Evaluation of Graft Compatibility for Taxonomical Study in Orange Subfamily Plants©

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Callus tissues induced from shoot of orange subfamily plants were grafted in vitro to evaluate the genetic relationship. The graft interface between two pieces of callus which are taxonomically of a close relation was not distinguishable by anatomical observation. On the other hand, in the combinations of a more distant relationship, the graft border interface was distinct. In a combination whose relation was further in taxonomical order, the border was clear and some deposits were accumulated. In these combinations cell wall decay was observed at the contact surface of both callus cells. The contacted callus cells showed the recognition response to the graft partners. The compatible and incompatible features in grafting can provide the information on taxonomy of orange subfamily plants.

In Vitro Propagation of *Cryptocoryne* Species©

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In vitro propagation protocol for *Cryptocoryne* species is reported. Shoot proliferation of *C. wendtii* and *C. pontederiifolia* were promoted on Murashige and Skoog (MS) agar solidified medium supplemented with BA at higher concentrations (1 to 20 μM). Compared to solid medium, shoot proliferation was improved in liquid-shake culture. However, abnormal shoot growth was observed in both species. In *C. pontederiifolia*, shoot grown in liquid-shake culture showed chlorotic leaf growth. In *C.wendtii*, leaves were needle-like in appearance. A double-layer culture method gave high yield of normal and healthy shoots. The volume of the additional liquid medium in double-layer culture affected shoot proliferation.

INTRODUCTION

Recently, market demand for ornamental aquatic plants has increased and efficient propagation techniques of aquatic plants is required. Although plant tissue culture techniques have been developed to propagate many horticultural plants, information is limited in aquatic plant species. (Kane et al. 1990, 1999; Jenks et al., 2000)

The genus *Cryptocoryne* contains some of the commercially important aquatic species. Most *Cryptocoryne* species are native to Southeast Asia and Indonesia and they are grown in either the submerged or emerged state. Because flower formation and subsequent seed production of *Cryptocoryne* occurs infrequently and rhizome division occurs slowly, the propagation of *Cryptocoryne* species is restricted.

This paper describes the in vitro propagation protocol for two *Cryptocoryne* species.

MATERIALS AND METHODS

Shoot tips, tuber, and stolon expants (10 mm long) were excised from donor plants (*C. wendtii* 'Tropica', and *C. pontederiifolia*) grown aerially in pots containing 1 peat : 1 vermiculite : 1 sand (by volume). Explants were rinsed in tap water and then surface sterilized by successive immersion in 70% ethanol for 5 min and 2 % NaOCl containing Tween 20 for 20 min, followed by three rinses in sterile distilled water. Surface sterilized explants were transferred into 150 × 25 mm culture tubes containing 10 ml of Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 5 μM benzylaminopurine (BA), 3% sucrose, and 0.8% agar and closed with transparent polyethylene caps. The pH was adjusted to 5.8 with 0.1 N KOH before autoclaving at 121 ºC for 20 min. Cultures were maintained 25 ºC under 30 μmol $m^{-2} \cdot s^{-1}$ and 16-h photoperiod provided by cool-white fluorescent lamps. Stock plant cultures were increased by subculturing the basal shoots produced at 30 days interval on MS medium supplemented with 5 μM BA, 3% sucrose, and 0.8% agar.

In preliminary experiments, tuber explants proved difficult to establish in vitro (60% contamination). Shoot tip and stolon explants were successfully surface disinfected and established (20% to 30% contamination). Therefore, shoot cultures from shoot tip explants were used for shock plant cultures.

Effect of BA Concentration in MS Solid Medium on Shoot Proliferation. Shoots of *C. wendtii* 'Tropica', and *C. pontederiifolia* were used. Shoots (about 10 mm long) were cultured on MS medium supplemented with BA at 0, 1, 5, 10, and 20 μM. After 30 days, cultures were evaluated for shoot proliferation and leaf growth. For each concentration, 15 replications were used and each experiment was repeated twice.

Shoot Proliferation in Liquid-Shake Culture. Shoots of *C. wendtii* 'Tropica', and *C. pontederiifolia* were used. Three shoots were cultured in 200-ml glass flasks that contained 80 ml of MS liquid medium supplemented with BA at 0, 5, 10, and 20 μM on a horizontal rotary shaker at 60 rpm. After 30 days, number of shoots and maximum leaf length were recorded. Each experiment was repeated three times.

