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MICROPROPAGATION OF *CODONOPSIS PILOSULA* (FRANCH.) NANNF BY AXILLARY SHOOT MULTIPLICATION

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Roots of *Codonopsis pilosula* (Franch.) Nannf. are among the most popular Chinese herbal medicines, exhibiting various beneficial activities which support immunity and stress resistance. The plant shows high intraspecific genetic variation. There is a need for effective vegetative propagation methods yielding high and sustainable quality. Here we report a micropropagation method using axillary shoot proliferation. Nodal segments from aseptically germinated plants were inoculated on modified MS media enriched with different concentrations of cytokinins: benzyladenine, kinetin (1, 4, 10 or 20 μM) or thidiazuron (1, 4 or 8 μM), with or without the auxin NAA (1 μM). Axillary bud break was initiated most efficiently on media with 1 or 4 μM BA and 1μM NAA. Shoot number increased markedly in subsequent cycles of harvesting and transfer to fresh 1 μM BA and NAA medium, leading to the maximum 69 shoots (mean 38.16±4.35) from a single nodal explant in the fourth harvest. The shoots were successfully (>98% efficiency) rooted in MS medium containing high sucrose (60 g/L) and 5 μM IAA, and acclimatized to soil cultivation with a survival rate of 90%. These results can be used to establish a simple and commercially viable protocol for mass propagation of *C. pilosula* for plantations or breeding.

Key words: *Codonopsis pilosula*, axillary bud, benzyladenine, in vitro rooting.

INTRODUCTION

Codonopsis pilosula (Franch.) Nannf. (Campanulaceae), a traditional Chinese herbal and health food crop, is gaining popularity in and outside Asia, and has been recommended for introduction into cultivation in, for example, the U.S.A. (Craker and Giblette, 2002). It is a perennial with twining stems and thick cylindrical roots. In the wild it grows in forest margins, thickets and scrub in several provinces in China (FLORA OF CHINA, 2011). Roots of *C. pilosula* have been used in traditional Chinese medicine (TCM) for centuries as an adaptogenic herb. The roots (*Codonopsis radix*, dangshen) are obtained from adult plants after about 3 years and used dried. The plant is gaining popularity in other countries around the world due to its therapeutic and preventive uses.

Nowadays most of the roots are obtained from cultivated plants because of habitat depletion and large market demand (Li et al., 2009a). The cultivated form, frequently referred to as *C. tangshen*, recently has been included in *C. pilosula* as *C. pilosula* subsp. *tangshen* (Oliv.) D. Y. Hong (Hong, 2010). According to TCM it has some similarity with Ginseng roots, boosting the Qi and nourishing the spleen and lungs. *Codonopsis* roots are often used as a substitute for the more expensive ginseng roots, hence the common name "poor man's ginseng" (Hu, 2005; Hempen and Fischer, 2009).

In addition to *C. pilosula*, several other species are used medicinally, including *C. subglobosa* W. W. Sm., *C. convolvulacea* Kurz. and *C. lanceolata* (Sieb. & Zucc.) Trautv. (Li et al., 2009b). Besides their use in herbal preparations, the roots are also used as a health food ingredient in the form of soup or broth with other herbs (Hu, 2005). Modern pharmacological, animal and human studies indicate that *C. pilosula*'s beneficial activities include improving adaptation of the nervous system to stress, prevention and healing of peptic ulcers, normalizing blood pressure, immunostimulation, as well as prevention of oxidative stress and alleviation of oxidative damage in diabetes (Liu et al., 1988; Chen and Chen, 2004; Chan et al.,

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Abbreviations: BA – N-6-benzyladenine; IAA – indole-3-acetic acid; NAA – naphthaleneacetic acid; MS – Murashige and Skoog (1962) medium; PGR – plant growth regulator.

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2008; Hempen and Fischer, 2009). The active principle of *Codonopsis radix* is rather complex and contains a variety of secondary metabolites such as triterpenoid, polyacetylene and phenolic glycosides, and specific water-soluble polysaccharides and pyrrholidine alkaloids – codonopsine and codonopsinine (Liu et al., 1988; Chen and Chen, 2004; Li et al., 2009b; Wakana et al., 2011).

