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Influence of genetic factors on the ephedrine alkaloid composition ratio of *Ephedra* plants [1]

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Abstract

We investigated the ephedrine alkaloid ((-)-ephedrine and (+)-pseudoephedrine) composition ratio of a crude Chinese herbal drug described in the Japanese Pharmacopoeia, “Ephedra Herb (Chinese name: Mahuang)”. There were marked changes in the alkaloid composition ratio of wild plants in areas where both male and female clusters coexisted. However, in genetically homogeneous areas with the growth of male or female clusters alone, all of these regression lines’ regression coefficients were positive, but each lean varied; This suggests that the alkaloid composition ratio has a clear tendency in each individual. Based on this, we cultivated individuals for vegetative propagation, and evaluated the alkaloid content ratio. Those propagated by separating the roots showed a specific tendency regardless of the cultivation area (Wakayama, Tanegashima). Those propagated by separating the herbaceous stem showed a specific tendency regardless of the soil or harvest time. In addition, we surveyed the (-)-ephedrine content ratio of 3- to 6-year-old strains. There was a high positive correlation coefficient between the previous and subsequent years. These findings suggest that the ephedrine alkaloid composition ratio of Ephedra Herb depends on genetic factors, but not on environmental factors or the growth period.

Key words: *Ephedra sinica* Stapf, ephedrine, pseudoephedrine, cultivation, division,

cuttage

Introduction

In the 16th Revision of the Japanese Pharmacopoeia [2], it is described that the total ephedrine alkaloid ((-)-ephedrine (Eph) and (+)-pseudoephedrine (P-Eph)) content of a Chinese herbal, crude drug, “Ephedra Herb (Chinese name: Mahuang)”, is 0.7% or more. The two components are diastereomer with similar structures, but their drug efficacy differs. However, their contents are not described. Concerning the drug efficacy, Eph shows more potent asthma-reducing [3][4] and blood pressure-increasing [5] actions compared to P-Eph. On the other hand, the anti-inflammatory effects of P-Eph are more marked than those of Eph [6]. Therefore, Ephedra Herb preparations should be selected in accordance with the content ratio of the two components. Zhang et al. recommended that Ephedra Herb, containing a higher level of Eph, should be used to resolve coldness through sweating or reduce cough/asthma (maoto, makyokansekitou)[7]. Furthermore, Ephedra Herb, containing a higher level of P-Eph, should be selected for anti-inflammatory/analgesic effects (dokkatsukakkonto)[8]. Previous studies reported Ephedra class/regional differences in the alkaloid content ratio. Concerning the former, the P-Eph content of *Ephedra intermedia* is higher than those of *E. sinica* and *E. equisetina* [9][10]. Concerning the latter, among commercially available products, Ephedra Herb, containing a higher level of P-Eph, is common in the Gansu and Qinghai Provinces in China [11]. Among wild plants, the alkaloid content of Ephedra Herb and P-Eph composition ratio are higher in areas with a low rainfall [12]. However, in these studies, the relative ratio of Eph and P-Eph in Ephedra Herb was reversed in some cases despite having the same place of origin. This was possibly associated with complex factors such as the growth environment/period and genetic factors; the details remain to be clarified. In this study, assuming that differences in the content ratio are primarily related to genetic diversity, we firstly surveyed wild Chinese plants in genetically diverse areas where both male and female clusters coexisted and genetically homogeneous areas with the growth of male or female clusters alone. The latter refers to areas with clusters consisting of the same individual connected at the rhizome, although they seem to be several individuals on the ground. For example, *E. sinica* extensively elongates its rhizome under the ground in comparison with other *Ephedra* plants such as *E.intermedia* and *E.equisetina*, forming such a cluster. In this study, we evaluated clusters in the Hebei Province and Inner Mongolia of China.

