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Evaluation of Sesquiterpenoids Content and Growth Characters in Clonal Lines from a Cross between *Atractylodes japonica* Koidz. ex Kitam. and *A. macrocephala* Koidz.

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ABSTRACT : Two *Atractylodes* species, *A. japonica* Koidz. ex Kitam. (AJ) and *A. macrocephala* Koidz. (AM) were used in this study. AJ population had higher amounts of sesquiterpenoids and stronger tolerance to root rot but less vigor of root growth than AM population. Two populations (AJ and AM) were crossed to make interspecific hybrid population. A total of 98 lines propagated clonally were selected from a cross of AJ and AM, and evaluated for contents of sesquiterpenoids, atractylon (ATLN) and atractylenolide III (AT3) using high performance liquid chromatography (HPLC), and growth characters such as plant height, stem number and root weight. HPLC profiles of the hybrids were compared with those of parent plants, and it demonstrated the production of introgression hybrid by crossing between AJ and AM. Of 98 clonal lines, 10 lines were selected by 10% level based on the growth vigor and tolerance to root rot, and AJM2102-51 line showed the heaviest root weight (117.1 g/plant) among them. A total of 98 hybrid lines contained on average 0.16 ± 0.10 ㎎/g of AT3, 2.00 ± 1.37 ㎎/g of ATLN, and 2.16 ± 1.40 ㎎/g of total sesquiterpenoids, showing high coefficients of variation (above 65 %). Ten lines having high contents of sesquiterpenoids were selected, and AJM2101-15 had the highest amount (9.83 ㎎/g) of ATLN, and showed 40.8 g/plant of root weight similar to mean value (39.9 g/plant) of hybrid lines. The result showed that the introgression of both characters of vigorous growth from AM and high sesquiterpenoids content from AJ could be possible to make new hybrid lines by crossing between AJ and AM.

***Key words* :** Atractylodes rhizome, hybrid population, sesquiterpenoids, *Atractylodes japonica*, *Atractylodes macrocephala*, atractylon, atractylenolide III

INTRODUCTION

Atractylodes rhizome has been used as herbal medicines mainly for the treatment of stomach disorders in Korea. They are roots of *Atractylodes* species and used as crude drugs of two types, 'Backchul' and 'Changchul' in Korea. 'Backchul' is the rhizome of *A. japonica* Koidz. ex Kitam. (AJ) and *A. macrocephala* Koidz. (AM), and 'Changchul' is that of *A. lancea* (Thunb.) DC. and *A. chinensis* Koidz. (AC) (The 8th Korean Pharmacopoeia, 2002). Recently, it is on record that AJ is the same species as *Atractylodes ovata* (Thunb.) DC. (Nat'l Plant List Committee, Korea National Arboretum), while AM has been written as *A. ovata* (Thunb.) DC. in most of reports. Many studies related to the molecular discrimination of *Atractylodes* species, 'Backchul' and 'Changchul' were reported because clear explanation for botanical origin of herbal medicines was very important in the herbal market and clinical use (Bang *et al.*, 2003; Shiba *et al.*, 2006). AJ has been used in Korea and Japan as a 'Backchul' while AM has been used in China. Two *Atractylodes* species are perennial herbal plants and can be cultivated by both seeding and vegetative propagations. Both species were known to contain volatile essential oil as bioactive constituents in their rhizomes (Chung *et al.*, 2004). The essential oil composed of sesquiterpenoids such as atractylon, atractylenolide I, II and III (Tang & Eisenbrand, 1992), and these sesquiterpenoids were evaluated as a quality factor in Atractylodes rhizome using high performance liquid chromatography (HPLC) and gas chromatography (GC) (Fukuta *et al.*, 1998; Suto *et. al*, 1998; Wang *et al.*, 2006). Wang *et al.* (2002) reported that atractylon and atractylenolide I were the major cytotoxic principle constituents of *A. ovata* (Thunb.) DC. on leukemia cell lines. In general, AJ population had stronger tolerance to Phytophthora rot and excess water injury of root than AM (Cho *et al.*, 2001) while AM had more vigorous growth characters in both of shoot and root to increase root yield. It has been known that two *Atractylodes*

