

## Article

# Seed Propagation of *Habenaria radiata*

## Morphological and Physiological Characteristics of Plants Derived from *in vitro* Cultured Seedlings

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### Abstract

The habitat and the number of *Habenaria radiata*, one of the vulnerable and endangered wild orchids in Japan, decreases year by year. It is necessary to develop an effective method to propagate this species. By applying *in vitro* germination and culture techniques, it was found that the seeds of this species germinated in a few weeks on artificial media and acclimated easily. Furthermore, the term of growth from seedlings to full-grown was significantly shorter than other Orchidaceae investigated. A large number of bulbs were obtained and the morphological and physiological characteristics of plants grown from them were examined in 1994 and 1995. Some characteristics of individual clones, e.g. number of leaves, size of the flowers, and arc of the lip remained stable over the two years. Positive comparative correlations were found between many characteristics, for example weight of the bulb and stem diameter, length of the stem and length of the leaf, length of the leaf and the number of leaves, the length of the leaf and the number of flowers, length of the leaf and stem diameter, width of the leaf and stem diameter, the number of leaves and the number of flowers. Several variant phenotypes such as albinos, dwarfs, variegated leaves, unopened flowers and green-striped flowers that are similar to reported mutants, were produced. The effectiveness of seed propagation and problems associated with variants is discussed.

**Key Words:** *Habenaria radiata*, vulnerable wild orchids, seed propagation, variants.

Recent progress in genetic engineering has allowed us to manipulation of genes and their transfer even among distantly related species. Since all species may ultimately be able to share genetic material through such manipulation, all existing genes may prove to be important. Thus, extinction of wild life reduces the pool of genetic information that is only now becoming exploitable.

One of the main functions of our museum is to collect gene bank data for conservation, and to increase the number of endangered and rare wild plants mainly growing in Hyogo prefecture.

In addition to categorizing plants with in Hyogo prefecture, we are surveying their habitats. We are also engaged in gathering and stocking seeds and cultivating several wild species, from seedlings and cuttings, in the experimental field at the museum.

A project was initiated to increase *Habenaria radiata*, one of the rare wild orchids in the prefecture ( Hyogo prefecture, 1995 ), which is also designated as one of the seven concentrated survey species of vulnerable plants in Japan ( WWFJ, 1989 ). *H. radiata* is one of the most popular wild orchids native to Japan. However, habitat destruction, due to development and human exploitation has threatened this species. In addition, since this flower is favored for its beautiful white flying-bird shape, many people pick it from its

natural habitat for personal use. As a consequence, the density and the number of this species decreases year by year.

If consumer varieties could be bred, it might help prevent the flower enthusiasts from picking the wild variety. For a long time, some wild orchids, including *H. radiata*, have been cultivated by flower enthusiasts. However, their cultivation has not been easy. It is therefore necessary to develop an effective methodology to propagate this species. Orchidaceae requires an atypical germination process, such as the help of specific orchid fungi. Thus, ordinary sowing of seeds in pots or seedbeds is not successful. However, it has been reported that if planted *in vitro* on artificial medium for sterile germination and culture, seedlings could be obtained without the aid of fungi ( Knudson, 1922, 1924 ). This study reports on the method of *in vitro* seed propagation of *H. radiata* and some interim results on morphological and physiological characteristics examined in 1994 and 1995.

## Materials and Method

Seeds of *Habenaria radiata* used this study were collected in open marshy fields to the north of Kobe and the Konda area. In August 1992 and 1993, artificial pollination was carried out and one month later, many capsules were collected.

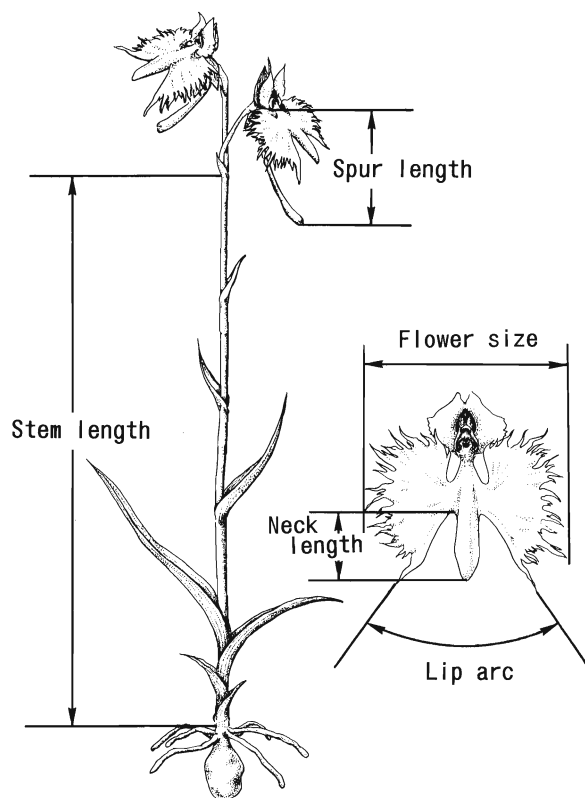
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**Table 1.** Composition of the Hyponex medium.

Hyponex(6.5-6-19/N-P-K)	3g
Sucrose	30g
Agar	10g
Organic compounds*	2g

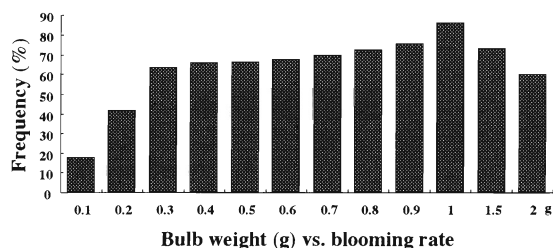
Fill up to 1 lit. with distilled water.

\* Yeast extract, Malt extract, Peptone, Casamino acid add one of them.



**Fig. 1.** Schematic picture of measured characteristics in *Habenaria radiata*.

From early September to late December 1992, seeds of *H. radiata* were sown on artificial medium under sterile conditions and cultured using the following method: first, the seeds were surface sterilized with one percent sodium hypochlorite for ten minutes and then rinsed three times with sterilized water; they were then placed on Hyponex medium (Table 1) and incubated at 25°C under fluorescent light at an intensity of 2000 lux. Within one or two weeks the seeds turned green and germinated. Seedlings produced several leaves and some had made bulbs in the medium by March. After acclimation in spring, seedlings were cultivated in a greenhouse to obtain bulbs. More than 650 bulbs were produced by the end of 1993. After one year the number of these bulbs had propagated to about 1400, and about 1100 new bulbs were produced from seeds using the same method.



**Fig. 2.** Frequency % of flowered plants and their bulb weights in 1995.

In 1994 and 1995 the bulbs were weighed and planted individually in 9 cm diameter and 8 cm deep plastic pots. Considering the possibility of breeding prominent varieties in the future, plants susceptible to disease were discarded in 1994; when some plants became infected, germicidal agents were not used. The plants grown from bulbs in 1995 survived the disease from the previous year. Many plants were again infected by disease in 1995, and this time germicidal agents were used.

The following characteristics were measured for blooming plants: date of germination, length of the stem, length and width of the leaf, the number of leaves per plant, stem diameter, date of blooming, the number of flowers per plant, size of the flower, length of the neck in the lip, arc of the lip, and length of the spur. Only blooming plants were included in the data. Weight of the bulb and blooming, blooming and non-blooming plants were included in the data.

Stem length was measured from the top of the bulb to the basal part of the first flower (Fig. 1). Length and width of the leaf were measured using the longest leaf of each plant. Stem diameter was measured over the leaf sheaths at the basal part of the stem. Blooming date was recorded at the day when the first flower bloomed. Size of the flower was measured between the edges of the lip of the flower blooming first (Fig. 1). These characteristics were measured when the first flower bloomed except for the sprouting date.

In 1994, out of 650 bulbs planted, 470 plants bloomed. Amongst them 378 plants were studied for these characteristics. In 1995, about 2500 bulbs were planted and more than 850 plants bloomed, amongst which 358 plants whose mother plants bloomed and were used in 1994 were studied again.

