Front. Biol. China 2009, 4(2): 217–221
DOI 10.1007/s11515-009-0006-9

RESEARCH ARTICLE

Effects of plant growth regulators on the rapid proliferation of shoots and root induction in the Chinese traditional medicinal plant *Atractylodes macrocephala*

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Abstract We developed an efficient plant regeneration protocol for rapidly propagating *Atractylodes macrocephala* Koidz, an important traditional Chinese medicinal plant, *via* shoot organogenesis. Shoot multiplication was induced on Murashige-Skoog (MS) medium supplemented with various concentrations of *N*-phenyl-*N*-1, 2, 3-thidiazol-5-ylurea (TDZ), 6-benzylaminopurine (BA) and α-naphthaleneacetic acid (NAA). Rooting was induced on half-strength MS medium supplemented with NAA and indolebutyric acid (IBA). The maximum mean number of shoots (5.61) was obtained from a single explant by the combined effect of 1.08 μmol/L NAA and 2.25 μmol/L TDZ. The longest roots and a minimum number of roots were produced when they were cultured in a medium without plant growth regulators. The shortest roots and the largest number of roots were observed in the medium supplemented with 2.7 μmol/L NAA.

Keywords Chinese traditional medicinal plant, *Atractylodes macrocephala* Koidz, multiple shoot, plant regeneration

1 Introduction

The dry rhizomes of *Atractylodes Macrocephala* Koidz (Baizhu) of the *Atractylodes* DC family have been used as a traditional Chinese herbal medicine for thousands of years. It was listed as a top grade medicine in one of the earliest works in Chinese pharmacy, *Shennong Herb-Root Classic*. The main active compounds of Baizhu are volatile oils such as hinesol, atractylone, and Rhizoma Atractylodis Macrocephalae lactone. These compounds have been used in drugs for abdominal pain, diarrhea, kidney, liver and spleen failure, inhibiting human rotavirus (HRV) replication and smooth uterine muscle movement (Zhang et al., 2000; He et al., 2001; Peng and Wang, 2004). Moreover, recent studies have shown that Baizhu has other medicinal properties such as anti-inflammatory activities (Li and He, 2006), inhibition of tumor cell proliferation (Huang et al., 2005; Liu et al., 2005), and suppression of diarrhea (Kim et al., 2005). Consequently, there is a growing commercial demand for Baizhu, which has caused such serious problems as unrestricted exploitation and nonselective cultivation, leading to the dwindling of wild sources and species degeneration. Additionally, conventional propagation of Baizhu through seeds is low due to poor germination. *In vitro* micropropagation can provide a more efficient approach for the rapid mass propagation of selected elite Baizhu varieties. However, to date, there have been only a few reports on the micropropagation of Baizhu (Zhu et al., 2006). The present study was carried out with the objective of developing an efficient micropropagation protocol for mass manufacturing of high-quality commercial Baizhu products and for conserving the existing germplasm. Specifically, we sought to establish an efficient regeneration system by optimizing the supplementation of plant growth regulators in the culture medium and comparing growth parameters of the micropropagated plantlets with the stock plants.

2 Materials and methods

2.1 Plant materials and growing conditions

The local variety of Baizhu produced in the Zhejiang Province of China, BZ1 (*Atractylodes macrocephala*

Received August 13, 2008; accepted October 21, 2008

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Koidz, BZ1) was used as the donor plant. Healthy rhizomes were selected and induced to sprout in sand. The terminal buds (about 0.5–1 cm) were isolated and washed well under running tap water and the surrounding leaflets were stripped off carefully. Explants were submerged in 70% ethanol for 45 s and sterilized with 0.1% (w/v) aqueous mercuric chloride for 6–10 min, or 10% aqueous solution of 5.4% sodium hypochlorite for 10–15 min, respectively, followed by six to eight rinses with sterile distilled water. Meristems of the aseptic shoots were cut off (about 0.1 cm) and cultured in MS basal medium (Murashige and Skoog, 1962). The survival rates of sterilized explants were determined after three weeks. All cultures were incubated at 25°C with a 16 h photoperiod (fluorescent, 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The same conditions were applied for all the experiments.