Codonopsis pilosula is a variable species, exhibiting substantial diversity not only in its phytochemistry (Li et al., 2009b; Yang et al., 2010), but also in molecular markers such as microsatellites and RAPD fragments (Zhang et al., 1999; Guo et al., 2007; Li et al., 2009a). Together, xenogamous free pollination and routine propagation from seeds can produce inconsistent and unpredictable crop quality, and can make it difficult to implement a rational breeding programs (Guo et al., 2007). There is a need for a simple, robust and efficient method of obtaining a large amount of uniform, high-quality plant material. Micropropagation in vitro may offer such an advantage.

In vitro approaches have been applied in a great number of medicinal plants which are rare or endangered (Rowntree, 2006) or widespread species from all over the world, including traditional Chinese herbs (Nalawade and Tsai, 2004, Zych et al., 2005; Sarasan et al., 2006). Widely cultivated and common species have also been studied in order to establish micropropagation methods employing a variety of techniques, including axillary bud induction, somatic embryogenesis, direct or indirect organogenesis, and adventitious shoot regeneration from different organs used as explants (Nalawade and Tsai, 2004).

Several species of the genus *Codonopsis* have been studied using in vitro approaches for different purposes, including mass propagation by somatic embryogenesis (Min et al., 1992), *Agrobacterium*-transformation for expression of the α-tocopherol methyltransferase gene (Ghimire et al., 2008) and an examination of the effects of in vitro conditions on genetic stability in *C. lanceolata* (Guo et al., 2006). Polysaccharide production has been studied in tissue cultures of *C. clematidea* (Rakhimov et al., 2003). There are only few reports of studies of *C. pilosula* in which indirect regeneration via callus and somatic embryos (Niu et al., 1991) or callus protoplasts (Li et al., 1993) were achieved. There are no published data on the use of axillary shoot induction for efficient micropropagation of this species.

In this paper we report an efficient method for regeneration and multiplication by induction of axillary bud break with benzyladenine treatment and subsequent rooting and ex vitro acclimatization of the regenerated plantlets. Repeated excision of axillary shoots and subculture of the primary explants markedly increased the number of axillary shoots. We investigated the effects of different hormonal regimes in basal MS medium on shoot regeneration. We report the conditions for regenerated shoot rooting and transfer to soil.

MATERIAL AND METHODS

CULTURE MEDIA

The basal medium used for the experiments was based on half-strength MS macroelements and full-strength microelements (Murashige and Skoog, 1962), and contained 20 g/l sucrose and 6 g/l agar (bacteriological grade agar N04, Biocorp, Poland). All media except seed germination medium were supplemented with other organic compounds: thiamine hydrochloride (2 mg/l), pyridoxine hydrochloride (0.1 mg/l), nicotinic acid (0.1 mg/l), myo-inositol (100 mg/l) and various concentrations of plant growth regulators (all from Sigma-Aldrich, U.S.A.). The pH was adjusted to 5.7–5.8 before autoclaving at 121°C under 1 atm for 17 min. The vitamins and temperature-sensitive PGRs were sterile-filtered through hydrophilic 0.22 μm syringe filters (Carl Roth GmbH, Germany) before addition to the autoclaved media.

EXPLANT PREPARATION AND INOCULATION

Aseptically grown seedlings were used for explant excision. The seedlings were obtained from surface-sterilized seeds collected from a single 6 year-old donor plant of *C. pilosula* subsp. *pilosula* cultivated in the Medicinal Plants Botanical Garden at the Medical University of Wroclaw, Poland. The seeds were soaked in 70% (v/v) ethanol solution for 1 min, followed by 15 min in 10% solution of commercial bleach (Clorox). The seeds were rinsed four times in an excess of sterile water and placed on agar media (6 g/l, Biocorp type N04 bacteriological agar) containing half-strength MS salts without organic additives. Decontamination efficiency was 100% and the germination percentage was 100% as well. The germinated seedlings were transferred to 35×300 mm glass test tubes containing the same medium and maintained in a growth room under a 16 h photoperiod (photon flux density 80 μmol m^{-2} s^{-1}) at 24±1°C until 8–10 nodes developed.

