Atractylodes lancea Autotetraploids Induced by Colchicine Treatment of Shoot Cultures

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Received November 20, 1997; accepted January 13, 1998

Two strains of autotetraploid plants of *Atractylodes lancea* DC. (Compositae) were raised from the *in vitro* colchicine-treated shoot cultures, and field trials were performed to evaluate their growth and the amount of essential oil components in the rhizome in comparison with the corresponding diploids. The tetraploid plants had larger leaves than the diploids. One of the selected tetraploid lines had about 1.5 times as heavy rhizomes as the diploid and contained atractylodin, hinesol and β -eudesmol in the rhizome to as great an extent or slightly less than the diploids. However, the contents of these constituents in the rhizome of the other tetraploid strain were lower than the diploids. The chloroplast number per guard cell, stomatal length, and stomatal density of leaf lower epidermis of the shoot cultures were good indicators for distinguishing tetraploids from diploids.

Key words *Atractylodes lancea*; tetraploid; micropropagation; colchicine treatment; hinesol; atractylodin

The addition of genomes sometimes results in more luxuriant growth in plants.¹⁾ Chromosome doubling also affects the yield of secondary metabolites, *e.g.* increased contents of tropane alkaloids in *Datura*,^{2–4)} of thebaine in *Papaver bracteatum* LINDL.⁵⁾ and of tanshinones in *Salvia miltiorrhiza* BUNGE,⁶⁾ as well as a decreased content of leptine glycoalkaloids in *Solanum chacoense* BITTER.⁷⁾ Considering these facts, ploidy breeding seems to be profitable for the production of certain organs of medicinal plants which are used as crude drugs.

Atractylodes lancea DC. is a perennial herb of the family Compositae which originated in China. Its rhizome is used as a crude drug in Chinese and Japanese traditional medicinal prescriptions. The plants cultivated in Japan are female and are propagated by division of the rhizome. The author successfully micropropagated the plant⁸⁾ and later reported that micropropagation yields clonal plants comparable to those obtained by conventional propagation methods.⁹⁾ In the present study, the author raised tetraploid *A. lancea* plants from colchicine-treated *in vitro* shoot cultures and compared their various morphological characteristics and essential oil component contents of the diploid and tetraploid plants cultivated in the field in order to establish strains of better botanical and/or chemical qualities. To obtain simple markers which make it possible to select tetraploids preliminarily out of colchicine-treated shoots prior to chromosome counting, the significance and variation of chloroplast numbers in a guard cell and the stomatal traits of leaf lower epidermis of plants at various developmental stages will also be discussed.

MATERIALS AND METHODS

Plant Materials Shoot cultures started from a shoot apex of *A. lancea* (strain At) were maintained on Linsmaier-Skoog agar medium¹⁰⁾ supplemented with 1 μ M benzylaminopurine by subculturing them at 1- to 2-month intervals, as described earlier.⁸⁾

Induction of Polyploid Plants Twenty 4-week-old shoot cultures were trimmed to 1 cm long. These shoots were incubated in 50 ml of growth regulator-free Linsmaier–Skoog liquid medium containing 0.3% colchicine on a reciprocating

shaker (90 strokes per min, 70 mm strokes) for 8 h, and then washed for 16 h in the same manner except the second medium lacked colchicine.¹¹⁾ Each treated shoot was multiplied on the agar medium mentioned above.