2.2 Multiplication of shoots

In vitro aseptic shoots were cut into individual seedlings (2–3 cm) and cultured in the proliferation MS medium supplemented with 4.4–22.0 μmol/L 6-benzylaminopurine (BA) and 0.45–4.5 μmol/L *N*-phenyl-*N*-1,2,3-thidiazol-5-ylurea (TDZ) alone or in combination with NAA (0, 1.08 and 2.7 μmol/L) for mass shoot indu-ction. In all assays, the culture medium pH was adjusted to 5.8 before autoclaving. Shoot proliferation efficiency was determined on the basis of the number of 'usable' shoots (> 1 cm long) produced per explant after four weeks of culture.

2.3 Rooting of shoots

Randomly selected individual seedlings (about 2–3 cm long) with intact apical buds and three to five leaves were detached from *in vitro* proliferating adventitious shoots and cultured on the rooting medium. The rooting medium was half-strength MS medium supplemented with sucrose (2%, w/v), agar (0.8%, w/v) and various concentrations of indolebutyric acid (IBA) (0 and 2.45 μmol/L) or NAA (0, 1.08 and 2.7 μmol/L) alone. The half-strength MS medium without plant growth regulators served as control. Data for root number and length were recorded after four weeks of incubation.

2.4 Acclimatization and transfer to the field

Rooted plantlets at 5–6 cm height were moved from the rooting medium and gently washed with sterile water supplemented with 0.01% (w/v) thiophanate methyl. In general, the micropropagated plantlets were transplanted into 72-hole flowerpots containing locally available sterile peat and vermiculite (3∶1), nourished with 1/10-strength MS's macro-element solution on every fifth day and grown under standard greenhouse conditions. After about one month, the plants were transferred to 4-inch pots.

2.5 Statistical analysis

All treatments were conducted in a randomized complete block design. Each experiment was repeated three times with 15 replicates. Data were expressed as mean ± standard errors (SE). Duncan's multiple-range test (SSR) was used with the Data Processing System (DPS) Statistical Software package (Tang and Feng, 1997).

3 Results and discussion

3.1 Optimal sterilization treatment

The best survival characteristics were obtained with mercuric chloride for 10 min with survival rates reaching 80% (Fig. 1). Our data shows that the contamination frequency was significantly reduced with increased mercuric chloride treatment time. There was a significant increase in death rates between ten, eight and six min of mercuric chloride treatment, while no differences were observed between the six and eight min treatments. Additionally, significant differences in survival rates were noted between mercuric chloride treatment for 10 min and all the other treatments. The contamination rates were lower using sodium hypochlorite treatment compared to that with mercuric chloride for six and eight min, though the death rates were higher for the former treatment (Fig. 1).

3.2 Optimal medium for shoot proliferation

Various combinations of plant growth regulators (PGRs) were used to determine the optimal medium for shoot

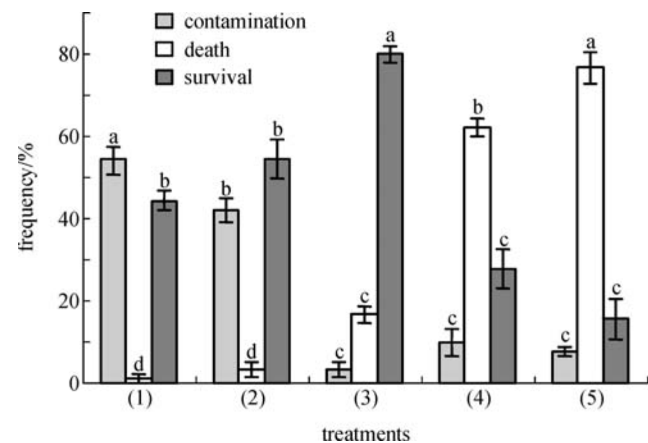

Fig. 1 Variations of growth parameters in response to different sterilization treatments. (1) Sterilized with 0.1% (w/v) aqueous mercuric chloride for six min; (2) Sterilized with 0.1% (w/v) aqueous mercuric chloride for eight min; (3) Sterilized with 0.1% (w/v) aqueous mercuric chloride for 10 min; (4) Sterilized with 10% aqueous solution of 5.4% sodium hypochlorite for 10 min; (5) Sterilized with 10% aqueous solution of 5.4% sodium hypochlorite for 15 min. Bars with different letters are significantly different ($P \leqslant 0.05$).