Secondly, we cultivated genetically stable clonal strains through cuttage/division to investigate the influence of growth environments and the alkaloid content ratio. As the aerial part is used to prepare a crude drug, Ephedra Herb, the rhizome remains, and it is possible to harvest this plant every year [13]. However, it is unclear whether the alkaloid content ratio is stable. We cultivated several genetically different strains, and investigated 3- to 6-year-old strains for a follow-up survey for a few years [1].

Materials

Ephedra sinica Stapf (wild plants)

Collection area: Chifeng 1 in Inner Mongolia (male and female clusters coexisted, alt.330m and 430 m in 2010 and in 2011, respectively), Sample No. 100623-3 to 4 (Several individuals in the same cluster), 110725A1-1 ~A1-4, 110725B1-2 ~ B1-5, Date of collection: June 23, 2010, July 25, 2011, Number of samples: 14, Deposite site: Kracie Pharma, Ltd.

Collection area: Chifeng 2 in Inner Mongolia (male and female clusters coexisted, alt.680m in 2010 and in 2011, respectively), Sample No. 100623-1 (Several individuals in the same cluster), 110726A1-1 ~ A1-4, 110726B1-1 ~B1-7, Date of collection: June 23, 2010, July 26, 2011, Number of samples: 14, Deposite site: Kanazawa University.

Collection area: Chengde in Hebei Province (male cluster consisting of a single individual, alt.: 1,350 m), Sample No. 02139, Date of collection: June 10, 2002, Number of samples: 9, Deposite site: Kanazawa University

Collection area: Baotou in Inner Mongolia (gender: unclear, cluster consisting of a single individual despite its appearance involving different individuals on the ground, alt. 1,380m), Sample No. 100720-10, Date of collection: July 20, 2010, Number of samples: 5, Deposite site: Kanazawa University

Collection area: Xilinhot in Inner Mongolia (gender: unclear, cluster consisting of a single individual despite its appearance involving different individuals on the ground, alt. 1,060m), Sample No.7081801, Date of collection: August 1, 2007, Number of samples: 5, Deposite site: Kanazawa University

Uncertain species (cultivated plants)

The EP-13 strain, which was introduced as *Ephedra distachya*, was used. Cultivation area: Wakayama or Tanegashima Division, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, Cultivation method: Division/outdoor cultivation, Date of collection: Wakayama, December 15, 2011, Tanegashima, December 13 to 14, 2011, Number of samples: Wakayama, 9;

Tanegashima, 26, Deposite site: Kracie Pharma, Ltd.

E. sinica (cultivated plants)

Collection area: Medicinal Plant Garden of Kanazawa University, Cultivation method: Cuttage/cultivation with wagnerpots for 2 years (annual transplantation), Soil: River/mountain sand, loamy soil (small, hard grains), Kanuma pumice (small grains), volcanic gravel in Kiryu, or commercially available soil (“Soil for Planters”, Akimoto Tensanbutsu Co., Ltd.), Date of collection: (1) November 22, 2011, (2) September 18, 2012, Number of samples: (1) 12, (2) 11. Deposite site: Kracie Pharma, Ltd.

Collection area: Medicinal Plant Garden of Kanazawa University, Cultivation method: Seedlings were planted in 1/5,000a or 1/2,000a wagnerpots, and cultivated from 2004 (Autumn) until 2010. Soil: River sand (The inferior layer of the soil was mixed with a chemical fertilizer (Futsukasei No. 8, Fujikawa Egg, N:P:K=8:8:8, 10 g/pot)) or the following 5 types: (1) commercially available soil (“Soil for Planters”, Akimoto Tensanbutsu Co., Ltd.), (2) loamy soil, (3) commercially available soil + loamy soil (volumetric ratio: 28:18), (4) commercially available soil + alkaline agent, and (5) loamy soil + alkaline agent, *: Alkaline agent (10 g/pot of magnesium-lime carbonate or nitrogen-lime), Watering conditions: Diluted artificial seawater was given once a week, and groundwater once a week (composition of artificial seawater: NaCl (special class) at 86.8 g, $MgSO_4/7H_2O$ (first class) at 20.8 g, $MgCl_2/6H_2O$ (special class) at 15.7 g, $CaCl_2/2H_2O$ (first class) at 4.5 g, and KCl (special class) at 2.2 g were dissolved in groundwater to prepare a volume of 3 L). Date of collection: September 2007, September 2008, September 2009, September 2010, Number of samples: 100 (a part of samples were blighted during the study. 5-year-old plant : 95samples. 6