Plant Regeneration and Field Trials At the 5th passage, shoots were rooted on plant growth regulator-free LS agar medium. Part of root tips of the plantlets were fixed for chromosome observation before they were potted. The potted plants were cultivated for 5 years. Among these plants, two plants (designated as 5B and 5D) of line 5 were selected. These two plants and the control plant (At), which had been maintained in a pot by division of the rhizome, were micropropagated by the methods described previously,⁹⁾ starting from a shoot apices. At the 13th passage after shoot culture initiation, shoots were rooted as mentioned above and the plantlets were potted and cultivated in a greenhouse for 4 months. The regenerated plants of the 3 lines were transplanted to an experimental field on November 7, 1992 according to a randomized block experiment. There were 30 plants per each line in a block and 4 blocks were designed. Forty plants of each line were randomly selected and growth measurements of the aerial parts were recorded on September 10, 1996 at their flowering stage. The data included the number of stems, plant height, number of leaf nodes, number of branches, number of capitula, and length and width of the leaf attached to the middle of the longest stem. The plants of two blocks were harvested on November 26, 1996 and the remainder on April 21, 1997. Forty plants of each line were randomly selected each harvest time. The rhizome and the root were weighed separately and root numbers were counted before they were dried at 60 °C for 5 d.

Data on Leaf Epidermis The leaf lower epidermis of cultured shoots, potted plantlets and field-grown plants was peeled off by a pair of forceps and mounted in water on a slide glass. For each lot of samples, 300 stomata (3 stomata $\times$ 10 plants) and 300 epidermal cells were randomly chosen and their lengths were measured and chloroplast numbers per guard cell counted under a light microscope. Stomatal density was determined by counting the number of stomata found in a microscopic field at $\times 400$ (0.42 mm²), observing 300 fields per each lot of samples.

Chromosome Observation The root tips of *in vitro* plantlets were treated with 2 mM 8-hydroxyquinoline for 4 h at 18 °C, rinsed twice with water, and fixed with Newcomer's fluid.¹²⁾ The slides for microscopic observation of chromosome number were prepared by the Feulgen squash method.

Quantification of Essential Oil Components Atractylon, β -eudesmol, hinesol, and atractylodin contents in the rhizome were determined by the gas chromatographic method reported earlier.¹³⁾ Forty samples of the 1996 harvest

and 20 samples of the 1997 harvest were analyzed.

Statistical Analysis The significance of difference between groups was calculated by applying Student's *t*-test.

RESULTS AND DISCUSSION

Selection of Tetraploid The colchicine-treated shoot cultures of *A. lancea* were micropropagated for 4 passages after the treatment and rooted at the next passage. The ploidy distribution based on the chromosome number in root tip cells is shown in Fig. 1. Four lines, whose numbers do not appear in the figure, deteriorated during subculture, probably because of the toxic effects of colchicine. Line 20 was not affected by the treatment as far as chromosome number is concerned. Four lines, 5, 6, 11, and 13, consisted solely of tetraploids. The other lines consisted of tetraploids and other ploidies. All the mixoploids observed here had triploid and tetraploid cells. Figure 2A shows the diploid, tetraploid, and mixoploid plants in pots. The tetraploid plants had broader leaves than the diploids. The mixoploid plant is characterized by undulatory leaves. Figure 2B shows chromosomes at metaphase in a root tip cell of a tetraploid plant. The basic number of chromosomes was 12 according to karyotype analysis of chromosomes in the mitotic metaphase of the control plants, as was the case in *A. macrocephala* Koidz. (*A. ovata* DC.).¹⁴⁾ Most chromosomes had centromeres at median or submedian positions, and no SAT-chromosomes, as observed in *A. macrocephala*, were found.

In order to select solid and elite tetraploid plants, regenerated plants of lines 5, 6, 11, and 13 were cultivated for 5

Fig. 1. Ploidy Distribution of *Atractylodes lancea* Plantlets Regenerated from Colchicine-Treated Shoot Cultures

Fig. 2. Characteristics of Diploid and Tetraploid *Atractylodes lancea*

A, Diploid, tetraploid, and mixoploid plantlets. B, 48 chromosomes in a root-tip cell of a tetraploid plant. C, Rhizomes of At (upper) and 5B (lower) plants harvested in April of the fifth year of cultivation. The lightest (left), average (center), and heaviest (right) rhizomes of each strain are shown. D, Lower epidermis of a diploid leaf. 1 unit of the micrometer = 2 μ m. E, Lower epidermis of a tetraploid leaf. 1 unit of the micrometer = 2 μ m. F, Leaves of At (diploid, top), 5B (tetraploid, middle), and 5D (tetraploid, bottom) plants grown in the field. G, A flowering tetraploid plant (line 5B).