In 1994, all plants were cultivated in the greenhouse. Most of the plants produced more than two bulbs (mostly three bulbs). In 1995, in such cases of more than two bulbs being available from one mother bulb, they were simultaneously cultivated in and outside of the greenhouse.

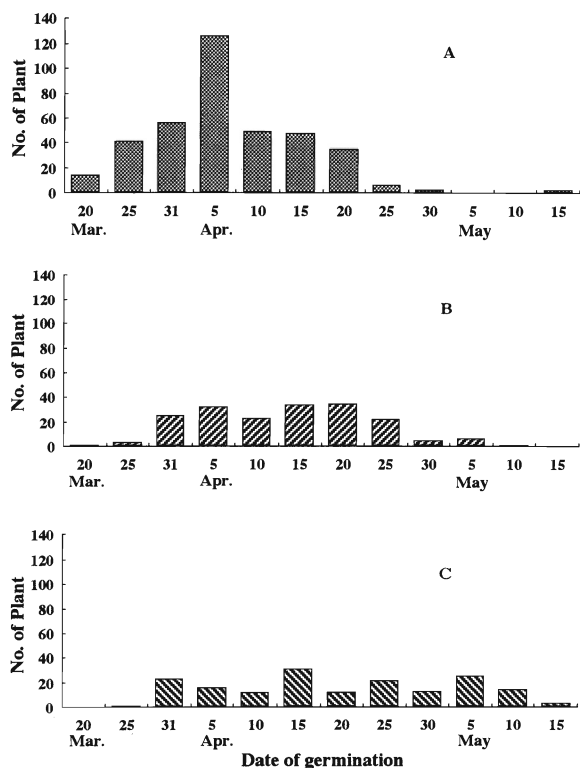


Fig. 3. Distribution of date of the germination. A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

## Results

### Bulb Weight vs. Blooming Rate

The mean weight of the bloomed bulbs was 560.3 mg (42 mg–1549 mg), whereas that of the non-blooming bulbs was 501.7 mg (33 mg–1900 mg) in 1995 (Fig. 2).

### Date of Germination

From early January to February, bulbs were planted in pots, and kept either in the greenhouse or outside. They germinated from late March to April (Fig. 3) and the date of germination was recorded.

### Length of the Stem

The mean stem length of plants that bloomed in 1994 was 238.9 mm (42 mm–404 mm). In 1995, it was 236.5 mm (93 mm–403 mm) in the group cultivated in the greenhouse and 132.5 mm (61 mm–249 mm) in the group cultivated outside (Fig. 4).

### Length and Width of the Leaf

Length and width of leaves varied greatly. The first one or two leaves and last two or three were very small. In some

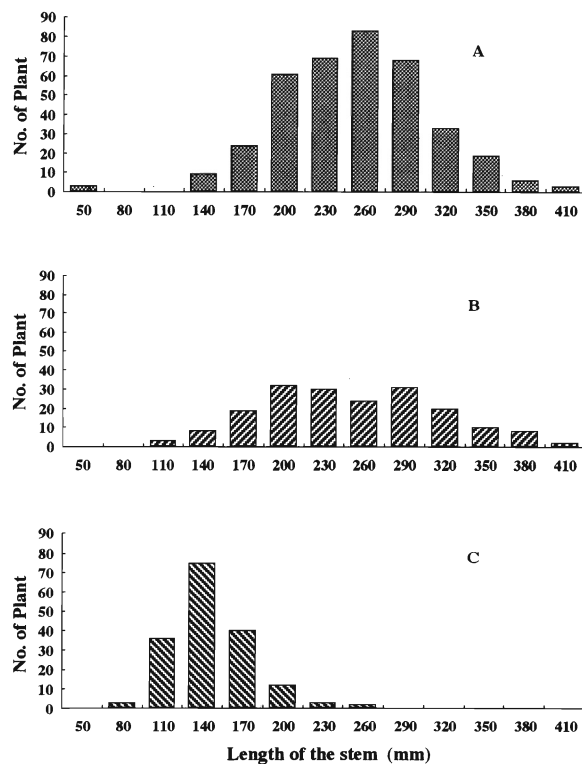


Fig. 4. Distribution of length of the stem(mm). A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995

cases, the first two leaves sprouted underground and died during their early growing stage. The mean length of the longest leaf was 97.8 mm (39 mm–193mm) in 1994. In 1995, it was 85.1 mm (33 mm–141 mm) in the group cultivated in the greenhouse and 52.2 mm (23 mm–80 mm) in the group cultivated outside (Fig. 5).

The mean width of the longest leaf was 7.7 mm (4.0 mm–12.0 mm) in 1994. In 1995, it was 6.8 mm (4.0 mm–9.0 mm) in the group cultivated in the greenhouse and 6.0 mm (3.0 mm–9.0 mm) in the group cultivated outside (Fig. 6).

### Number of the Leaves per Plant

The mean number of leaves per plant was 6.9 (4–11) in 1994. In 1995, it was 7.9 (5–12) in the group cultivated in the greenhouse and 7.3 (5–10) in the group cultivated outside (Fig. 7).

### Stem Diameter

When the stem diameter was measured, the stems were covered tightly with the leaf sheaths. Thus, the measured diameter included the thickness of the leaf sheaths. The mean stem diameter thus measured was 3.7 mm (1.1 mm–7.6 mm) in 1994. In 1995, it was 3.7 mm (1.7 mm–6.1 mm) in the group cultivated in the greenhouse and 3.0 mm (1.8 mm–8.7 mm) in the group cultivated outside (Fig. 8).

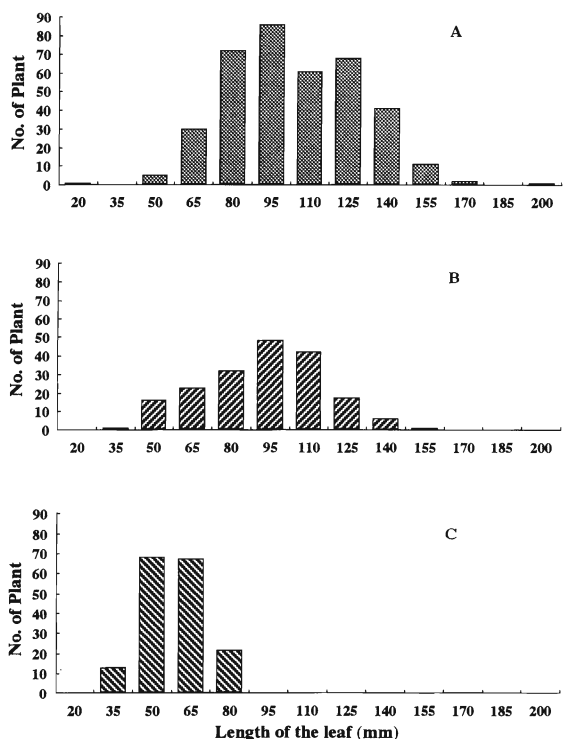


Fig. 5. Distribution of length of the leaf(mm). A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

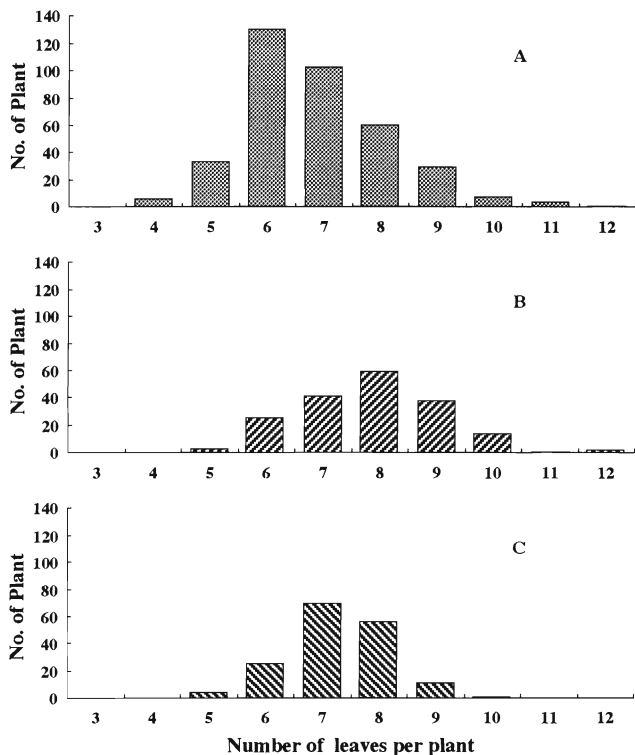


Fig. 7. Distribution of number of the leaves. A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

