots at the wounds on tissue-culture seedlings
A, B, C: induced hairy roots at the wounds; D: propagated hairy roots

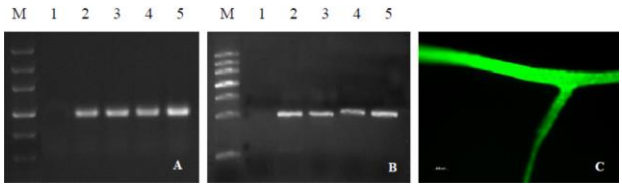

Fig. 3: PCR identification and fluorescence microscopic observation of hairy roots
A: *rolB* gene primer amplification; B: *gfp* gene primer amplification; C: fluorescent hairy roots emitting strong fluorescence; M: DNA standard molecular weight; 1: common root; 2: recombinant. *rhizogenes* K599 (with pRI101-AN-gfp); 3-5: three different hairy roots

in MS medium with 0.5–2.0 mg/L of IAA and the root inducing effect is the best in MS with 1.0 mg/L of IAA medium. In the MS medium with different concentrations of NAA, the base became callus and failed to differentiate root (Table 3). The process of regenerating whole plants of Wenshanyao is shown in Fig. 1.

Induction of Hairy Roots

A. rhizogenes K599 infects the stems of tissue-culture seedlings *in vivo*, and typical hairy roots can appear around the wound after 10–15 days of incubation (Fig. 2A, B and C). When the hairy roots were 2–3 cm long, the hairy roots were transferred to a subculture medium supplemented with 500 mg/L Cef. A large number of proliferating hairy roots were obtained in 20–30 d (Fig. 2D).

PCR Analysis and Fluorescence Microscopic Observation of Hairy Roots

The genomic DNA of hairy roots and common roots (*i.e.* non-transgenic roots), as well as *A. rhizogenes* K599, were separately amplified by PCR (Fig. 3A and B). No bands

were amplified by common roots, indicating that the obtained hairy roots are transgenic roots containing the exogenous gene *rolB* and *gfp*. Under blue excitation light (filter: FITC), the transgenic hairy roots emitted a strong green fluorescence (Fig. 3C), while the common roots showed no fluorescence, indicating that the *gfp* gene was successfully expressed in the transgenic hairy roots.

Discussion

Plant tissue culture has been used for conservation, micropropagation and in planta overproduction of some pharma molecules of medicinal plants (Niazian, 2019). Hairy root cultures could be furtherly used for future medical purposes, especially as anti-cancer agents. These areas of plant biotechnology will be surely promising (Xiang *et al.*, 2016; Jeziorek *et al*., 2018). Now, *in vitro* plant regeneration and A. *tumefaciens*/*rhizogenes*-mediated transformation techniques were developed for some medicinal plants (Du *et al*., 2015; Guo *et al*., 2018; Rajewski *et al*., 2019). Stable transformation methodology of these species would be advantageous for future genetic studies.

In this study, the stem segments with leaf axils were used as explants to highly efficiently regenerate plants, which provided a basis for genetic improvement of Wenshanyao varieties. On the other hand, the use of hairy roots as a reactor is an effective way to produce secondary metabolites of medicinal plants (Srivastava and Srivastava, 2007). Wenshanyao is rich in medicinal ingredients such as flavonoids, saponins, and choline phytate (Sun *et al.*, 2014). In this study, *A. rhizogenes* K599 was used to infect the stems of sterile seedlings of Wenshanyao, successfully inducing the generation of transgenic hairy roots, which provided a basis for the future industrialized production of medicinal components of Wenshanyao by hairy roots. The fluorescence microscopic observation of the hairy roots indicated that the exogenous marker gene *gfp* was successfully expressed in the transgenic hairy roots, which will serve as a technical basis for producing recombinant protein drugs using hairy roots as reactors.

Conclusion

The stem segments with leaf axils of yam cv. Wenshanyao were used as explants to highly efficiently regenerate plants. *A. rhizogenes* K599 was used to infect the stems of sterile seedlings of Wenshanyao, successfully inducing transgenic hairy roots of Wenshanyao. This is the first report that *in vitro* plant regeneration and hairy roots induction in Wenshanyao.

Acknowledgement

The work was supported by grants from Undergraduate Innovation Ability Improvement Project of Hangzhou Normal University.

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[Received 11 Mar 2019; Accepted 03 Apr 2019; Published (online) 20 Aug 2019]