years, since it was thought from the data on chromosome counts that these had a higher possibility of being solid tetraploids than the other lines. It will be possible to shorten the pot-cultivation period for the evaluation of plant growth to a few years instead of 5 years employed in the present study. Two plants, named 5B and 5D, both of which were tillers of tetraploid line 5, were selected because of the normal appearance of their aerial parts and better growth habit. Figure 2G shows a flowering plant of line 5B thus selected.

Micropropagation and Field Trials Since the proliferation rate of *A. lancea* by division of the rhizome is very low, 5B, 5D, and a control plant (At) were multiplied through micropropagation to obtain enough plant materials for field trials within a short period. The average monthly shoot multiplication rates of lines At, 5B and 5D during the first 12 passages were 2.3, 1.7, and 1.9, respectively, and there were no significant differences between the control and either of the tetraploid lines. The rooting ratios of lines At, 5B and 5D at the 13th passage were 99 (248/250), 95 (237/250) and 99% (247/250), respectively. After a cultivation period of 4 years, 93% (112/120) of At, 93% (111/120) of 5B, and 87% (104/120) of 5D plants survived in the field.

Characteristics of Tetraploids Various morphological characters of the aerial parts of diploids and tetraploids were recorded at the flowering stage in September of the fourth year of cultivation in the field, and the underground parts were harvested in November when the aerial parts withered. The plants of lines 5B and 5D were significantly ($p < 0.001$)

taller and significantly ($p < 0.05$) shorter than the At plants, respectively. No significant difference was found in the number of shoots per plant among the three lines (Fig. 3A). The node numbers on the longest stem were significantly different between the diploid and either of the tetraploid lines (Fig. 3B). Marked differences were found in leaf size: small slender leaves of At and large wide leaves of 5B and 5D plants (Figs. 2F, 3C, D). These differences were reflected in the length/width ratios of the leaf; those of At, 5B and 5D were 3.7 ± 0.6 , 2.8 ± 0.3 , 2.4 ± 0.4 , respectively. Although the number of capitula was nearly the same (about 20) between the control and 5B, that of 5D was extremely small (7), reflecting significantly ($p < 0.001$) fewer branches of 5D plants than those of At or 5B. The tetraploid plants of both lines flowered 1–2 weeks earlier than the control diploid plants.

As for the underground parts, the rhizome harvested in 1996 was significantly heavier (about 50%) in 5B than in At, but not in 5D (Figs. 2C, 3E). The rhizome dry/fresh weight ratio was similar (about 30%) among the three lines. The root number decreased when a ploidy level increased. Roots of the tetraploids were thicker, especially in 5B, than those of the diploid. Since the atractylon contents of the rhizome were negligible (not more than 0.05% of dry weight) in all three lines, the contents of atractylodin, hinesol, and eudesmol in the rhizomes harvested in 1996 are shown in Figs. 3F–H. The plants of line 5B contained the same levels of these three components. On the other hand, the contents of all these components were significantly lower in line 5D than in At.

Fig. 3. Evaluation of *Atractylodes lancea* Tetraploids

A–D: Recorded on September 10, 1996. E–H: Harvested on November 26, 1996. I–L: Harvested on April 21, 1997. Each bar represents the mean $\pm$ S.E. of 40 (A–H) or 20 (I–L) plants. Significantly different from At (2x): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 4. Ploidy Markers of Abaxial Epidermis of the Leaf Discriminating Tetraploid (5B and 5D) from Diploid (At)
 —, At shoot culture; ----, At potted plant; ·····, 5B shoot culture; ---, 5B potted plant; ·····, 5D shoot culture.