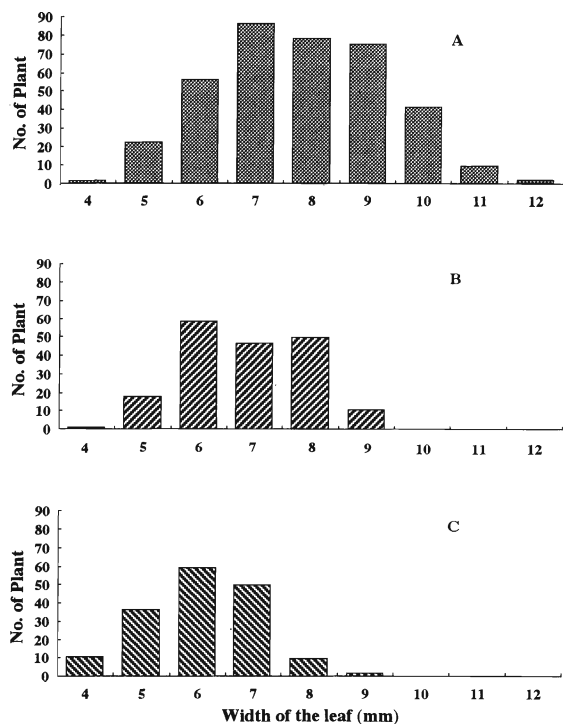


Fig. 6. Distribution of width of the leaf(mm). A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

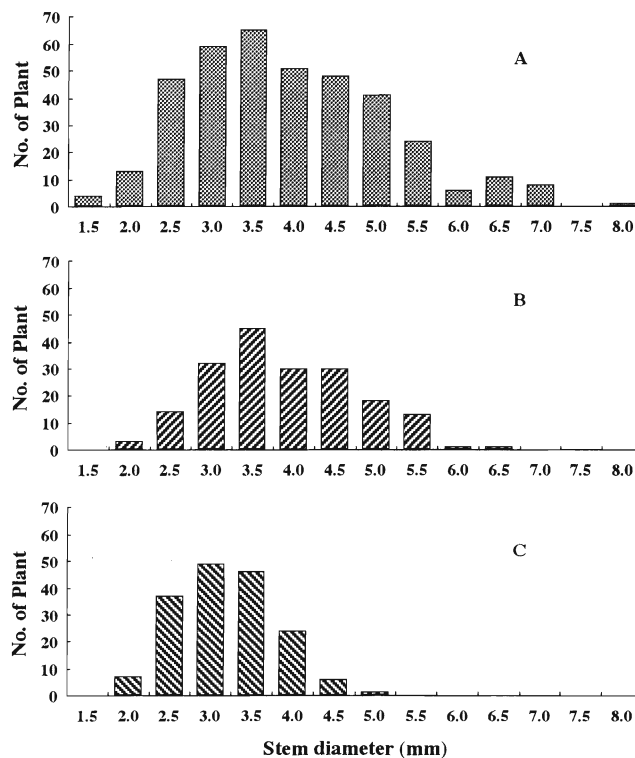


Fig. 8. Distribution of diameter of the stem(mm). A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

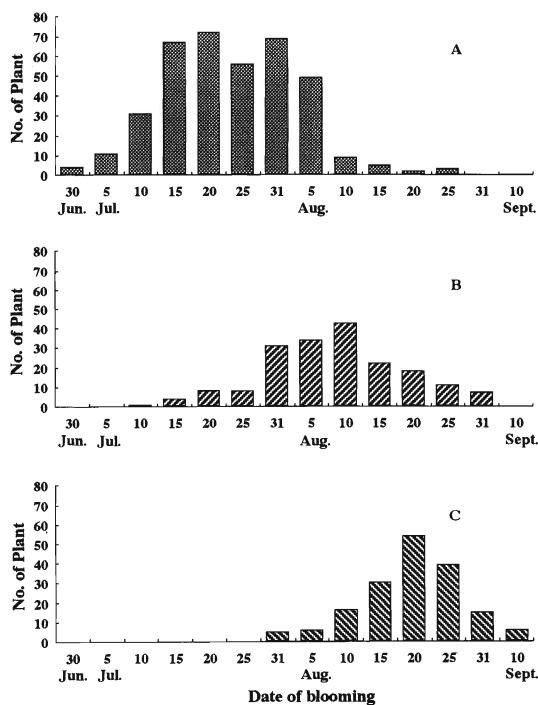


Fig. 9. Distribution of date of blooming. A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

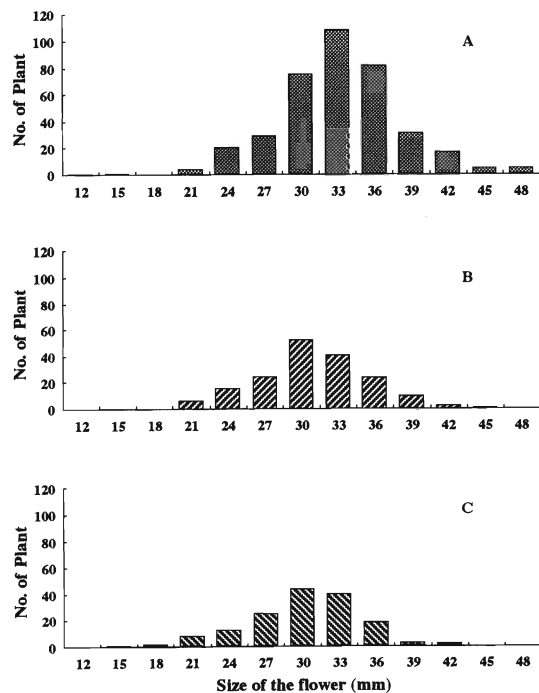


Fig. 11. Distribution of size of the flower (mm). A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

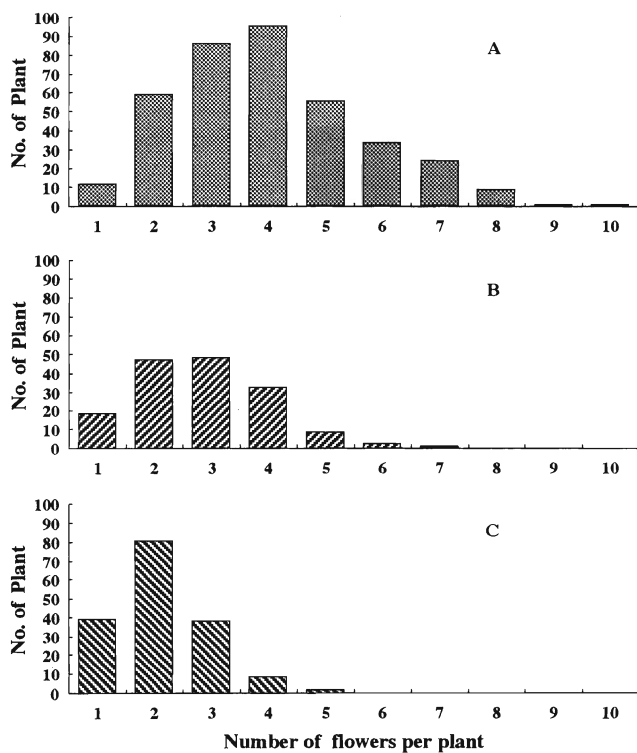


Fig. 10. Distribution of number of the flowers. A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