proliferation from aseptic seedlings. Typically, shoots were observed after a one-week culture, although significant differences in the induction of the number of adventitious shoots were noted, depending on the PGRs used (Table 1, Fig. 2A). A significant difference in the increase of shoot proliferation was observed in the MS medium supplemented with 2.25 μmol/L TDZ and 1.08 μmol/L NAA, with an average of 5.61 shoots per explant (Fig. 2B). A significant difference in shoot proliferation was also noted with BA ($P = 0.0001$) and TDZ ($P = 0.0007$), while no increases were observed with NAA. Interactions between NAA and TDZ ($P = 0.0001$) were also significant for shoot proliferation, while no significant additive effect was noted between NAA and BA ($P = 0.236$).

Previous studies with other plants have suggested that TDZ, BA and NAA have similar proliferation stimulating effects (Visser et al., 1992; Caglar et al., 2005; Ramakrishnan et al., 2005). Other workers have, however, reported that TDZ is more effective than BA (Fiola et al., 1990; Malik et al., 1992; Bedir et al., 2003). Caglar et al. (2005) reported that the induction capacity of TDZ was stronger than that of BAP, resulting in a mean of 47.5 shoots per explant in caper (*Capparis spinosa* L.). Similar results were obtained by Visse et al (1992), who reported that auxins were involved in the induction and/or expression of TDZ-induced morphogenic differentiation of geranium (*Pelargonium* x *hortorum*). Our observations show that the medium containing lower concentrations of TDZ or BA (0.45 μmol/L TDZ, 4.4 μmol/L BA) resulted in a non-significant increase in the number of induced adventitious shoots (Table 1), although plants grew, flourished and were stout. While the medium containing slightly higher concentrations of TDZ or BA (2.25 μmol/L TDZ, 13.2 μmol/L BA) resulted in a large number of induced adventitious shoots, and plants were non-luxuriant. High concentrations (4.5 μmol/L TDZ, 22.0 μmol/L BA) also resulted in a large number of shoots, but plants were misshaped, with swollen, transparent and vitrified leaflets. Additionally, plants had lower survival rates in culture. Different plant varieties display different responses to

Table 1 Effect of PGRs on shoot regeneration of Baizhu

PGRs /μmol·L^{-1}	NAA	number of shoots per explant
BA		
4.4	0	2.20 ± 0.03 h
4.4	1.08	2.25 ± 0.05 h
4.4	2.7	2.24 ± 0.05 h
13.2	0	5.00 ± 0.08 c
13.2	1.08	5.00 ± 0.11 c
13.2	2.7	4.80 ± 0.04 c
22	0	3.67 ± 0.10 d
22	1.08	3.53 ± 0.09 de
22	2.7	3.33 ± 0.11 ef
TDZ		
0.45	0	2.85 ± 0.05 g
0.45	1.08	3.29 ± 0.16 f
0.45	2.7	2.83 ± 0.04 g
2.25	0	5.39 ± 0.06 b
2.25	1.08	5.61 ± 0.09 a
2.25	2.7	5.27 ± 0.05 b
4.5	0	3.70 ± 0.03 d
4.5	1.08	3.24 ± 0.04 f
4.5	2.7	3.56 ± 0.07 d

Data are expressed as mean ± standard error (SE). Mean values followed by different lowercase letters are significantly different ($P \leqslant 0.05$).

Fig. 2 Development of a regeneration system for Baizhu through shoot multiplication. A: Proliferation of regenerated shoots (bar = 1 cm); B: Cluster of shoots proliferated from bud explant on MS medium containing 2.25 μmol/L TDZ and 1.08 μmol/L NAA after 4 weeks in culture (bar = 1 cm); C: Plantlet with *in vitro* regenerated root system before transfer to soil (bar = 1 cm); D: Plant in the greenhouse 2 months after transplantation (bar = 2 cm); E: Mature normal plants produced in the greenhouse (bar = 4 cm); F: Flower bud of the *in vitro* regenerated plant (bar = 1 cm); G: Mature flower of *in vitro* regenerated plant (bar = 1 cm).

hormones although. Although PGRs generally regulate dedifferentiation, redifferentiation and proliferation of plant cells. In the present study, although explant cells were triggered to reproduce by PGRs, the effect was weak, with low PGR concentrations resulting in a small number of induced adventitious shoots.