The explants were prepared from middle nodes (3 or 4 depending on seedling size) by cutting off the leaf blades and isolating the node with ~5 mm of the internodes on both sides of it. The explants were placed horizontally on the different bud induction media in polystyrene Petri dishes (100×20 mm).

AXILLARY SHOOT PROLIFERATION

For the initial experiment aimed at selecting media for best bud break rate, the following PGR combina-

TABLE 1. Effect of growth regulators on axillary bud break in nodal explants from *Codonopsis pilosula* after 42 days of culture (means of four replicates, each consisting of 10 explants). PGR concentrations are μmol/liter. Means followed by the same letter within column do not differ significantly at P<0.05; ± SE

BA	KIN	TDZ	NAA	% of responding explants	Mean number of axillary shoots
–	–	–	–	0	0
–	–	–	1	0	0
1	–	–	–	98.3 ±1.2ab	2.32±0.06a
1	–	–	1	100.0±0.0a	2.36±0.09a
4	–	–	–	96.0±2.8b	2.41±0.10a
4	–	–	1	100.0±0.0a	2.22±0.06a
10	–	–	–	88.3±3.6b	1.81±0.09b
10	–	–	1	83.4±4.9b	1.83±0.09b
20	–	–	–	95.0±2.2b	1.52±0.08c
20	–	–	1	100.0±0.0a	1.66±0.06c
–	1	–	–	86.0±3.0b	2.44±0.09a
–	1	–	1	90.0±4.4b	2.36±0.13a
–	4	–	–	77.0±4.4b	6.23±0.40d
–	4	–	1	77.2±6.7b	2.51±0.23a
–	10	–	–	90.0±0.0b	1.36±0.08e
–	10	–	1	93.3±3.6b	1.35±0.06e
–	20	–	–	85.0±5.4b	2.41±0.10a
–	20	–	1	85.0±4.7b	2.17±0.04ab
–	–	1	–	0	0
–	–	1	1	0	0
–	–	4	–	22.0±5.2c	0.48±0.10f
–	–	4	1	8.0±2.2cd	0.45±0.09f
–	–	8	–	8.0±2.7cd	0.27±0.08f
–	–	8	1	2.0±1.3d	0.29±0.06f

tions were used: cytokinins BA or kinetin at 1, 4, 10 or 20 μM, or thidiazuron at 1, 4 or 8 μM alone or in combination with NAA (1 or 5 μM).

For further proliferation, only media enriched with BA and 1 μM NAA were selected.

After 42 days, the first few elongating axillary shoots were excised with a scalpel, leaving about 2 mm of the stub. The original explant was then transferred to fresh medium and allowed to grow for another 6 weeks. Then the operation was repeated, and the number of microshoots was counted before each excision, considering only shoots longer than 10 mm with at least two clearly visible leaf pairs.

The shoots from the fourth (final) harvest were collected for the rooting experiment.

ROOTING AND EX VITRO ACCLIMATIZATION

Regenerated microshoots with at least two fully visible nodes were excised and transferred to rooting media supplemented with IAA (5 or 10 μM), IBA (5 μM) or increased sucrose (60 g/l). Addition of active charcoal (5 g/l) was also tested in both control (PGR free) and selected IAA and IBA media (Tab. 3). Each treatment was represented by 50 explants. The shoots were maintained in 35×300 mm glass tubes filled with 25 ml agar medium at a 45 slope. The basal end of the shoot was submerged in the medium to 3–5 mm depth for up to six weeks. The developing roots were visually assessed for length, thickness and lateral branching.

For ex vitro transfer, only the plantlets from the three optimal media were used: 20 g/l sucrose with 5 μM IBA (25 plantlets) or IAA (20 plantlets) and 60 g/l sucrose with 5 μM IAA (30 plantlets). Plantlets having developed root systems with lateral roots were removed from the agar, washed thoroughly but gently with sterile tap water and transferred to peat pot strip sets with autoclaved potting soil mix (soil: sand:peat:perlite, 5:2:2:1 v/v/v/v). The plantlets in the pots were initially covered with glass jars and maintained in the growth room under the same conditions as for the in vitro cultures. The jars covering the plantlets were temporarily removed every day for