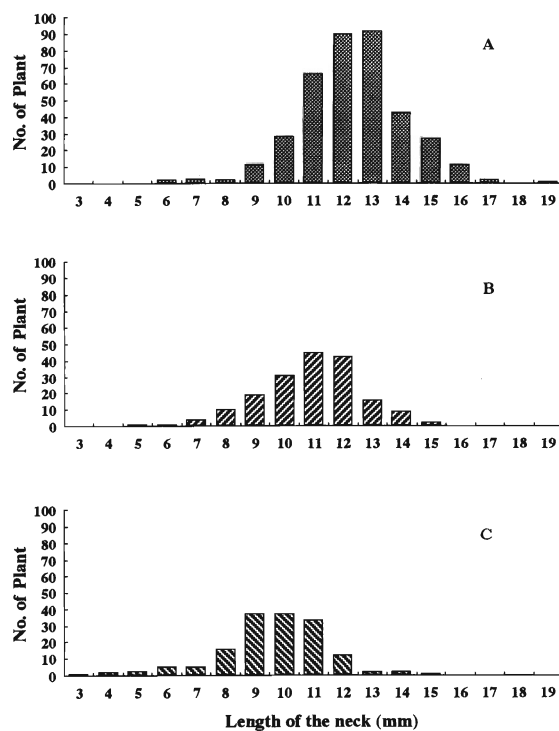


Fig. 12. Distribution of length of the neck (mm). A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

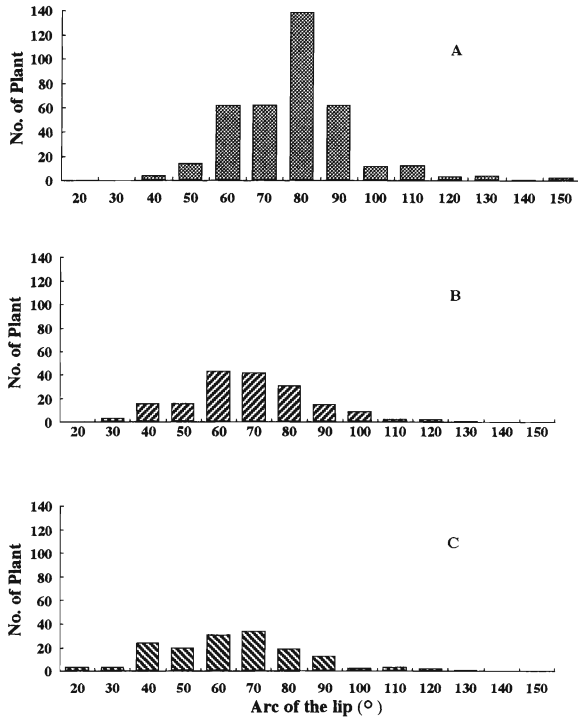


Fig. 13. Distribution of arc of the lip(°). A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

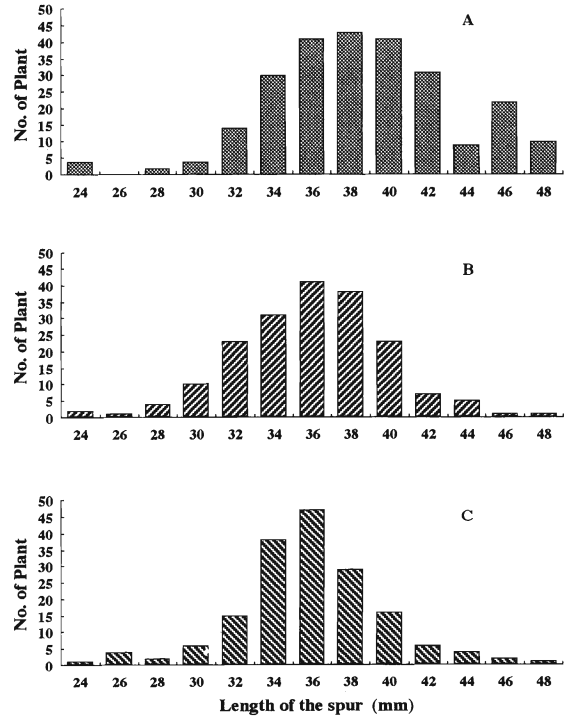


Fig. 14. Distribution of length of the spur(mm). A: In the greenhouse 1994. B: In the greenhouse 1995. C: Outside 1995.

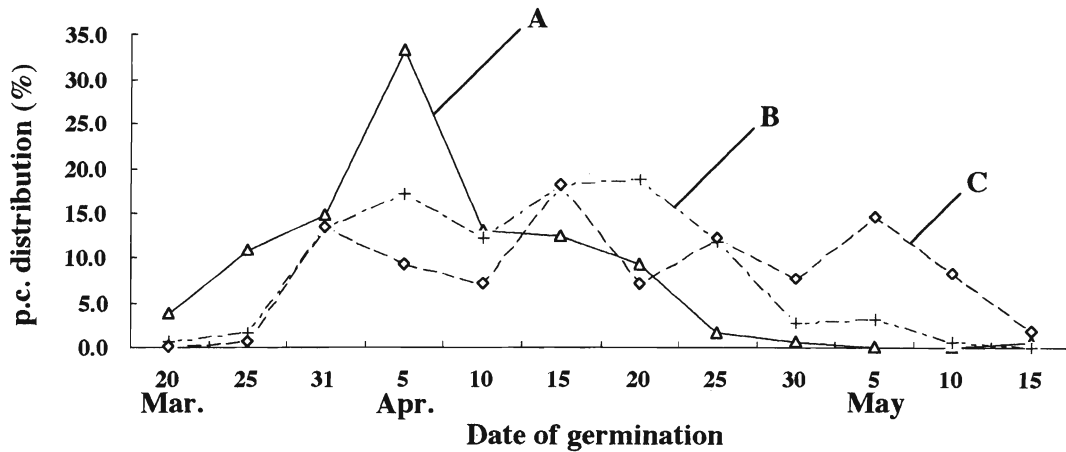


Fig. 15. Percent distribution of date of the germination, A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

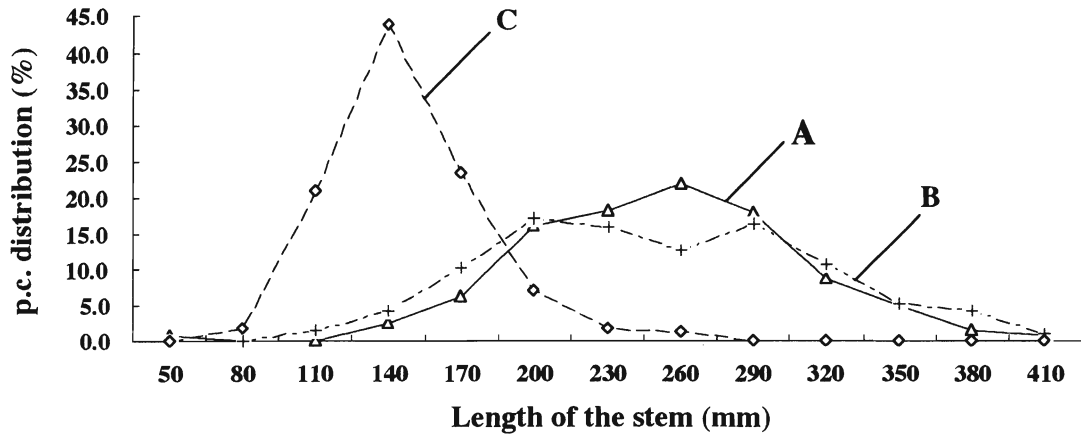


Fig. 16. Percent distribution of length of the stem(mm), A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