year-old-plants : 90samples), Deposite site: Kanazawa University

Methods

Quantification of alkaloids

Quantification was performed as described below. Some tests were conducted in accordance with the quantification method described in the Japanese Pharmacopoeia. In some samples, we compared differences in the Eph/P-Eph ratio between the two methods, and confirmed that the rate of difference was 5% or less.

Preparation of sample solution: The mobile phase of HPLC at 5.0 mL (described below) was added to each sample powder at 100 mg, and placed at room temperature for 20 minutes. Subsequently, ultrasonic extraction was performed for 25 minutes. Each solution was centrifuged at 3,000 rpm for 15 minutes. The supernatant was filtered using a 0.45-μm membrane filter (Minisart RC25, Sartorius Stedium Biotech), and used as the sample solution.

HPLC conditions: An L-2130 pump, L-2200 autosampler, L-2400 UV detector, D-2500 integrator (Hitachi), and Handy ODS column (4.6 mm I.D. x 250 mm, No. 14562)(Wako Pure Chemical Industries, Co., Ltd.) were used. Column temperature: room temperature, Flow rate: 1.0 mL/min, Detection wavelength: 210 nm, Mobile phase: 27 mM sodium lauryl sulfate (SDS) solution/MeCN/H_3PO_4 (305:195:0.8)

Result

Correlation coefficient of Eph and P-Eph in wild plants

In two areas where both male and female clusters coexisted (Inner Mongolia Chifeng), all of these regression lines' regression coefficients were negative (ex. The regression equation in Chifeng 1 (2010) was y= -0.85639x+0.5013 (r= -0.9335), that in Chifeng 2 (2011) was y= -0.8817x+1.4036(r= -0.7605) (regression equation: y=ax+b, a: regression coefficient); there were individual differences in the alkaloid content ratio (Fig. 1). In samples obtained in 2010, the alkaloid content was lower than in 2011. This was possibly because samples were collected earlier (late June) in 2010. There are seasonal changes in the alkaloid content. A study reported that the alkaloid content of samples collected in June was lower than in July [14].

On the other hand, in individuals obtained in an area with male clusters (Hebei Province) and the herbaceous stem of the same individual connected at the rhizome (two areas in Inner Mongolia), all of these regression lines' regression coefficients were positive, but each lean varied. The regression equation in Baotou was y=2.2874x-0.0703 (r=0.9544), that in Xilinhot was y=0.0707x-0.0007(r=0.9597), and that in Hebei was y=0.9931x-0.084(r=0.8603) (Fig. 2). In those from Hebei Province, the correlation coefficient was markedly lower than in those from Baotou and Xilinhot.

Eph-to-P-Eph content ratio in cultivated plants (division, outdoor cultivation, cuttage, cultivation with wagnerpots)

There was a high correlation coefficient in the alkaloid content ratio even when *Ephedra* plants were cultivated using the division method in the two distant areas of Wakayama and Tanegashima (The regression equation was y=0.1318x+0.0002 (r=0.9799)(Fig. 3). When the cuttage method was used, there was also a high correlation coefficient in the alkaloid content ratio, although the type of soil and harvest time differed (The regression equation was y=0.9837x-0.0233 (r=0.9799)) (Fig. 4).

