

Cold Storage of *Atractylodes ovata* Shoot Cultures and Evaluation of the Regenerated Plants

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Abstract

Atractylodes ovata (Compositae) shoot cultures were put into cold storage and the regenerated plants then evaluated. All the *in vitro* shoot cultures of *A. ovata* DC. survived cold storage at 2 °C for up to 24 months. The cold-stored shoots were then multiplied and rooted under normal culture conditions. The regenerated plants were cultivated in the field for one or two years and various biological and biochemical characteristics were compared with a control plant. Statistical analysis of the data showed no significant differences between the two groups in most morphological traits and the contents of the atractylon, a sesquiterpene component of essential oil in the rhizome. These results demonstrate that non-frozen cold storage of *in vitro* shoot cultures can be used as a germplasm preservation system for short- or medium-duration without deterioration of their biological and biochemical characteristics.

Key words: atractylon, Compositae, essential oil, germplasm preservation, micropropagation, refrigeration.

Abbreviations

BA, 6-benzyladenine; IAA, indole-3-acetic acid; LS, Linsmaier and Skoog (1965) medium; NAA, 1-naphthaleneacetic acid.

Atractylodes ovata DC. (*A. macrocephala* Koidzumi) is a valuable perennial medicinal plant distributed in China. Its rhizome is listed in Japanese and Chinese Pharmacopoeia and used as a digestive, diuretic, and antihidrotic (Tang and Eisenbrand, 1992). Atractylon is a major sesquiterpenoid component of essential oil in the rhizome of *A. ovata* and has various pharmacological activities, including antihepatotoxicity (Kiso *et al.*, 1983; Yamahara *et al.*, 1983), antiulcer action (Matsuda *et al.*, 1991) and cytotoxic activity (Wang *et al.*, 2002). *A. ovata* is generally propagated by seeds (Fukuda *et al.*, 1992) and requires cross fertilization for seed production. Consequently, a wide range of variation has been observed in the morphological and chemical characteristics of this species (Fukuda *et al.*, 1997). Furthermore, *A. ovata* is easily hybridized with other *Atractylodes* species (Fukuda *et al.*, 1995). Therefore, micropropagation seems an alternative option for producing true to type plants and

also for preserving the traits of a mother plant. Sung and Zheng (1989) and Hatano *et al.* (1990) reported the clonal propagation of *A. ovata*. However, no attempt has been made for *in vitro* preservation and assessment of regenerated plants. In our previous study using *A. lancea*, we reported plant regeneration starting from floral buds or shoot tips (Hiraoka *et al.*, 1984; Hiraoka and Tomita, 1990), cold storage of shoot cultures (Hiraoka, 1992) and biological and chemical evaluation of plants regenerated from refrigerated shoots (Hiraoka and Kago-shima, 1993). The present communication describes the non-frozen method for medium-term preservation of *in vitro* *A. ovata* shoots and assessment of regenerated plants. The primary focus was the effect of long-term cold storage of shoots on accumulation of secondary metabolites or pharmacologically active principles in plants raised from cold-stored shoot cultures.

A. ovata plants were a kind gift from Dr. T. Kimura, Daich College of Pharmaceutical Sciences and were maintained at the Medicinal Plant Garden, Niigata University of Pharmacy and Applied Life Sciences in Niigata city, Japan (Plant registration No. 913).

Throughout the present study, Linsmaier and

Skoog (1965) medium containing 3% sucrose and 0.8% agar was used as the basal medium. A culture tube (o.d. 13 x 130 mm) containing 20 ml of agar medium was capped with a silicone sponge and autoclaved for 20 min at 121 °C. All the cultures were incubated at 25 °C under fluorescent lamps producing an irradiance of $37 \mu\text{mol m}^{-2} \text{s}^{-1}$ at culture level over a 16 h photoperiod, except where otherwise stated. The meristematic parts having two leaf primordia of about 0.5 mm diameter were removed aseptically from two shoot tips of a single plant of *A. ovata* and inoculated on the basal

medium fortified with $10 \mu\text{M}$ NAA and $1 \mu\text{M}$ BA. Each 5 mm long shoot emerged was transferred to the shoot proliferating medium consisting of basal medium containing $1 \mu\text{M}$ IAA and $10 \mu\text{M}$ BA. Shoot cultures were maintained by subculturing every 1–2 months on the shoot proliferation medium under normal culture conditions. Shoot inocula were trimmed to about 1 cm in length (weight about 0.06 g) and inoculated singly. Ten shoots were cultured on shoot proliferation medium for 11 days before they were cold-stored at 2 °C under continuous illumination from a fluorescent lamp at