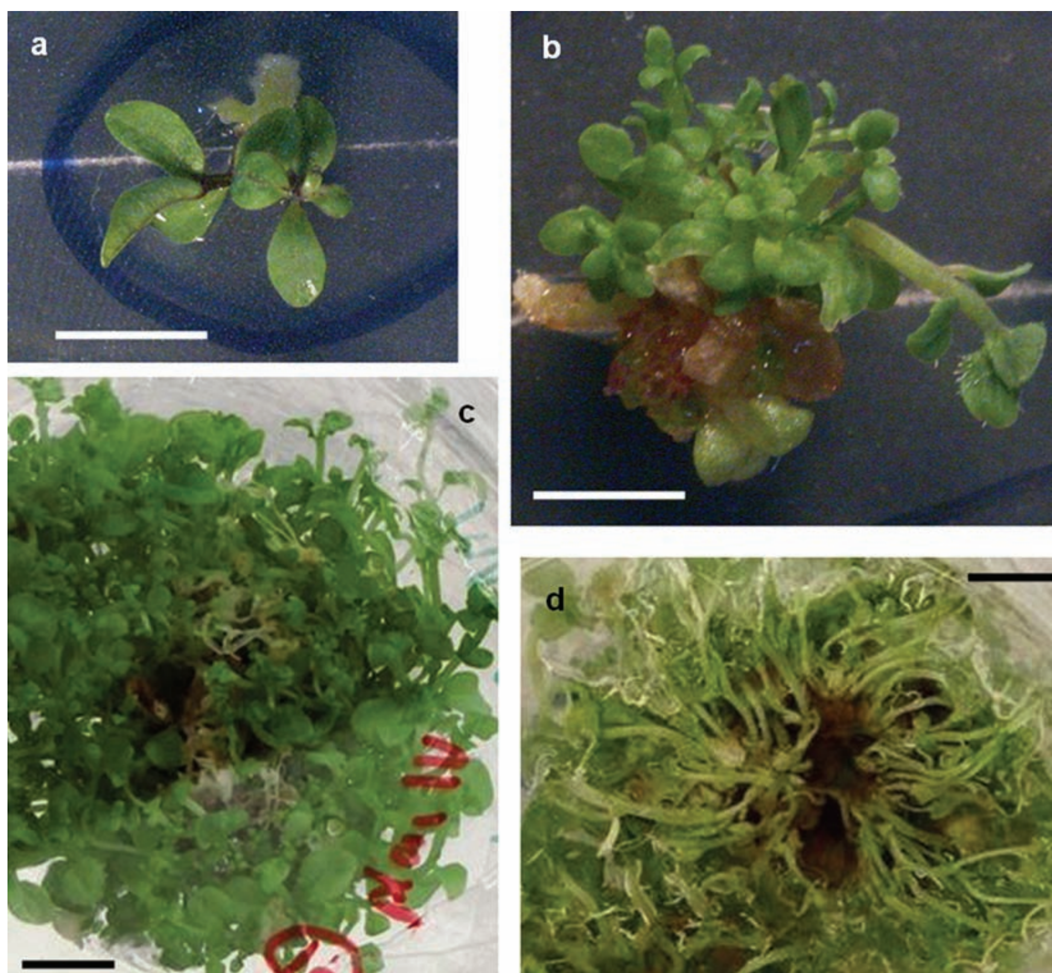

Fig. 1. Axillary shoot regeneration in *Codonopsis pilosula* nodal explants. (**a**) First subculture/excision cycle – only two shoots emerging from the node, (**b**) Third cycle – numerous shoots from the node, (**c**) Fourth cycle – explant covered with many shoots and branches of various sizes, (**d**) The same explant viewed from below to show the numerous branching stems. Bar = 1 cm.

increasing periods. After three weeks the potted ex vitro plantlets were transferred to a greenhouse under ambient daylight and after another two weeks they were repotted to larger containers (20 cm diam) and maintained for at least 60 days before successive transplanting to the garden during the vegetation season (first decade of May). The percentage of surviving plants was calculated at the potting and garden phases of ex vitro adaptation.