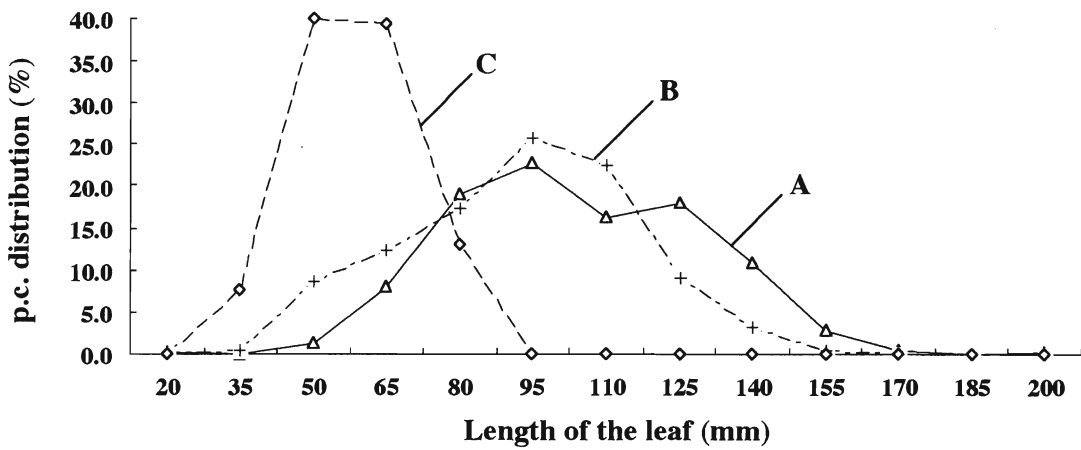


Fig. 17. Percent distribution of length of the leaf(mm), A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

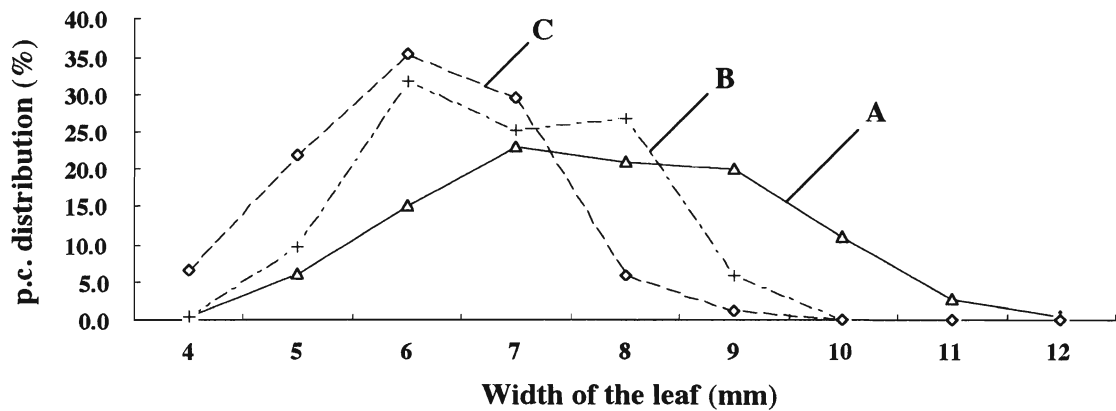


Fig. 18. Percent distribution of width of the leaf(mm), A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

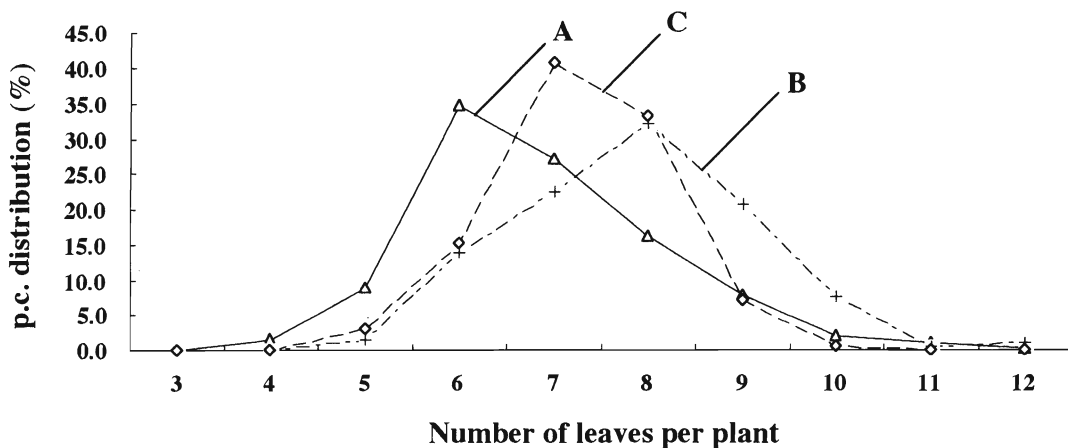


Fig. 19. Percent distribution of number of the leaves, A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

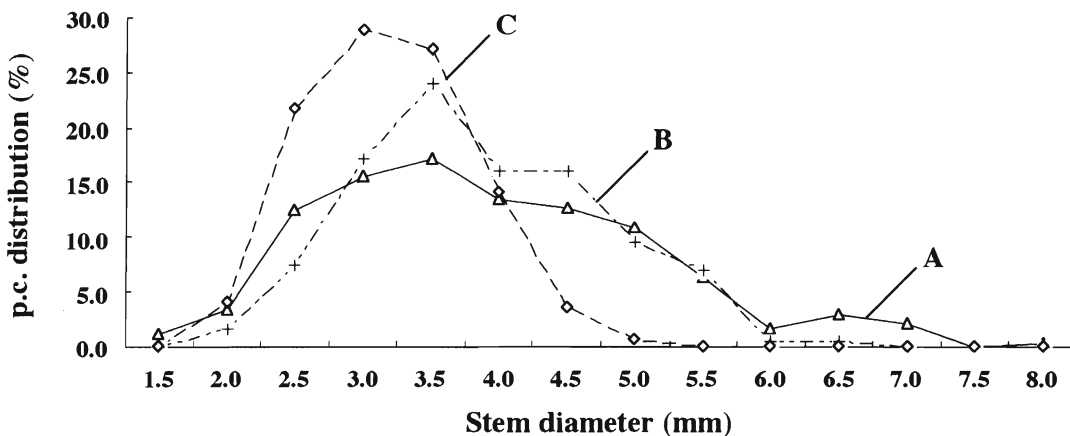


Fig. 20. Percent distribution of diameter of the stem (mm), A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

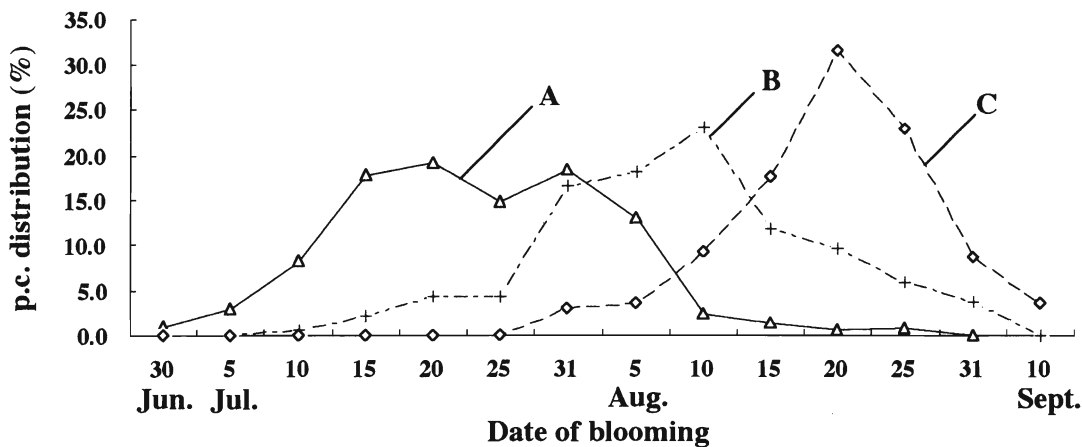


Fig. 21. Percent distribution of date of the blooming, A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.