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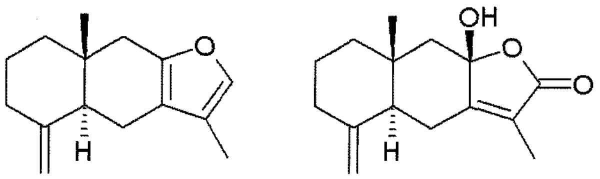
species are allogamous plants. AJ and AM populations had great variation of agronomic characters and diverse degree of susceptibility to Phytophthora root rot in the filed (Cho *et al.*, 2001; Huh *et al.*, 2002; Kim *et al.*, 2002). Individual variation of the external morphological characteristics, growths, and the amounts of chemical substances such as essential oil and sesquiterpene compounds were evaluated for *A. ovata* (Thunb.) DC. population (Fukuta *et al.*, 1997). Fukuta *et al.* (1995) reported that *A. ovata* (Thunb.) DC. produced hybrids through pollen contamination with *A. japonica* Koidz. ex Kitam. and *A. lancea* (Thunb.) DC., and that the morphological characteristics of the hybrids were all intermediate in those of their parent plants. Low yield of individual root due to susceptibility to root rot was the limiting factor in cultivating Atractylodes rhizome. So it is imperative to develop good variety having tolerance to root rot and high root yield, and also with enhanced content of sesquiterpenoids.

The objectives of this study were to evaluate growth characters and sesquiterpenoids content in interspecific hybrid population between AJ and AM, and to select elite lines having high amount of sesquiterpenoids and high root yield from a cross population.

MATERIALS AND METHODS

Production of hybrid lines from a cross population

Parent plants used for crossing were selected from two populations of genus *Atractylodes*, *A. japonica* Koidz. ex Kitam. (AJ) and *A. macrocephala* Koidz. (AM). AJ population was collected in Korea and AM was introduced from China. Two populations have been maintained under selection for growth characters and disease tolerance at the experimental field, National Institute of Crop Science, RDA, Suwon, Korea. Superior lines with stronger tolerance to root rot were selected from two population, respectively, and used for crossing, which were AJ2106 in AJ as pistillate parent and AM9912 in AM as pollen parent. Two lines, AJ and AM, were crossed and then a total of 527 F_1 seeds were harvested in 2001. Interspecific hybrid population (434 plants of F_1) was grown in non-heated plastic film house in February 2002. A total of 98 elite lines from hybrids were selected for growth characters and disease tolerance, and maintained clonally at the field in 2002 and propagated to make breeding lines in 2003. In 2004, Ninety-eight clonal lines were investigated for growth characters such as root weight, stem diameter, and contents of sesquiterpenoids such as atractylon, atractylenolide III, in addition to 10 lines of AJ and 2 lines of AM population including parent lines used for crossing.



Atractylon
Mw = 216

Atractylenolide III
Mw = 248

Fig. 1. Chemical structures of atractylon and atractylenolide III isolated from *Atractylodes japonica* Koidz. ex Kitam. roots.

HPLC analysis

For determination of two sesquiterpenoids, atractylon and atractylenolide III, approximately 25 g of each line was freeze-dried and homogenized using a homogenizer. Samples (2 g) were extracted in 30 mL of 80% methanol in water for 1 day with shaking at 100 rpm, and filtered. The residues were extracted two times more, and final volume of each extract solution was exactly adjusted to 100 mL. Methanol extract was stored for 1 day at freezing room, and the supernatant was transferred to 2 mL autosampler bottle before HPLC injection for sesquiterpenoids analysis. The HPLC instrument (HPLC Agilent 1100 Series, Agilent Technologies Co., USA) equipped with an ultraviolet-visible detector (Agilent 1100 Series Diode-Array Detector) at 220 ㎚ and a reversed-phase column, Capcell Pak C18 UG 120 (5 ㎛, 4.6×150 ㎜, Shiseido Co., Japan) was used. The mobile phase was a linear gradient from solvent A, acetonitrile: water (50 : 50, v/v), to solvent B, acetonitrile: water (90 : 10, v/v), in 40 min and flow rate was set at 1.0 mL/min. Running time of each sample was within 60 min. Sesquiterpenoids, atractylon (ATLN) and atractylenolide III (AT3) (Fig. 1), were isolated from hexane extract of *Atractylodes japonica* Koidz. ex Kitam. rhizome using open column chromatography, and used as standard compounds for HPLC analysis. Total sesquiterpenoid was the sum of ATLN and AT3 contents.