Fig. 1 Cold storage of *in vitro* shoots and field performance of regenerated plants of *Atractylodes ovata*. (A) Eleven day old precultured shoots for cold storage. (B) 24-month-old cold stored shoots. (C) Recultured shoots. (D) Rooted shoots. (E) Regenerated plants in planters and (F) 2-year-old regenerated plants cultivated in the field. (C–F) Left to right: control and cold stored.

irradiance of $5 \mu\text{mol m}^{-2} \text{s}^{-1}$. After one and two years of storage, each of 5 cultures was withdrawn from the cold room. During this period, control cultures were maintained by regular subculture under normal conditions. The cold-stored shoots were recultured for shoot proliferation. After three passages of subculturing on the shoot proliferation medium, cold-stored shoot cultures were inoculated on the rooting medium (LS agar medium containing no plant growth regulators) and incubated for 40 days. Shoots without cold storage inoculated into same rooting medium served as the control.

The rooted shoots raised from the shoots of both groups were transplanted into planters filled with a mixture of soil-vermiculite-sand (1:1:1 by volume) and covered with vinyl sheets for the first 10 days to maintain high humidity. After one month of cultivation in planters, the plantlets were transplanted to an experimental field in April 2001 for the first year field trial. The crops were harvested in early November of the same year and various traits were recorded. Rhizomes were dried at 60°C and stored at room temperature in a vinyl bag with "AGE-LESS" (Mitsubishi Gas Chemical Co. Inc.), an oxygen consuming reagent, till chemically analyzed. On the last day of November of the same year, all the remaining rhizomes were dug out of the soil and the roots discarded. The rhizomes were

arranged stem-side up in a wooden box, which was buried underground outdoors until the following spring. In early March 2002, the rhizomes were taken out of the wooden box and planted again for the second year field trial. All the plants were harvested on the last day of October of the same year and treated as above (Ministry of Health and Welfare, Japan, 1996). The atractylon content in the dry rhizome was determined by the gas chromatographic method, as reported previously (Hiraoka, 1995). The statistical significance of differences between the control and cold-treated groups were assessed using Student's *t* test.

The shoot culture of *A. ovata* was successfully established from shoot tips and subcultured into shoot proliferation medium (LS + $10 \mu\text{M}$ BA + $1 \mu\text{M}$ IAA). The average shoot proliferation rate in a one-month culture period was 3.2–4.0 shoots/explant during the first five years, which decreased to 2.0–3.0 shoots/explant thereafter. Shoots maintained for one year served as the plant material for the cold preservation experiments. Shoots were precultured for 11 days on the shoot proliferation medium before the start of cold storage at 2°C (Fig. 1A). The control shoot culture was maintained by subculturing under normal culture conditions without refrigeration.

Cold-stored shoots for 1 year were 5.0 cm long,

Table 1 Field trials of *Atractylodes ovata* plants regenerated from cold-stored shoot cultures.

Characteristics	First year of cultivation (n=12)		Second year of cultivation (n=10)	
	Control	Cold-stored	Control	Cold-stored
Aerial parts				
Plant height (cm)	22 ± 5	20 ± 3	31 ± 3	29 ± 6
Fresh weight (g)			59 ± 31	57 ± 44
Diameter of a stem (mm)	6.3 ± 1.3	6.4 ± 1.5	7.5 ± 2.1	8.1 ± 2.2
Number of nodes			17.5 ± 2.5	19.6 ± 2.3
Number of branches	3.1 ± 2.4	1.7 ± 1.8	8.2 ± 3.9	5.9 ± 3.2
Leaf length (mm)	82 ± 17	78 ± 8	95 ± 11	96 ± 8
Leaf width (mm)	28 ± 5	26 ± 4	55 ± 12	48 ± 14
Capitulum length (mm)	35 ± 2	33 ± 3	41 ± 4	39 ± 3
Capitulum width (mm)	22 ± 1	20 ± 2	18 ± 2	19 ± 3
Floret length (mm)	34 ± 1	33 ± 1	30 ± 2	29 ± 2
Rhizome				
Fresh weight (g)	10.6 ± 3.1	$15 \pm 5.2^*$	42.2 ± 14.3	42.7 ± 15.2
Dry weight (g)	4.3 ± 1.4	$6.2 \pm 2.3^*$	12.2 ± 5.0	13.2 ± 5.8
Dry wt./Fresh wt. ratio	40.3 ± 1.4	40.9 ± 2.1	29.3 ± 8.2	31.1 ± 6.9
Atractylon content (% DW)	0.7 ± 0.21	0.62 ± 0.14	1.83 ± 0.32	1.73 ± 0.89