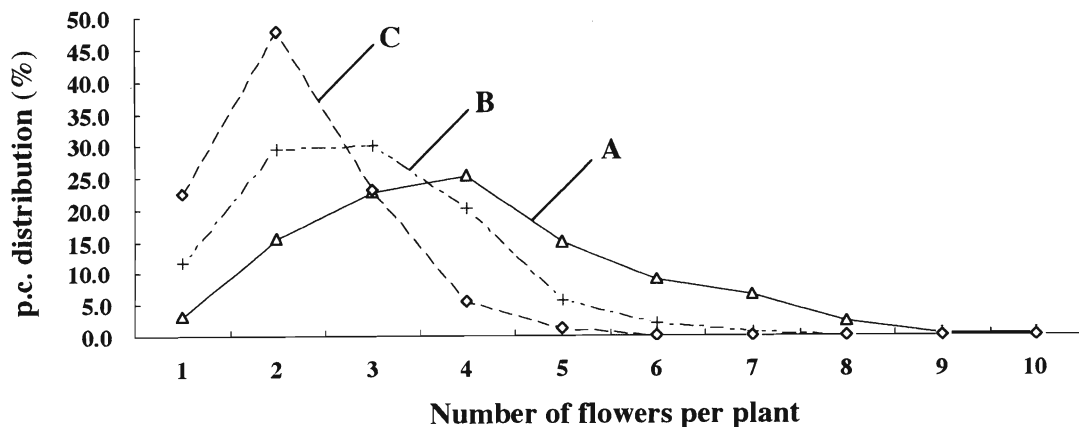


Fig. 22. Percent distribution of number of the flowers, A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

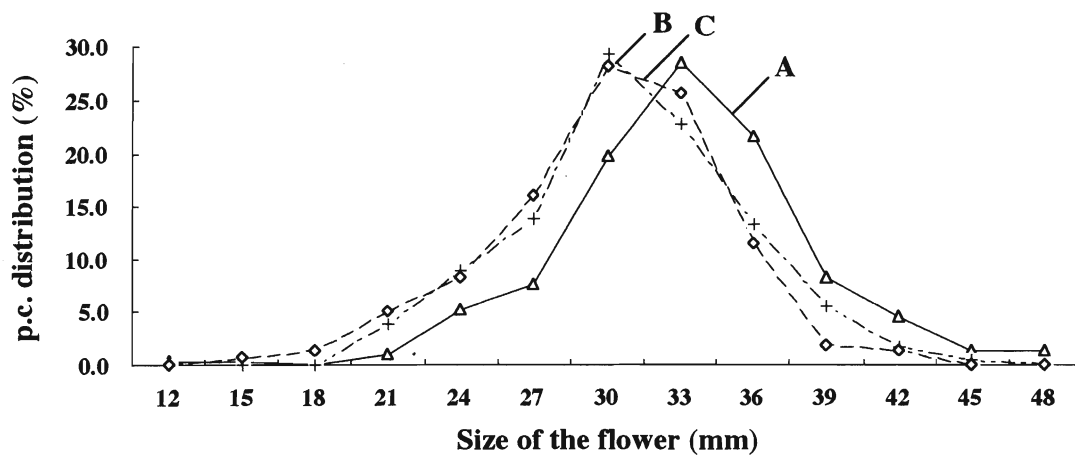


Fig. 23. Percent distribution of size of the flower (mm), A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

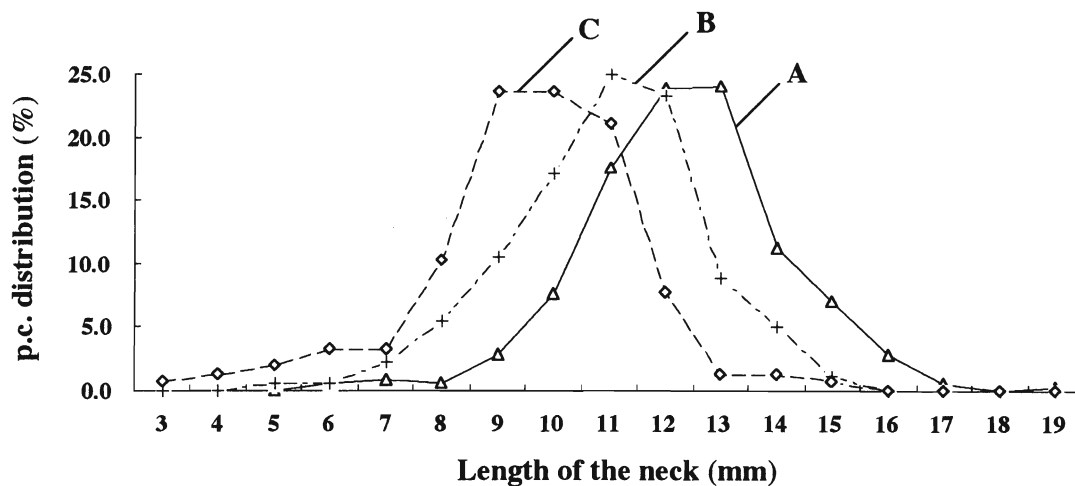


Fig. 24. Percent distribution of length of the neck (mm), A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

### Date of Blooming

Flowers started to open in early July in the group cultivated in the greenhouse and late July in the group cultivated outside ( Fig. 9 ). When there were more than two buds per plant, they usually bloomed either every other or every third day.

### Number of the Flowers per Plant

The mean number of flowers per plant was 4.0 (1–10) in 1994. In 1995, it was 2.9 (1–7) in the group cultivated in the greenhouse and 2.1 (1–5) in the group cultivated outside ( Fig. 10 ).

### Size of the Flower

The mean size of the flowers was 32.0 mm (12 mm–47 mm) in 1994. In 1995, it was 30.0 mm (19 mm–45 mm) in the group cultivated in the greenhouse and 29.1 mm (13 mm–41 mm) in the group cultivated outside ( Fig. 11 ). Sizes of the first flowers were almost the same as those of the second or third one blooming in the same plant, but later flowers were a little smaller.

### Length of the Neck in the Lip

The mean length of the neck in the lip ( Fig. 1 ) was 12.2 mm (5.5 mm–19.0 mm) in 1994. In 1995, it was 10.8 mm (5.0 mm–15.0 mm) in the group cultivated in the greenhouse and 9.5 mm (2.0 mm–15.0 mm) in the group cultivated outside ( Fig. 12 ).

### Arc of the Lip

The mean arc of the lip ( Fig. 1 ) was 76.1° (35°–150°) in 1994. In 1995, it was 67.7° (30°–130°) in the group cultivated in the greenhouse and 62.8° (10°–130°) in the group cultivated outside ( Fig. 13 ).

### Length of the Spur

The mean length of the spurs ( Fig. 1 ) was 38.2 mm (20 mm–53 mm) in 1994. In 1995, it was 35.4 mm (23 mm–47 mm) in the group cultivated in the greenhouse and 35.3 mm (24 mm–47 mm) in the group outside ( Fig. 14 ).

Fig. 15, combining the three graphs 3A, 3B, and 3C shows the percent distribution of characteristics studied. Figs. 16–26 also shows the combined three graphs of 4–13 in the same manner. Group ( A ) represents all plants from 1994, group ( B ) represents greenhouse plants from 1995, and ( C ) outside plants from 1995. When the cultivating environments were considered the same ( A and B groups ), stem length of was almost the same over the two years. However, when the cultivating environments were different ( B and C groups ), the stem length was different ( e.g. C group showed remarkably shorter stem length ). The result shows that the stem length of this species is influenced by its environment ( Fig. 16 ). The same tendency was shown in the length of the leaf ( Fig. 17 ) and the number of flowers per plant ( Fig. 22 ), however, there were no significant differences in the leaf width between B and C groups ( Fig. 18 ). As to flower size and spur length, there were no differences between B and C groups ( Figs. 23, 26 ). The length of the neck was longer in B group than in C group ( Fig. 24 ).