In several 3- to 6-year-old, genetically different seedlings, there was a high correlation coefficient in the content ratios (Eph/Eph + P-Eph) of individuals between each year and the previous year over 4 consecutive years despite different soil/water compositions (The regression equation between 3-year-old and 4-year-old was $y=0.9519x+0.0666$ ($r=0.9534$) . That between 4-year-old and 5-year-old was $y=0.9284x+0.0687$ ($r=0.9719$). That 5-year-old and 6-year-old was $y=0.9565x+0.0373$ ($r=0.9835$)) . And the regression equation between 3-year-old and 6-year-old was $y=0.8786x+0.1421$($r=0.9564$) (Fig. 5). There was also a high correlation coefficient in the alkaloid content ratios (Eph/Eph + P-Eph). However the regression lines' regression coefficient was slightly lower and intercept was higher than that between each year and the previous year. This suggests that the Eph content ratio slightly increased in comparison with the previous year.

Discussion

In areas where male and female clusters coexisted, the alkaloid content ratio varied (Fig. 1). This was possibly because several individuals with different genetic information through sexual reproduction coexisted in such areas. This is also supported by the results of a study investigating serial changes in cultivated plants (Fig. 5). The test strains were genetically different seedlings. The alkaloid content ratio (Eph/Eph + P-Eph) ranged from 0.2 to 1.0. On the other hand, there was a high positive correlation coefficient of the regression line in areas with the same individual male clusters alone (Fig. 2). This suggests that the alkaloid composition ratio has a clear tendency in each individual. In the area of Hebei Province surveyed in this study, almost all strains had male flowers, whereas there were no female flowers or flower-free individuals. In areas with male clusters, plants undergo asexual propagation. Therefore, a cluster may be derived from a single individual elongating its rhizome. However, the correlation coefficient was markedly lower than those of a single individual or cultivated clonal strain. This may be associated with the following factors: the possibility of multiple strains and growth period-related changes in the content ratio in the same individual. Concerning the latter, a 4-year follow-up survey showed that the Eph content ratio (Eph/Eph + P-Eph) slightly increased in comparison with the previous year (average of the Eph content ratio in 3-year-old strain: 0.72, in 4-year-old strain: 0.75, in 5-year-old strain: 0.76, and in 6-year-old strain: 0.77). Even in the same individual, there may be a variation between two points with a more than 10-year interval. A long-term survey should be performed every 10 years. The scale of the area with male clusters in Hebei Province was relatively large, but it was very rare autogenesis ground. The scale of areas with the cluster of a single individual was extremely small. Based on these findings, it may be difficult to continuously obtain Ephedra Herb in which the component content ratio is stable, from wild or seed propagation-mediated, cultivated plants. On the other hand, the alkaloid content ratio in cultivated plants multiplied through division or cuttage was constant despite different cultivation areas/conditions (Figs. 3 and 4). Briefly, the alkaloid content ratio depends on genetic factors, but not on the environment or growth period. We can obtain target Ephedra Herb by selecting a strain with an alkaloid content ratio at which the target pharmacological activity is more potent than those of other alkaloids, and cultivating clonal strains using the vegetative propagation method.

On the other hand, as a limitation, the ephedrine alkaloid contents (Eph + P-Eph) of genetically equivalent, clonal strains cultivated in the same area ranged from 0.1 to 1.0% (Figs. 3 and 4). Even among wild strains connected at the rhizome, the ephedrine alkaloid content of the aerial parts differed (Fig. 2), suggesting the influence of differences in growth. However, it is very difficult to standardize all individuals' growth; a method to increase the content on average should be examined.

Using these methods, a stable, crude drug, Ephedra Herb, may be obtained.

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Fig.1 Relationship between content of ephedrine of wild *Ephedra* plants (*Ephedra sinica* Stapf) increased by sexual production and the pseudoephedrine. Regression equation; Chifeng 1(2010) (n=6) y=-0.85639x+0.5013(r=-0.9335), Chifeng 1(2011)(n=8) y=-0.2811x+0.195(r=-0.2841), Chifeng 2(2010)(n=3) y=-0.2242x+0.5465(r=-0.6687), Chifeng 2(2011)(n=11) y=-0.8817x+1.4036 (r=-0.7605).