The plants were raised from shoot cultures stored at 2°C for 24 months. Data represent mean $\pm$ SD.

Fresh weight and the number of nodes were not recorded in the first year.

* Significantly different from control at $p < 0.05$.

weighed 0.87 g, and consisted of 7.8 shoots over an average of 5 cultures. They appeared healthy and proliferated as vigorously as the control group when recultured. At the third passage of reculture following the cold storage, no significant difference in proliferation rate was found between the cold-stored strain (3.9 ± 2.4 shoots/culture) and the control (3.2 ± 1.5 shoots/culture).

After two years of cold storage, the remaining five cultures were withdrawn from the cold room (Fig. 1B). All the cultures except one, which was contaminated, survived the cold storage, although the leaves were partly deteriorated. The average weight, height, and number of shoots per culture were 1.17 ± 0.65 g, 3.8 ± 1.9 cm, and 4.3 ± 4.0 , respectively. No significant difference was found in the proliferation rate between the cold-stored strain (5.4 ± 1.9) and the control (4.4 ± 2.3) at the second passage of reculture (Fig. 1C). The rooting ratio of the shoots on the rooting medium was 98% (97/99), whereas that of the control was 87% (84/97). The number of roots per shoot was significantly ($p < 0.05$) higher in cold-stored group (4.0 ± 1.6) than the control (2.8 ± 1.8), however, no significant difference was found in root length (control, 40 ± 17 mm and cold-stored strain, 48 ± 11 mm) (Fig. 1D). The percentage of control plants that survived in the planters was 24% while 42% of the cold stored strain survived (Fig. 1E). The data from the first and second year of cultivation are shown in Table 1. The characteristics of both groups were very similar, except for rhizome weight (Fig. 1F). The second year crops were larger and heavier than the first year ones in vegetative organs. In particular, rhizome weight and atractylon content were markedly higher. No deviation was observed in the morphology of the leaf, bract and floret or rhizome of the two groups. The capitulum was bigger than the other species of *Atractylodes* and had fish-bone shaped bracts and reddish purple florets as in the original plants.

The reproducibility of any protocol depends on successful field performance and assessment of micropropagated plants. In previous studies on *A. lancea*, we found that a duration of 24 months seemed to be the limit of cold storage of *in vitro* shoot cultures under the same conditions as in the present study, since leaves began to deteriorate around this time (Hiraoka and Kagoshima, 1993). In the present study using *A. ovata*, the control and cold stored group showed the same morphological features including compound leaf shape at the middle and lower parts of the stem, characteristic bone shaped bracts, and red-purple florets. Although a difference in rhizome weight was observed

between the two groups in the first year crop, this was not the case in the second year crop. Flowering time was also the same between the two groups. The gas chromatogram of *n*-hexane extract of the rhizome was almost the same for the two groups and showed one main peak of atractylon and several small unidentified peaks. No significant differences in content were observed, as previously reported (Hatano *et al.*, 1990; Mizukami *et al.*, 1996; Fukuda *et al.*, 1997; Hiraoka *et al.*, 1997).

The present findings coincide with the data obtained with *A. lancea* (Hiraoka and Kagoshima, 1993) and clearly demonstrate that non-frozen cold storage of *in vitro* *A. ovata* shoots does not affect the morphological and biochemical features of the regenerated plants. Consequently, slow growth storage of shoot cultures at a temperature of a little above 0°C provides a dependable method for the medium-term preservation of plant resources without adverse effects on various characteristics, including secondary metabolite accumulation.

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