The correlation coefficients between the data in 1994 and 1995 are shown in Tables 2 and 3. Amongst the characteristics in 1994, there were positive correlations between bulb weight and stem diameter, stem length and the number of leaves, length of the leaf and stem diameter, length of the leaf and the number of leaves, length of the leaf and the number of flowers, leaf width and stem diameter, and the number of leaves and number of flowers. In 1995, there were positive correlations between stem length and the length of the leaf, the length of the leaf and the number of flowers. In addition there were some positive correlations between the data collected the previous year.

Germinating frequency and frequency of variants varied among capsules.

## Discussion

The seeds of *Habenaria radiata* easily germinated on the artificial media. By adding different organic compounds to the media, e.g. either yeast extract, malt extract, casanino acid or peptone, some differences resulted. Those results will be discussed in another report.

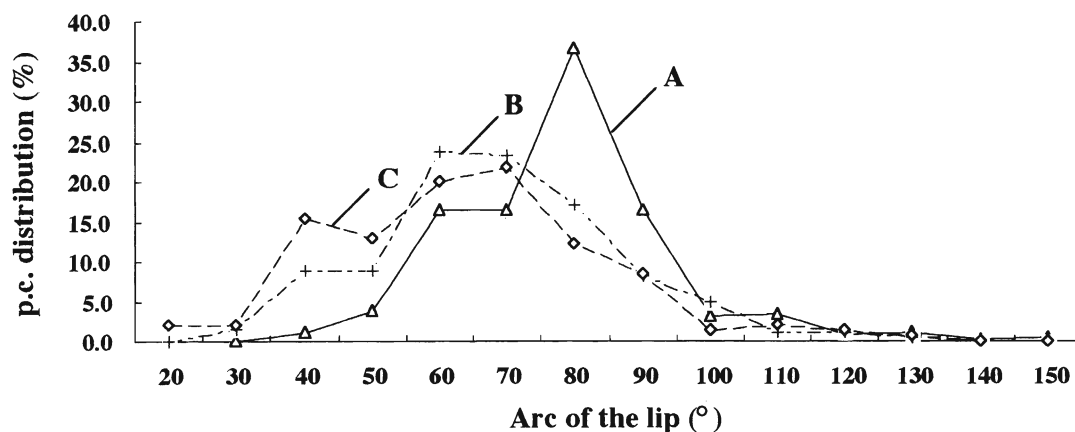


Fig. 25. Percent distribution of arc of the lip (°), A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

Table 2. Correlation coefficients between characteristics measured in 1994.

	Bulb weight	Stem length	Leaf length	Leaf width	Stem diameter	Leaf number	Flower number	Flower size	Neck length	Lip arc	Spur length
Bulb weight	-										
Stem length	0.35	-									
Leaf length	0.55	0.53	-								
Leaf width	0.43	0.04	0.43	-							
Stem diameter	0.60	0.20	0.63	0.64	-						
Leaf number	0.34	0.60	0.60	0.08	0.33	-					
Flower number	0.41	0.54	0.62	0.30	0.48	0.60	-				
Flower size	0.03	0.32	0.16	0.11	0.08	0.23	0.11	-			
Neck length	0.07	0.37	0.30	0.03	0.04	0.32	0.25	0.54	-		
Lip arc	0.08	0.02	0.00	0.10	0.07	0.03	0.10	0.09	0.18	-	
Spur length	0.40	0.19	0.32	0.45	0.45	0.01	0.05	0.02	0.20	0.20	-

Table 3. Correlation coefficients between characteristics measured in 1995.

	Bulb weight	Stem length	Leaf length	Leaf width	Stem diameter	Leaf number	Flower number	Flower size	Neck length	Lip arc	Spur length
Bulb weight	-										
Stem length	0.34	-									
Leaf length	0.48	0.62	-								
Leaf width	0.39	0.17	0.41	-							
Stem diameter	0.50	0.26	0.58	0.52	-						
Leaf number	0.45	0.58	0.50	0.18	0.34	-					
Flower number	0.46	0.50	0.69	0.43	0.55	0.42	-				
Flower size	0.06	0.42	0.33	0.05	0.11	0.31	0.22	-			
Neck length	0.30	0.40	0.43	0.16	0.23	0.38	0.23	0.57	-		
Lip arc	0.18	0.01	0.25	0.11	0.25	0.13	0.08	0.01	0.30	-	
Spur length	0.24	0.18	0.36	0.17	0.34	0.24	0.11	0.21	0.24	0.14	-

In bulbous plants, generally, blooming is strongly influenced by bulb volume. In the case of *H. radiata*, when the bulb was heavier than 201 mg, the blooming frequency was high (68.7 %). By contrast, if the bulb weight was lighter than 200 mg, the frequency of bloomed plants was low (35.0 %). It is possible that the lighter bulbs may not grow to adult phase (Fig. 2).

Takeda (1995) reported that seeds of *H. radiata* gathered in October, then sowed in January the following year, germinated and grew seedlings of 3–5 cm by the end of June. They entered the dormant stage in September. In the next spring 150 bulbs were potted and one plant bloomed in September. Thus, it took almost two years for the first flower to bloom. Tegarayama Botanical Garden (1991) also reported similar results. However, using our method, it took only one or two years to obtain adult phase bulbs, some of which bloomed within one year. Thus, sowing seeds in autumn, incubating them throughout the winter, and acclimatizing them in spring shortens the growing period.

Comparing the blooming dates between 1994 and 1995 of the plants cultivated in the greenhouse, the former group

was 2–3 weeks earlier than the latter (Fig. 21). Higuchi et al., (1987) reported that flowering of *Habenaria* depends on temperature only. When comparing the daily temperatures from April to August of the two years, a maximum difference of 1.3°C and a minimum difference of 0.9°C was observed. It was warmer in 1994 than in 1995 (Fig. 27).

For the past thirty years, there have been several mutants reported in the leaf characteristics of this species (Kimura, 1988). Most of these were discovered in the field. Concerning flower characteristics, some mutants were reported during the past fifteen years (Kimura, 1995). In the present investigation, several variants were produced from seedlings, e.g. albinos, dwarfs, variegated leaves, unopened flowers, and green-striped flowers, etc.. Some of these variants appeared the same as the reported mutants. Usually this species propagates vegetatively in the field. Thus, there might have been accumulated recessive genes in the population of this species in the field.

Whether specific variants result from developmental conditions, or genetic components is currently under investigation.

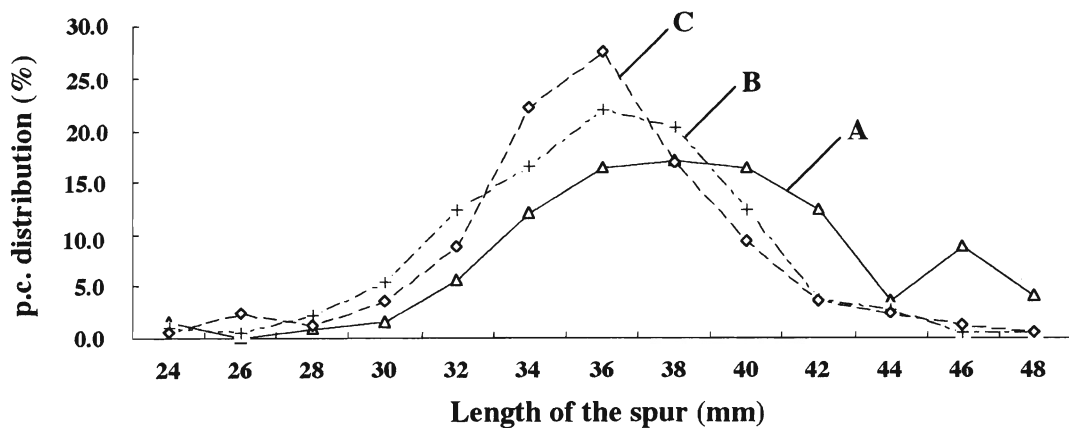


Fig. 26. Percent distribution of length of the spur (mm), A in the greenhouse 1994, B in the greenhouse 1995, C outside 1995 respectively.