Fig.2 Relationship between content of ephedrine of wild *Ephedra* plants (*Ephedra sinica* Stapf) increased by asexual reproduction (genetically homogeneous areas with the growth of male or female clusters alone) and the pseudoephedrine. Regression equation and correlation coefficient; Baotou (■)(n=5) y=2.2874x-0.0703 (r=0.9544), Xilinhot (▲) (n=5) y=0.0707x-0.0007(r=0.9597), Hebei (○)(n=9) y=0.9931x-0.084(r=0.8603).

Fig.3 Relationship between content of ephedrine of cultivated *Ephedra* plants (Uncertain species, EP-13) (dividing roots / raising outdoors) and the pseudoephedrine. Wakayama (●) (n=9), Tanegashima (○) (n=26).

Fig.4 Relationship between content of ephedrine of cultivated *Ephedra* plants (*Ephedra sinica* Stapf) increased by (dividing shoots / cultivating in wagnerpot) in Kanazawa and the pseudoephedrine. Collecting in 2011.11.22 (●) (n=12), Collecting in2012.9.18 (○) (n=11).

Fig.5 Relationship between alkaloid rates (content of ephedrine / the total content of ephedrine and pseudoephedrine) of 3-year-old cultivated *Ephedra* plants (*Ephedra sinica* Stapf) in Kanazawa and the 6-year old. 3-year-old(n=100), 6-year-old(n=90).

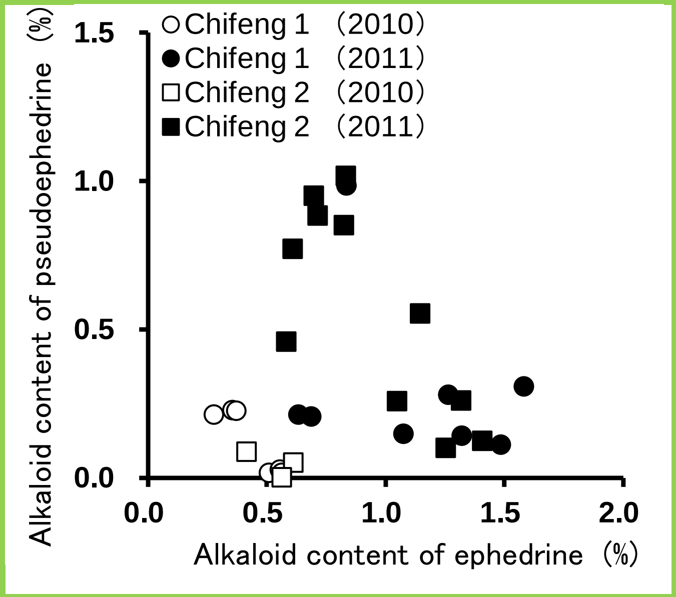

Chifeng 1 （2010）
Chifeng 1 （2011）
Chifeng 2 （2010）
Chifeng 2 （2011）
Alkaloid content of pseudoephedrine （%）
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
Alkaloid content of ephedrine （%）