RESULTS AND DISCUSSION

HPLC profiles

Chromatographic profiles of a hybrid and two parents (AJ and AM) were shown in Fig. 2. Peaks of AT3 and ATLN appeared at 6.4 min and 28.0 min, respectively. HPLC profile at 220 ㎚ of ultraviolet-visible wavelength in AJ was similar to that of AM, and peak heights of AT3 and ATLN in AJ were higher than those in AM. Three peaks (S1, S2 and S3) on each chromatogram made differences between parents and a hybrid. The peak of S2 at 10.0 min and the peak of S3 at 15.2 min appeared in AJ and AM, respectively, and each peak showed

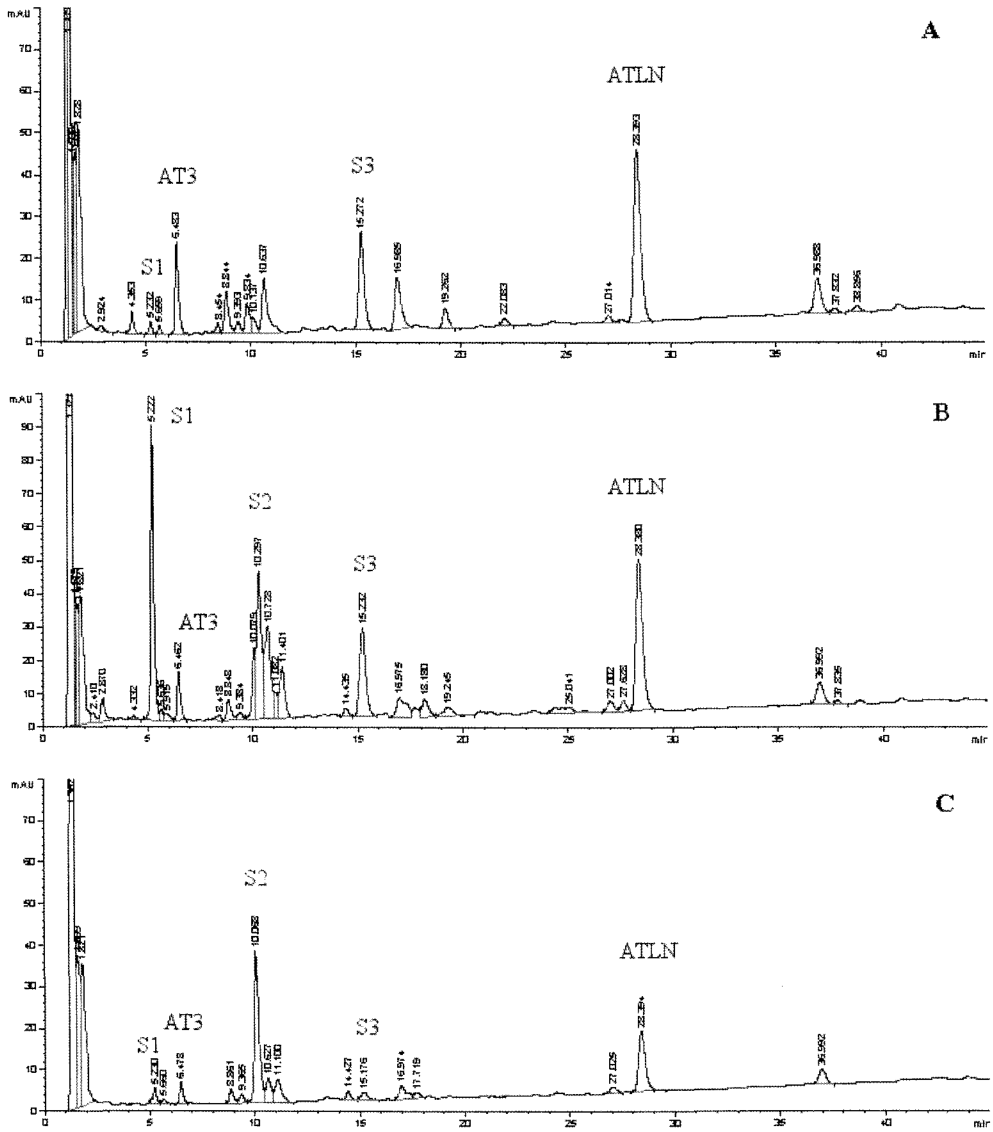


Fig. 2. HPLC profiles of 80% methanol extract solutions from roots of a hybrid plant (AJM2103-12; B) and two populations, *Atractylodes japonica* Koidz. ex Kitam. (Hamyang; A) and *A. macrocephala* Koidz. (AC2201; C). ATLN, atractylon; AT3, atractylenolide III; S1, S2 and S3 were explained in text.