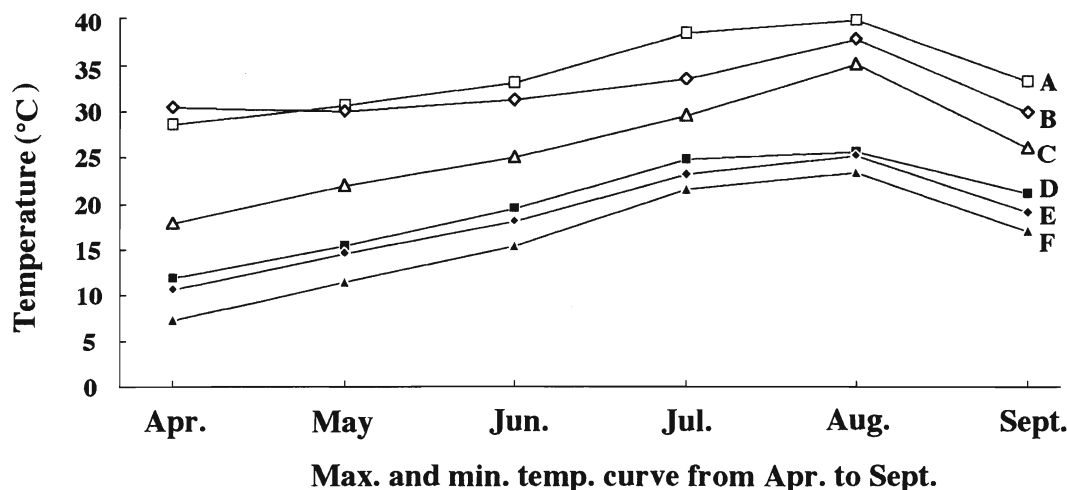


Fig. 27. Temperature curve from April to September (° c), A maximum temp. in the greenhouse 1994, B maximum temp. in the greenhouse 1995, C maximum temp. outside 1995, D minimum temp. in the greenhouse 1994, E minimum temp. in the greenhouse 1995, F minimum temp. outside 1995 respectively.

Table 4. Mean values and correlation coefficients of various morphological and physiological characteristics between parent and offsprings.

Generation year	Characteristics									
	Stem length	Leaf length	Leaf width	Stem diameter	Leaf no.	Flower no.	Flower size	Neck length	Lip arc	Spur length
1994	238.9 mm	97.8 mm	7.7 mm	3.7 mm	6.9	4.0	32.0 mm	12.2 mm	76.1°	38.2 mm
1995	236.5 mm	85.1 mm	6.8 mm	3.7 mm	7.9	2.9	30.0 mm	10.8 mm	67.7°	35.4 mm
R=	0.46	0.34	0.26	0.41	0.59	0.18	0.57	0.47	0.54	0.44

\*The mean value in 1995 was totaled only cultivated in the greenhouse. R : Correlation coefficients

All bulbs investigated in 1995 were vegetatively propagated. Thus, the same genotypes were used in the investigations in these two years. The characteristics measured over the two years were compared ( Table 4 ). There were some positive correlations between the two years data in number of leaves ( $r=0.59$ ), size of the flower ( $r=0.57$ ), and arc of the lip ( $r=0.54$ ). There were weak correlations between the two years data in stem length ( $r=0.46$ ), stem diameter ( $r=0.41$ ) and spur length ( $r=0.44$ ). The results indicated that once bulbs were obtained from seed, various characteristics might remain stable.

The authors wish to express their thanks to Mr. Daniel Albert for his review of the manuscript.

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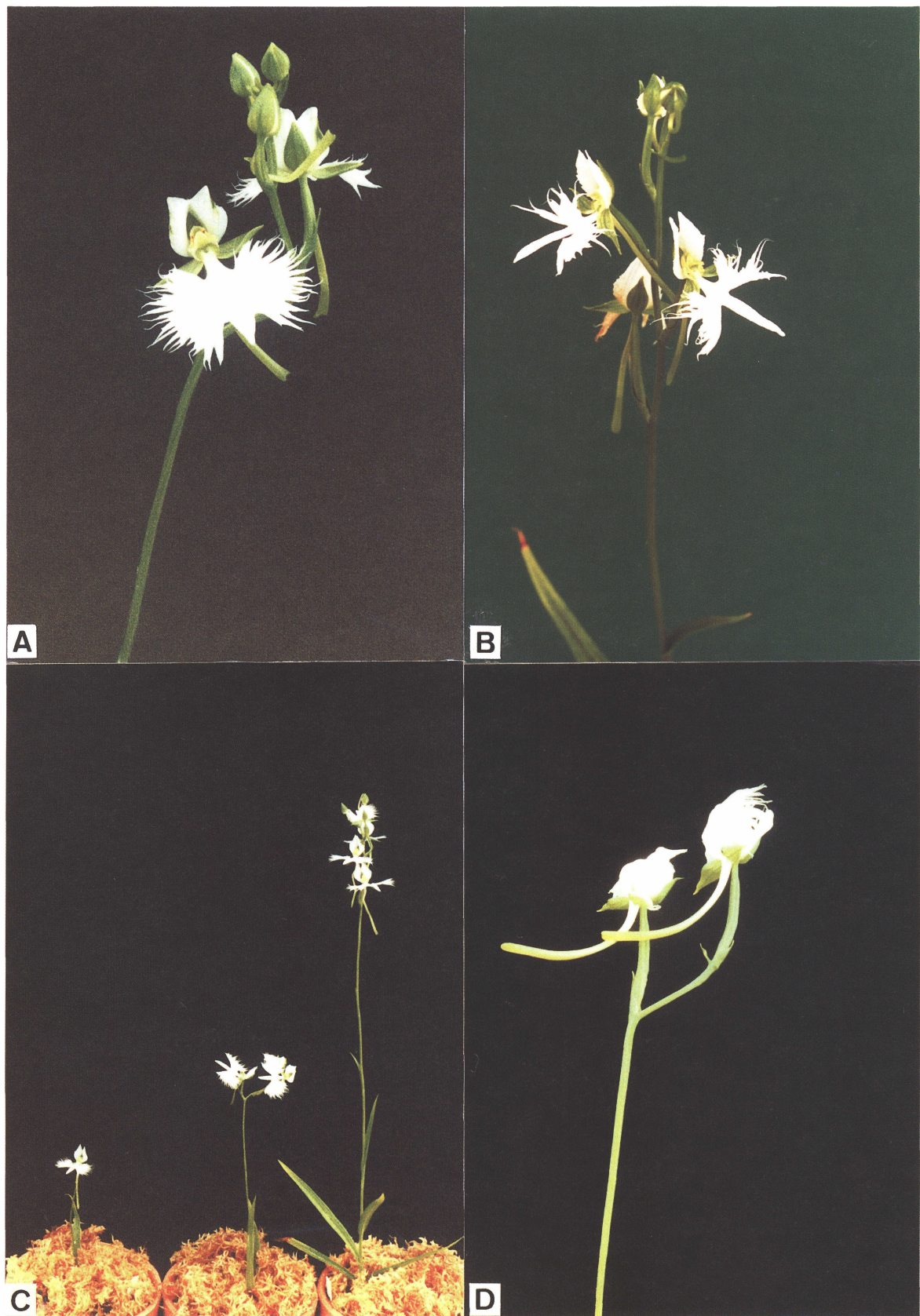


Photo. 1. Variants derived from seedlings. A: Short neck B: Long neck C: Dwarfs (center and left) D: Unopened flower



Photo. 2. Variants derived from seedlings. E: Multi-bud F: Albinos G: Twin flowers H: Cross lip flower