Shoots formation by rhizome culture of *Anemone keiskeana*©

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INTRODUCTION

Anemone keiskeana Maxim. is a perennial plant classified in the *Ranunculaceae*, that grows in western part of Japan. The standard flower has 12-22 pale bluish purple sepals, lacks petals, though the flower color has rich variations, including white, pale pink, and pale yellowish brown.

The leaves emerge from the rhizomes in autumn; the scapes appear and bloom in March. Leaves wither and the rhizomes go into a dormant period in May and are therefore summer dormant. *Anemone keiskeana* often develops "brood buds" in the axils of the rhizomes (Figure 1). Brood buds are microtuber-like bodies that originate from lateral buds and in Japanese are called "mukago". These buds don't develop into lateral branches on the rhizome and easily separate from the rhizome. In pot culture separated brood buds sprout after 1 or few years from separation and form small rhizomes.

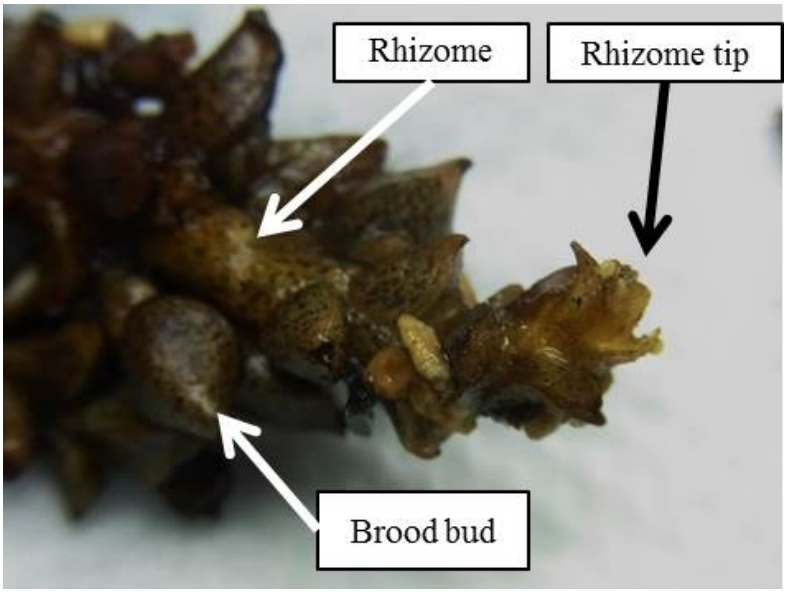

Figure 1. Rhizome, rhizome tip and brood buds of *Anemone keiskeana* in dormant period.

In this study, we aimed to establish *A. keiskeana* in a sterile culture system from cultured rhizomes segments and brood buds and develop a multiplication system.

MATERIALS AND METHODS

For this experiments, rhizomes of four clones ('Akehasu', Unknown, 'Ruri-ichige', and 'Yanadani') and brood buds of 'Yanadani' clone were used in the primary culture (Table 1). The rhizomes and brood buds were washed in tap-water and laid on moist pumice (diameter of 1-2 mm) used in gardening and called "kanuma" soil in the bottom of shallow storage containers. For keep wet condition, the containers were put in a plastic tank, and covered by plastic tray, incubated under 20±2°C, 16 h day^{-1} white fluorescent lamp

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illumination (about 1,000 Lux) condition. To reduce risk of contamination, the rhizomes and brood buds were sprayed with 1,000 mg L^{-1} Benlate wettable powder(Sumitomo Chemical Co., Ltd., Japan, containing 50% Benomyl) solution, every 3-5 days according to label directions.

Table 1. Tested organs, characteristic of sepal and origin of tested 4 clones of *Anemone keiskeana.*

Name of clone	Tested organs	Color and characteristic of sepal	Origin
Akehasu	Rhizomes	Pale pink	Ehime, collected by Akehasu
Unkown	Rhizomes	Pale yellowish brown	Unknown
Ruri-ichige	Rhizomes	Bluish purple	Ehime, collected by Y. Hiraoka
Yanadani	Rhizomes and brood buds	Pale yellowish brown plenty sepals	Yanadani, Kuma-kogen, Ehime, collected by S. Ogawa

About 20 days after start of Benlate spray treatment, the rhizomes and brood buds were used as materials of tissue culture. They were washed by tap-water, and dipped in sodium hypochlorite solution (1% available chlorine, with spreader) for about 9 mins., and then rinsed with sterilized water. Explants were prepared by cutting the rhizome into sections including rhizome tip and nine additional 1 mm thick sections. The brood buds were used intact as explants.

Basal medium for all cultures was MS medium (Murashige and Skoog, 1962) with inorganic salt used at half strength, sucrose at 30 g L^{-1}, and a combination of 1-naphthylacetic acid (NAA) and 6-benzylaminopurine (BA) at 0, 0.1, or 1 mg L^{-1} each as plant growth regulators. The pH was adjusted to 5.8±0.1 and 2.5 g L^{-1} gellan gum (Wako pure Chemical Industries, Ltd., Japan) was added before dispensing 10 mL test $tube^{-1}$ (25 mm diameters; 120 mm height).

The explants of rhizome tip sections and brood buds were placed with the cutting plane down, in case of rhizome round sections, put the cutting planes horizontally, and placed one per test tube on each medium, and then observed for every 30 days after inoculation for contamination, callus formation and shoot formation.