trace inversely in chromatogram, whereas both peaks of S2 and S3 showed highly on HPLC profile of a hybrid. Further analysis with LC-MS (HCTultra, Bruker Daltonics Inc., USA) elucidated that the peaks of S2 and S3 were sesquiterpenoids similar to ATLN and AT3 although their chemical structures were not clearly identified. The peak of S1 at 5.2 min was relatively great in all profiles of hybrid lines, and also appeared in two parent plants showing various height of that peak. However, we think it may be an oxidized substance derived from other sesquiterpenoids due to their chemical unstability. These HPLC profiles provided the possibility of introgression hybrid with increased and varied compounds by crossing of AJ and AM.

Growth characters of parents and hybrid lines

Growth characters of parent lines, AJ2106 and AM9912, were displayed in Table 1. AM had stronger vigor in overall growth characters such as stem diameter, branch number, leaf area, root weight, size of inflorescence than AJ, while AJ had stronger tolerance to Phytophthora root rot than AM, as reported by previous study (Cho *et al.*, 2001). Flower color of AJ population was white while that of AM was reddish, and most of hybrids had reddish flower.

A total of 98 clonal lines were selected based on growth vigor and disease tolerance for interspecific hybrid population. Table 2 showed growth characters of clonal lines derived from

Table 1. Growth characters of two parents used for crossing between *Atractylodes japonica* Koidz. ex Kitam. and *A. macrocephala* Koidz. plants.

Parents	Plant height (cm)	Stem diameter (mm)	No. of stems	No. of branches	Length of branches (cm)	Leaf length (cm)	Leaf width (cm)	Length of inflorescence (mm)	Disease incidence rate† (%)	Root weight‡ (g/plant)
AJ2106 (♀)	35.1	1.7	4.8	1.3	2.4	6.2	2.9	19.3	8.3	28.9
AM9912 (♂)	35.3	6.6	2.0	9.0	20.3	8.4	3.6	33.1	17.3	36.2

AJ2106 line as a pistillate parent selected from AJ (*Atractylodes japonica* Koidz. ex Kitam.) population; AM9912 line as a pollen parent selected from AM (*A. macrocephala* Koidz.) population; †Disease was rhizome rot caused by *Phytophthora drechsleri* Tucker (Cho et *al*., 2001); ‡Expressed as grams of fresh root.

Table 2. Mean, standard deviation and coefficients of variation of growth characters in the hybrid population between *Atractylodes japonica* Koidz. ex Kitam. and *A. macrocephala* Koidz. plants.

Characters (n = 98)	Plant height (cm)	Stem diameter (mm)	No. of branches	Leaf length (cm)	No. of stem	Top weight† (g/plant)	Flowering date	No. of flowers	Length of inflorescence (mm)	Root weight‡ (g/plant)
Mean	32.0	3.2	2.6	6.1	6.3	17.3	17 Oct.	16.1	23.0	39.9
STD	6.5	1.2	1.5	1.1	2.9	10.8	5.5	6.3	3.1	20.6
Min.	13.0	1.3	1.0	3.3	1.0	2.9	1 Oct.	8.0	15.1	11.8
Max.	48.0	7.5	7.2	8.5	15.0	54.2	25 Oct.	42.0	33.5	117.1
CV	20.4	36.4	56.4	17.3	46.7	62.2	54.1	39.4	13.5	51.6

†Expressed as grams of fresh shoot; ‡Expressed as grams of fresh root; CV, coefficients of correlation, which was expressed as percentage.

Table 3. Means of growth characters of 10 clonal lines selected by 10% level based on the disease tolerance and growth vigor in hybrid populations of *Atractylodes* species.