High adventitious shoot proliferation efficiency was induced by high concentrations of PGRs. However, care must be taken not to surpass the 'threshold value' of hormonal regulation, as cells may be injured or induced to go into teratogeny. We also observed similar responses to hormone regulation and 'threshold value', although there was a negative correlation between the bud number and growth conditions. Large numbers of induced adventitious shoots corresponded with a feeble plant growth. Consequently, the establishment of a regeneration system should take into consideration the relationship between proliferation increment and plant quality to ensure the most economical production of high quality seedlings. The enrichment culture medium MS + 2.25 μmol/L TDZ + 1.08 μmol/L NAA was utilized in all further studies.

3.3 *In vitro* rooting

The optimal medium for root differentiation was determined using various combinations of auxins. Roots were induced after four weeks of culture with the best root formation obtained in half-strength MS medium containing 2.7 μmol/L NAA, and significant effects in root formation were observed following PGR treatments (Table 2 ; Fig. 3). The maximum number of roots was observed in half-strength MS supplemented with 2.7 μmol/L NAA, with each shoot regenerating a mean of 14.67 roots, a significantly higher number compared to the other media used (Fig. 3D). The smallest number of roots was observed in the hormone-free half-strength MS medium (each shoot regenerated four roots, Fig. 3A). There were significant differences in the number of induced roots by media containing 2.45 μmol/L IBA (Fig. 3B), 1.08 μmol/L NAA (Fig. 3C), 2.7 μmol/L NAA (Fig. 3D), and the culture medium that did not contain any hormone (Fig. 3A). The longest roots were induced in the medium without any hormone (average 4.48 cm, Fig. 3A), and the shortest in the medium supplemented with 2.7 μmol/L NAA (average 1.50 cm, Fig. 3D). Additionally, the difference in induced root length was significant among all the treatments. The best rooting was obtained using 2.7 μmol/L NAA.

Table 2 Effect of PGRs on root formation from shoots

PGRs/μmol·L^{-1}		number of roots per shoot	root length/cm
IBA	NAA		
0	0	4.0 ± 0.58c	4.48 ± 0.05A
2.45	0	10.33 ± 0.88b	2.45 ± 0.10C
0	1.08	9.67 ± 0.88b	3.34 ± 0.06B
0	2.7	14.67 ± 0.33a	1.50 ± 0.07D

Data are expressed as mean ± standard error (SE). Mean values within columns followed by different lower case letters are significantly different at $P \leqslant 0.05$; Those followed by different capital letters are significantly different at $P \leqslant 0.01$.

3.4 Plant establishment

All the rooted plantlets showed good survival rates when transplanted to a greenhouse. More than 95% of plants put on new growth (Fig. 2D). After five to six months, micropropagated plants produced flowers and were fertile, with similar growth characteristics as the stock plants (Fig. 2). There was no difference in survival rates among seedlings with different root lengths and numbers, although seedlings with roots of 1–2 cm in length were easily transplanted and well protected from mechanical damage during transplanting.

4 Conclusions

We present here a reliable regeneration protocol for the mass-propagation of Chinese traditional medicinal plant Baizhu for different purposes. The mass micropropagated plantlets can provide large quantities of material for elite

Fig. 3 Effects of plant growth regulators on root formation after four weeks of culture. Rooting of regenerated shoots in half-strength MS medium, A: without plant growth regulators; B: containing 2.45 μmol/L IBA; C: containing 1.08 μmol/L NAA; D: containing 2.7 μmol/L NAA. Bars = 1 cm.

variety selection and cloning of superior individual genotypes. This provides an avenue for the direct introduction of novel traits into Baizhu through genetic engineering without the need for numerous back-crossings in breeding programs that slow down cultivar improvement.

Acknowledgements We thank Roy B. Mugiira for proofreading the manuscript. This work was partially funded by the Science and Technology Department of Zhejiang Province, China (Nos. 2005C13016 and 2005C22080).

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