Fig.1

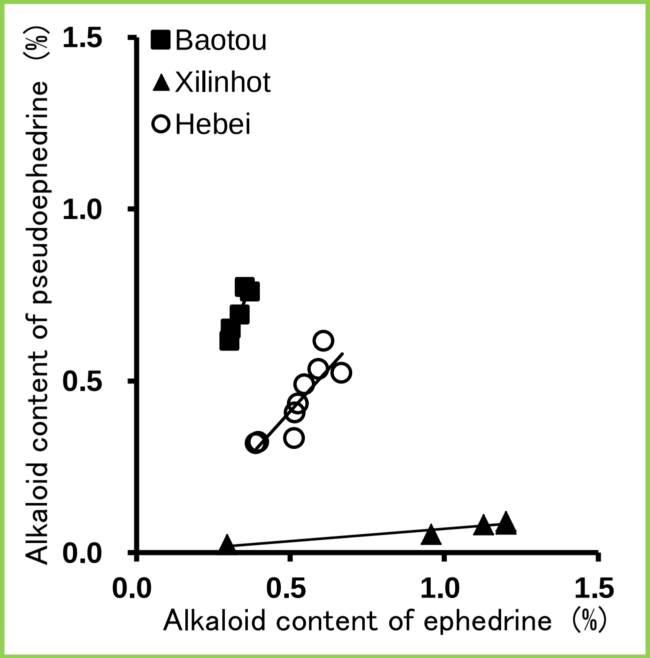
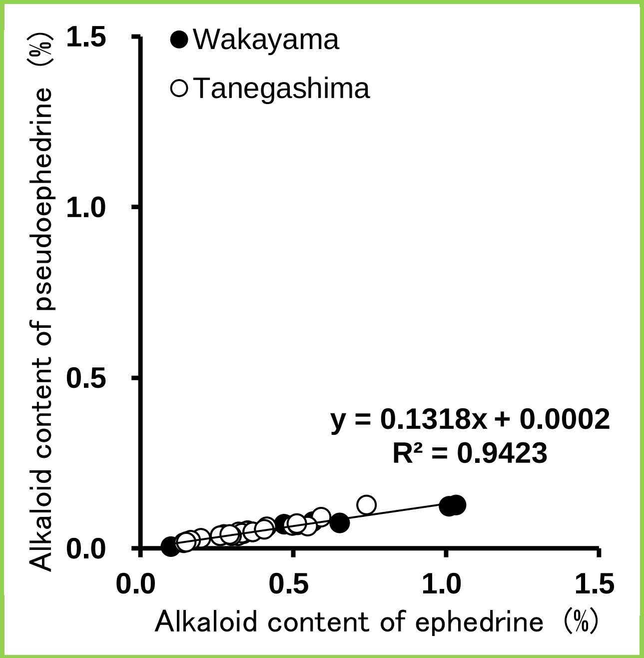
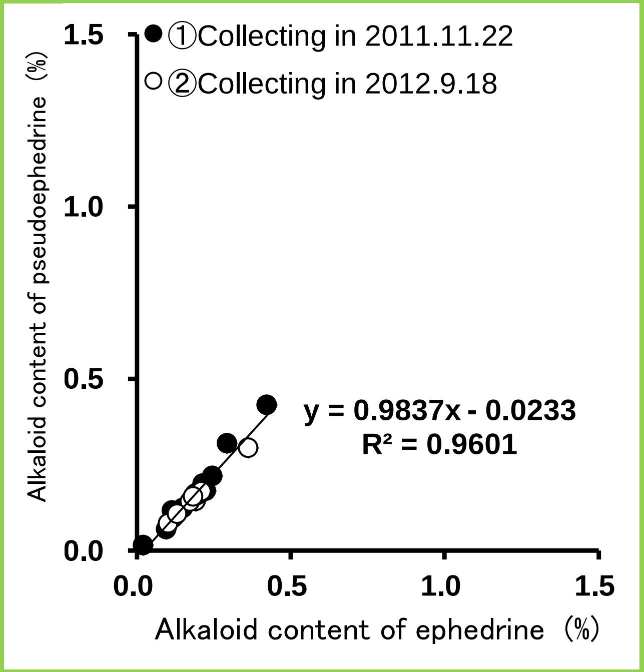

Baotou
Xilinhot
Hebei
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
Alkaloid content of pseudoephedrine （%）
Alkaloid content of ephedrine （%）