STATISTICAL EVALUATION

The treatments were arranged in a random design. Each treatment was represented by at least four replicates consisting of 10 or more explants. The significance of differences between means of the results of each treatment was assessed by one-way ANOVA followed by post-hoc analysis with Duncan's multiple comparison test. The percentage data on axillary bud break were arcsine-transformed before analysis. For in rooting percentage data the differences between percentages were analyzed pair-wise with the two-tailed Z-test. For all tests, differences were considered significant at $P<0.05$. All calculations were done in Statistica 8.0 (Statsoft, Poland).

RESULTS AND DISCUSSION

Explants cultured on media without growth regulators did not produce new shoots. Preliminary experiments using three cytokinin PGRs gave optimal results with BA. With kinetin the shoots grew more slowly and were frequently overgrown by callus. However, the average number of shoots induced from a single explant tended to be higher on kinetin-enriched media than on the corresponding BA media; most of those differences were not significant

Fig. 2. (**a**) Rooting plantlet on higher-sucrose medium (IAA 5 μM, sucrose 60 g/l), (**b**) Plant established ex vitro before transferring to field conditions. Bar = 2 cm.

TABLE 2. Multiple shoot induction in consecutive 42-day cycles of harvesting and explant re-inoculation on medium enriched with 1 μM BA and NAA. Shoots longer than 10 mm consisting of at least two visible nodes were counted; ±SE

Harvest	Numwer. of shoots/explant	Highest number of shoots/single node
1st	2.22±0.16	4
2nd	3.61±0.97	10
3rd	5.71±1.23	21
4th	38.16±4.35	69

(Tab. 1). On the other hand, the proportion of responding explants was significantly lower (for all explants from both cytokinins: 84% on kinetin vs. 95% on BA). Adding TDZ did not induce axillary bud break in the majority of explants (less than 25% on average) and the growing shoots were stunted and showed hyperhydricity. Therefore the experiments using kinetin and thidiazuron were discontinued. Callus formed at the cut edges of explants at all stages of culture. Callusing was most intensive on kinetin/NAA media but also occurred on media with BA.

The optimal medium for shoot number and elongation contained 1 μM BA and NAA. This was unexpected, as most of the literature suggests that higher cytokinin than auxin is usually favorable. In our study, increasing the BA concentration to 20 μM significantly decreased the explant survival percentage. At the intermediate BA concentrations the number of shoots was comparably high, but they were shorter and difficult to count and excise individually for subculture.

The most striking observation was the remarkable increase in the number of axillary shoots in the subsequent 42-day cycles of subculture, reaching over 50 shoots per explant in the fourth harvest (mean 38.1 shoots per explant). There was wide variation of shoot number between individual explants: the highest number was 69 (compare Tab. 2), the explant with the lowest number gave only 18 fully formed shoots, and most of the explants had more than 30 shoots (mean 38.16±4.35). We did not count the tiny, not fully developed shoots and branches. The average total number of shoots theoretically obtainable from the single node explant in four rounds of harvesting would exceed 1500 (Fig. 1). The propagation rate can be further increased by using the harvested axillary shoots as source of nodal explants. This approach would yield an enormous number of regenerated shoots within a short time. In a parallel small-scale experiment (data not shown) we confirmed the axillary bud break ability of the regenerated shoots on the same media with BA for at least 6 consecutive harvesting and nodal explant inoculations. This would exponentially increase the number of plantlets obtainable in each cycle. An increase of axillary shoots in consecutive excision-subculture cycles has also been reported in *Ocimum kilimandscharicum* recently (Saha et al., 2010).

The physiological mechanisms of the increase of number and quality of regenerating shoots in subsequent cultures remain unknown. They could be based on removal of the apical dominance of the excised axillary shoots, for which the addition of exogenous cytokinins alone was not sufficient.

In another medicinal species from the Campanulaceae, *Adenophora triloba*, Chen et al. (2001) achieved the most efficient shoot bud induction using internodal primary explants on MS medium with 8.88 μM BA and 0.54 μM NAA. In the second step of culture they observed enhanced axillary shoot multiplication (30 shoots per explant) from nodal segments of the adventitious microshoots. The optimal BA concentration was then 17.75 or 35.51 μM. They also tested a similar liquid medium; it gave an even higher number of axillary shoots (44), but strong hyperhydricity occurred.