Clonal lines	Plant height (cm)	Stem diameter (mm)	No. of stems	Flowering date	Leaf length (cm)	No. of branches	Top weight† (g/plant)	Root weight‡ (g/plant)
AJM2101-20	33	1.9	3	18 Oct.	7.5	8	23.3	75.5
AJM2101-27	32	3.2	1	25 Oct.	6.0	9	14.7	70.8
AJM2102-01	47	7.2	5	25 Oct.	6.3	8	53.9	89.0
AJM2102-11	35	3.1	1	15 Oct.	5.3	7	47.7	71.5
AJM2102-51	32	2.2	4	3 Oct.	5.2	3	52.4	117.1
AJM2102-52	31	7.5	3	2 Oct.	5.1	4	30.5	71.7
AJM2103-03	28	2.3	4	1 Oct.	6.8	4	28.4	78.8
AJM2103-15	41	3.8	4	12 Oct.	6.3	10	48.0	80.7
AJM2106-02	31	3.9	3	24 Oct.	6.7	4	22.5	81.7
AJM2107-06	37	4.0	3	15 Oct.	6.7	6	36.0	72.8
Mean	34.7	3.9	3.1	14 Oct.	6.2	6.3	35.7	81.0

†Expressed as grams of fresh shoot; ‡Expressed as grams of fresh root; Number of hybrid lines was ninety-eight.

a cross of AJ and AM population. Mean value of each character was 32.0 ± 6.5 cm of plant height, 3.2 ± 1.2 mm of stem diameter, 6.3 ± 2.9 of stem number, 17.3 ± 10.8 g of top weight per plant, and 39.9 ± 20.6 g of root weight per plant, showing very large variation. Flowering date was on average 17 October and number of flowers was approximately 16. Of 98 clonal lines, 10 lines as elite lines were selected by 10% level based on growth vigor and tolerance to root rot, and AJM2102-51 line had the highest root weight (117.1 g/plant) among them (Table 3).

We could observe that the size and shape of rhizome and number of small rhizomes were so varied in the field as growth years increased (data not shown). There was little information related to genotypic and environmental effects on

Table 4. Means and standard deviation of sesquiterpenoids contents of parents and hybrid populations of *Atractylodes* species.

Populations	N	ATLN (mg/g)	AT3 (mg/g)	Total (mg/g)
AJ	10	1.992 ± 1.038	0.178 ± 0.140	2.169 ± 1.073
AM	2	0.635 ± 0.592	0.082 ± 0.012	0.717 ± 0.603
Hybrid	98	2.002 ± 1.369	0.155 ± 0.100	2.157 ± 1.394
Mean	110	1.976 ± 1.339	0.156 ± 0.104	2.132 ± 1.366

N, number of lines; AJ, the population of *Atractylodes japonica* Koidz. ex Kitam.; AM, the population of *A. macrocephala* Koidz.; Hybrid, the hybrid population between AJ and AM; ATLN, atractylon; AT3, atractylenolide III; Total, total sesquiterpenoid, which was the sum of ATLN and AT3.

Table 5. Mean contents of sesquiterpenoids of 10 clonal lines selected by 10% level based on the high sesquiterpenoids contents in hybrid populations of *Atractylodes* species.

Rank	Clonal lines	ATLN (mg/g)	AT3 (mg/g)	Total (mg/g)
1	AJM 2101-15	9.830	0.102	9.932
2	AJM 2113-15	5.241	0.144	5.385
3	AJM 2107-08	5.223	0.161	5.384
4	AJM 2107-07	4.770	0.280	5.050
5	AJM 2103-12-01	4.841	0.119	4.960
6	AJM 2106-02	4.412	0.203	4.614
7	AJM 2103-12	4.210	0.266	4.475
8	AJM 2103-16	3.845	0.181	4.026
9	AJM 2109-01	3.652	0.242	3.894
10	AJM 2107-35	3.683	0.202	3.885
Mean		4.971	0.190	5.161

ATLN, atractylon; AT3, atractylenolide III; Total, total sesquiterpenoid, which was the sum of ATLN and AT3; Number of hybrid lines was ninety-eight.

variation of growth characters and accumulation of chemical constituents in Atractylodes plants. Therefore, to understand the heritabilities of useful characters investigated in this study, we think that it is very important to know environmental factors; aging and morphological changes of root influencing on root yield and sesquiterpenoids content, and seasonal changes of growth and constituents of root as growing time passes. These are the questions to be solved in the future in order to select superior lines with genetic trait of high root weight in Atractylodes plants.