Fig.2



Wakayama
Tanegashima
y = 0.1318x + 0.0002
R² = 0.9423
0.0
0.5
1.0
1.5
Alkaloid content of pseudoephedrine (%)
Alkaloid content of ephedrine (%)

Fig.3



1.5
1.0
0.5
0.0
Alkaloid content of pseudoephedrine （%）
● ①Collecting in 2011.11.22
○ ②Collecting in 2012.9.18
y = 0.9837x - 0.0233
R² = 0.9601
0.0
0.5
1.0
1.5
Alkaloid content of ephedrine （%）

Fig.4

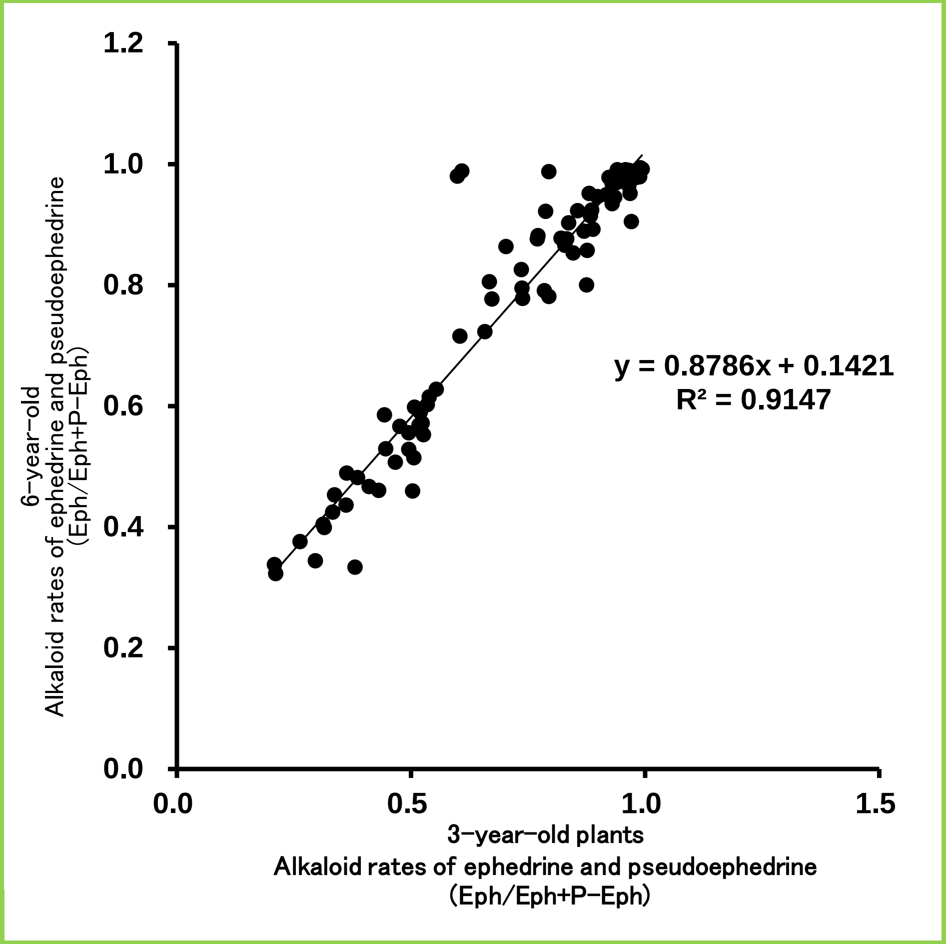

1.2
1.0
0.8
0.6
0.4
0.2
0.0
6−year−old
Alkaloid rates of ephedrine and pseudoephedrine
(Eph/Eph+P−Eph)
y = 0.8786x + 0.1421
R² = 0.9147
0.0
0.5
1.0
1.5
3−year−old plants
Alkaloid rates of ephedrine and pseudoephedrine
(Eph/Eph+P−Eph)

Fig.5