These were incubated under 21±2°C and 16 h day^{-1} white fluorescent lamp illumination (about 2,000 Lux) condition.

RESULTS AND DISCUSSION

Contaminations were observed in three clones except in the 'Akehasu' clone, at 210 days after inoculation. The contaminated explant rates of 'Ruri-ichige' clone were 44.4% in rhizome tip sections and 25.0% in rhizome round sections; these rates were higher than 'Yanadani' and Unknown clones (Table 2). In each explant types, the total contaminated explants rate of rhizome round section explants was 6.3%, this value was lower than rhizome tip section explants (19.4%) and brood explants (17.8%). We consider that the cause is the difference of the ratio of epidermis area to explant's surface area.

In this experiment, frequency of contamination was less than expected and we consider that one of the factors was the Benlate spray treatment. To corroborate the hypothesis, rhizome round sections and brood buds of "Yanadani" clone were treated 0, 1 or 4 times with Benlate solution spray every 2-3 days, and inoculated on hormone-free medium, in the same way. Although in all Benlate spray treatments the contaminated explants were little or nothing about 3 weeks after inoculation (data not shown).

Table 2. The relation between rate of contamination and explant types in rhizome culture of *Anemone keiskeana* (about 210 days after inoculation).

Name of clone	Rhizome tip sections			Rhizome round sections			Brood buds		
	No. of explants	No. of contaminated explants	Rate of contamination (%)	No. of explants	No. of contaminated explants	Rate of contamination (%)	No. of explants	No. of contaminated explants	Rate of contamination (%)
Akehasu	9	0	0	36	0	0	-	-	-
Unknown	9	1	11.1	36	0	0	-	-	-
Ruri-ichige	9	4	44.4	36	9	25.0	-	-	-
Yanadani	9	2	22.2	36	0	0	45	8	17.8
Total	**36**	**7**	**19.4**	**144**	**9**	**6.3**	**45**	**8**	**17.8**

Callus and shoot formation occurred from all clones, however, the frequency and amount varried depending on the clone.

Callus arose from the xylem of section planes, and as a result the callus formation happened at higher frequency in the rhizome round sections and required addition of BA (Table 3).

Shoot formation occurred even on hormone-free medium in particular rhizome tip sections (Table 4). It was noted that shoots originating from the shoot apical meristem on the rhizome tip and the axillary bud were larger than those that originated via adventitious buds. The addition of NAA inhibited shoot formation especially from shoot apical meristem and the addition of BA promoted adventitious shoot induction via callus.

After this, we will study establishment of multiplied shoots and regeneration from callus, in parallel, try interspecies crossing with other species of *Anemone*.

Table 3. Effects of plant growth regulators for callus formation on rhizome culture of *Anemone keiskeana* (about 210 days after inoculation).

PGR[1]		Rhizome tip sections			Rhizome round sections			Brood buds		
NAA (mg L^{-1})	BA (mg L^{-1})	No. of explants	No. of explants formed callus	Rate of explants formed callus (%)	No. of explants	No. of explants formed callus	Rate of explants formed callus (%)	No. of explants	No. of explants formed callus	Rate of explants formed callus (%)
0	0	3	0	0	14	0	0	5	0	0
0	0.1	2	0	0	14	1	7.1	5	0	0
0	1	3	0	0	15	6	40.0	4	1	25.0
0.1	0	3	0	0	15	0	0	3	0	0
0.1	0.1	3	1	33.3	16	8	50.0	3	0	0
0.1	1	4	1	25.0	15	11	73.3	4	1	25.0
1	0	3	0	0	16	1	6.3	4	0	0
1	0.1	4	2	50.0	15	7	46.7	5	0	0
1	1	4	1	25.0	15	9	60.0	4	2	50.0
Total		**29**	**5**	**17.2**	**135**	**43**	**31.9**	**37**	**4**	**10.8**

[1]PNG: Plant growth regulator.

Table 4. Effects of plant growth regulators for shoot formation on rhizome culture of *Anemone keiskeana* (about 210 days after inoculation).

PGR[1]		Rhizome tip sections			Rhizome round sections			Brood buds		
NAA (mg L^{-1})	BA (mg L^{-1})	No. of explants	No. of explants formed shoots	Rate of explants formed shoots (%)	No. of explants	No. of explants formed shoots	Rate of explants formed shoots (%)	No. of explants	No. of explants formed shoots	Rate of explants formed shoots (%)
0	0	3	3	100	14	3	21.4	5	2	40.0
0	0.1	2	2	100	14	1	7.1	5	3	60.0
0	1	3	1	33.3	15	1	6.7	4	3	75.0
0.1	0	3	1	33.3	15	0	0	3	0	0
0.1	0.1	3	1	33.3	16	1	6.3	3	1	33.3
0.1	1	4	3	75.0	15	6	40.0	4	2	50.0
1	0	3	0	0	16	0	0	4	0	0
1	0.1	4	0	0	15	0	0	5	2	40.0
1	1	4	0	0	15	3	20.0	4	4	100
Total		**29**	**11**	**37.9**	**135**	**15**	**11.1**	**37**	**17**	**45.9**

[1]PNG: Plant growth regulator.

Literature cited

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. *15* (*3*), 473–497 https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.