For the preparation of crude drugs, rhizomes of *A. lancea* are generally harvested in late autumn in Japan,¹⁵⁾ and twice a year, in spring and autumn, in China.¹⁶⁾ Therefore, in the present study, the rhizomes were harvested also in April. Figures 3I—L give the data on the 1997 harvest. Contrary to the previous results, the rhizome atractylodin content of line 5D was comparable to that of At and the hinesol and eudesmol contents of line 5B were significantly ($p < 0.05$) lower than those of At. The hinesol and eudesmol contents of line 5D were markedly ($p < 0.001$) lower than those of At, as was the case in the 1996 harvest.

Ploidy Markers For exploiting simple markers to select tetraploid shoots preliminarily among the colchicine-treated shoot cultures, several leaf traits were examined. As the stomata of the leaf were distributed scarcely in the upper epidermis, all the data were collected from lower epidermis. Figure 4 gives typical examples of leaf epidermis characteristics. The chloroplast number per guard cell was 9.6 ± 1.6 (mean $\pm$ S.D.), 14.9 ± 3.0 and 18.2 ± 2.9 in At, 5B and 5D shoot cultures, respectively, making it possible to discriminate between diploid and tetraploid shoots (Fig. 4A). These shifted to smaller numbers when the plants were cultivated in pots, especially in the tetraploids. The plants cultivated in an experimental field gave similar frequency distribution data on chloroplast number per guard cell as the potted plants. Stomatal length was a good ploidy marker, too (Fig. 4B). Its frequency distribution patterns resembled those of chloroplast number per guard cell, except that almost no distribution shift occurred in the diploid plants of different cultivation stages. Also in this category, line 5D gave results similar to line 5B. These findings coincide with the data by Francis *et al.*¹⁷⁾ that heritable differences were found for the leaf mesophyll cell plan area and chloroplast numbers of ryegrass, and those by Pyke and Leech,¹⁸⁾ that the chloroplast number in leaf mesophyll cells is determined by the size of the cell. Both ploidy levels were distinguishable also by the stomatal

density in the lower epidermis of the leaf, as shown in Fig. 4C, although the highest frequency differed depending on the line of the tetraploids. These three kinds of markers did not change among the shoots of different passages of the same strain, but changed from strain to strain, though this fluctuation did not reduce their usefulness as ploidy markers (*e.g.* Figs. 4A and C). The chloroplast number per guard cell and stomatal length of the leaf epidermis of potted or field-grown plants shifted to smaller ranges than those of shoot cultures, indicating that these markers should be compared between shoots/plants of the same developmental stage. The decrease in chloroplast number per guard cell of the potted plants and field-grown plants may be attributed to a decrease in stomatal size.¹⁸⁾ It is well known that tetraploid plants have bigger cells than diploids.¹⁾ However, epidermal cell size was not a good marker for this purpose since its distribution among the tetraploids of the present plant materials overlaps with that of the diploids to a considerable extent (Fig. 4D).

Other leaf traits failed to discriminate ploidy levels. For example, the leaf width of shoot cultures was similar, irrespective of ploidy levels, although it was clearly different between diploids and tetraploids in the case of potted or field-grown plants. The chlorophyll content exhibited no significant difference among different ploidy genotypes (data not shown).

In conclusion, an autotetraploid line of *A. lancea* bearing bigger rhizomes than, and containing comparable amounts of essential oil components with, diploid plants was obtained from colchicine-treated shoot cultures. Prior to chromosome counting, tetraploid shoots can be preliminarily selected on the basis of chloroplast number per guard cell, stomatal length, and stomatal density in the lower epidermis of the leaf as ploidy markers. Since autotetraploids contain no foreign genes, there are no risks of gene contaminations when they are cultivated in the field. The selected strain with bigger rhizomes in comparison to diploid lines will be profitable

as an agricultural product, providing that it satisfies the standards of Japanese Pharmacopoeia such as ash and acid-soluble ash, which are not evaluated in the present study.

Acknowledgements The author acknowledges the financial support of Kampo Iyaku Kenkyu Shinko Zaidan.

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