In the rooting experiment (results are given in Tab. 3), the use of full-strength MS medium gave a lower percentage of rooting shoots, so the experiment was continued using only 50% MS macroelements. Also, adding IBA did not yield a satisfactory

TABLE 3. Effect of media composition on rooting efficiency in shoots derived from axillary shoot explants. Rooting was recorded 42 days after inoculation. Percentage data are means of all plantlets for each treatment (n=50); ±SE

MS macroelement strength	Sucrose (g/L)	Auxin (μM)	Charcoal (g/L)	% of rooting shoots	Average root number/ rooted plantlet	% of surviving explants	% of rooted explants surviving after potting
1/2	20	–	–	20	6.9±0.2	50	
			+	10	1.4±0.1	66	
		IBA 5	–	52	4.9±0.3	100	64
		IAA 5	–	56	3.0±0.1	68	36
		IAA 10	–	100	callus overgrowth	100	
	40	–	–	34	5.0±0.0	100	
			+	0	0	100	
		IAA 5	–	80	4.0±0.0	92	
			+	20	1.2±0.1	96	
		IAA 10	–	88	callus overgrowth	94	
	60	–	–	22	4.5±0.2	90	
		IAA 5	–	98	4.5±0.1	100	90
			+	0	0	100	
		IAA10	–	100	callus overgrowth	100	
full	20	–	–	18	7.3±0.1	80	
			+	8	1.5±0.1	56	
		IBA 5	–	48	6.2±0.4	84	

rooting percentage, even though it was significantly higher than the control (up to 50% and 20.0% respectively). Callus formed at the base in most of the shoots. IAA was more efficient, but the results were best not with IAA added alone but when the sucrose concentration was increased to 40 or 60 g/l.

The rooting frequency was highest (above 85%) for four media – IAA10S20, IAA5S60, IAA10S60 and IAA10S40 – but both roots and shoots became shorter and thicker with higher sucrose (40 or 60 g/l), which was favorable for transfer to soil (Fig. 2a). Moreover, the roots developed faster and were seen after two weeks, as compared to over four weeks in control medium. In the media with lower auxin and sucrose the rooting percentage was significantly lower and the roots were long and thin. Adding activated charcoal reduced both the rooting frequency and shoot growth. The rooting frequency dropped to 20% in IAA5S60 medium, and to 10% in the control (PGR-free, low sucrose). Moreover, in the media with charcoal, many shoots (10–20%, data not shown) died within 42 days.

Callus tended to form at higher auxin concentrations (10 and 20 μM) but the rooting frequency was not affected. However, the callus grew fast and often overgrew the shoot base, hindering transfer to soil. For that reason, only plantlets from the 5 μM auxin treatments were potted. The obtained efficiency was high, and sufficient for subsequent ex vitro acclimatization. The survival rate was significantly lower for plants from the low-sucrose medium (35%) than from the high-sucrose medium (90%). Probably the delicate root system in those plantlets was unable to cope with the soil conditions. The further development of the survivors after transfer to the greenhouse was similar. All plants survived transplanting to the garden plot (Fig. 2b).

Codonopsis pilosula presents a relatively high level of genetic diversity, even among cultivated populations (Zhang et al., 1999; Guo et al., 2007). In the closely related and more often investigated species *C. lanceolata*, molecular markers revealed significant genetic instability in plants regenerated from callus derived from a single donor plant (Guo et al., 2006). In this respect, using axillary bud induction should be advantageous, but so far no such data are available for *C. pilosula*. The present study will be continued after the micropropagated plants growing in the garden reach crop maturity (2–3 years), with the aim of determining the genetic and phenotypic fidelity of the regenerants and comparing their crop quality to that of conventionally grown plants. Previously only callus-based methods for in vitro propagation have been reported for *C. pilosula* (Niu et al., 1991; Li et al., 1993).

Using nodal segments as explants and applying repeated excision and subculture on BA-supplemented media, we developed an efficient protocol for mul-

tiplication of *C. pilosula* axillary shoots. The rooted plantlets were successfully transferred to soil. These results can be used to establish a simple and commercially viable protocol for mass propagation of *C. pilosula* for plantations or breeding purposes.

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