Sesquiterpenoids content of hybrid lines

Table 4 showed sesquiterpenoids contents of parents and hybrid lines that had been propagated clonally. Ten lines of AJ and two lines of AM including parent lines used for crossing were evaluated for sesquiterpenoids, ATLN and AT3, and also a total of 98 hybrid lines selected for elite characters were evaluated using HPLC. Means of ATLN and AT3 were 1.99 ± 1.04 mg/g and 0.18 ± 0.14 mg/g in AJ lines, and 0.64 ± 0.60 mg/g and 0.08 ± 0.01 mg/g in AM lines, respectively. Total sesquiterpenoids content (2.17 mg/g) of AJ was higher than that (0.72 mg/g) of AM. A total of 98 hybrid lines contained 0.16 ± 0.10 mg/g of AT3, 2.00 ± 1.37 mg/g of ATLN, and 2.16 ± 1.40 mg/g of total sesquiterpenoid, showing high coefficients (over 65%) of variation (Fig. 3). Sesquiterpenoids contents of 10 lines selected by 10% level were shown in Table 5. Both of clonal lines selected by 10% level based on the growth characters and sesquiterpenoids content did not overlap each other, being an unexpected result. However, AJM2101-15 had the highest content (9.83 mg/g) of ATLN, showing 40.8 g/plant of root weight that was similar to mean value (39.9 g/plant) of 98 clonal lines. Elite lines selected in this study should be tested for tolerance to root rot and probably can be used as parent for crossing to breed high quality line of Atractylodes plant, considering plant vigor.

In conclusion, the result showed that both characters of vigorous growth from AM and high sesquiterpenoids content from AJ could be simultaneously introduced to make new

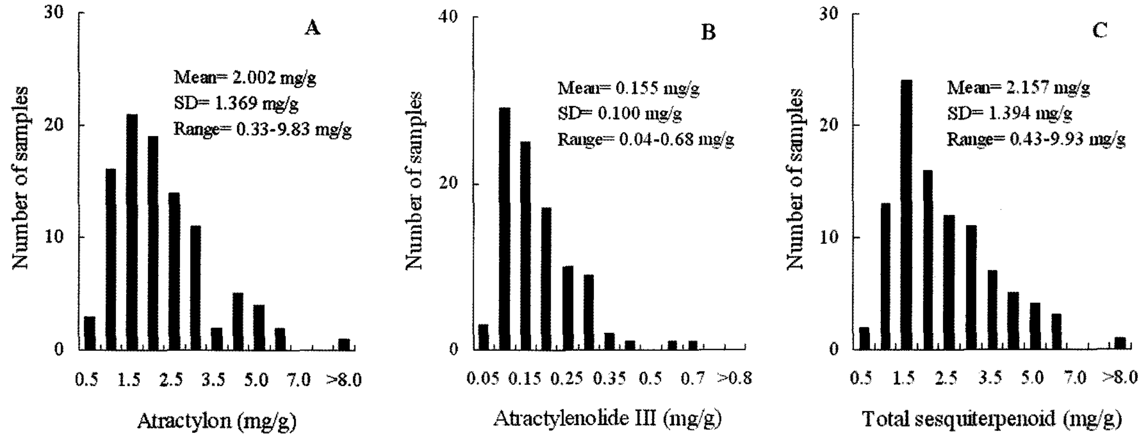


Fig. 3. Frequency distributions for contents of atractylon (A), atractylenolide III (B) and total sesquiterpenoids (C) in roots of hybrid population of *Atractylodes* species. Total sesquiterpenoid was the sum of atractylon and atractylenolide III contents.

hybrid lines by crossing between *Atractylodes japonica* Koidz. ex Kitam. (AJ) and *A. macrocephala* Koidz. (AM). Although superior lines having simultaneously both characters were not yet selected in this study, our results indicated that good lines with stronger tolerance to root rot and higher amount of sesquiterpenoids in addition to more vigorous root growth could be selected from a cross of AJ and AM in *Atractylodes* species.

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