Effect of Double-Layer Culture Method on Shoot Proliferation. In the double-layer method, shoots were cultured in 150 × 25 mm culture tubes containing 10 ml of MS solid medium supplemented BA at 0, 1, 5, 10, and 20μM and then, MS liquid basal medium (without BA) was added to the level of shoot height (shoot tips appeared just above the surface of liquid medium in a merged state). Effect of liquid medium volume added on MS solid medium was also examined. In this experiment, MS liquid medium was added on solid medium until the shoot was cultured in complete submerged state (twice of shoot height). For each treatment, 15 replications were used and each experiment repeated twice.

RESULTS AND DISCUSSION

Effect of BA Concentration in MS Solid Medium on Shoot Proliferation. The data is shown in Table 1. On MS solid medium, shoot proliferation of *C. wendtii* and *C. pontederiifolia* were promoted on the medium supplemented with BA at higher concentration. However, number of shoots in *C. pontederiifolia* cultured on the medium with BA at 25 μM was 2.7 (data not shown). Therefore, optimum BA concentration for shoot proliferation was 20 μM in *C. pontederiifolia*. Shoots grown on MS solid medium appeared normal and healthy.

Table 1. Effect of BA concentration in solid medium on shoot proliferation.

	Cryptocoryne pontederiifolia		*Cryptocoryne wendtii* 'Tropica'	
(μM)	Shoots (no.)	Max. leaf length (cm)	Shoots (no.)	Max. leaf length (cm)
0	1.1±0.1[z]	2.3±0.2	1.6±0.4	1.1±0.2
1	1.7±0.3	1.5±0.2	1.8±0.4	1.1±0.2
5	2.9±0.6	1.0±0.1	3.6±0.2	1.2±0.2
10	2.2±0.4	1.2±0.2	4.8±0.6	0.8±0.1
20	4.0±0.7	1.2±0.1	5.0±1.0	1.0±0.1

[z]Each value is the mean±standard error.

Table 2. Effect of BA concentration in liquid medium on shoot proliferation.

	Cryptocoryne pontederiifolia		*Cryptocoryne wendtii* 'Tropica'	
(μM)	Shoots (no.)	Max. leaf length (cm)	Shoots (no.)	Max. leaf length (cm)
0	1.4±0.2[z]	3.1±0.6	2.8±1.1	1.3±0.7
5	4.6±0.7	1.9±0.3	7.8±1.0	1.7±0.1
10	7.6±1.6	0.9±0.1	8.6±2.3	1.7±0.1
20	1.8±0.6	0.9±0.1	12.7±1.5	1.4±0.1

[z]Each value is the mean±standard error.

Shoot Proliferation in Liquid-Shake Culture. In general, shoot proliferation was improved in liquid-shake culture (Table 2). However, in *C. pontederiifolia*, shoot proliferation in MS liquid medium supplemented with BA at 20 μM was remarkably decreased. Furthermore, when shoots of *C. pontederiifolia* were cultured in MS liquid media without shaking, most shoots died (data not shown). The results indicated that shoot growth and proliferation of *C. pontederiifolia* might require more oxygen supply than those of *C. wendtii.* In liquid-shake culture, abnormal shoot growth was observed in both species. In *C. pontederiifolia*, shoot grown in liquid-shake culture showed chlorotic leaf growth. In *C. wendtii*, leaves were narrow and had a needle-like appearance. When these abnormal shoots were transferred on MS solid media supplemented with BA, shoot growth occurred very slowly.

Effect of Double-Layer Culture Method on Shoot Proliferation. Viseur (1987) reported that double-layer culture method gave high yield of axillary shoot, while avoiding vitrification in pear. In this paper, we tested this culture method to avoid shoot abnormality. The result is shown in Table 3. In both species, shoot proliferation was promoted on MS medium supplemented with BA at 1 to 20 μM. Shoots cultured by double-layer culture method showed normal and healthy growth. The volume of the additional liquid medium affected shoot proliferation. When a large quantity of liquid medium was added on solid medium and shoots were cultured in submerged state, shoot proliferation of *C. pontederiifolia* was re-

Table 3. Effect of BA concentration on shoot proliferation.

	Cryptocoryne pontederiifolia		*Cryptocoryne wendtii* 'Tropica'	
(μM)	Shoots (no.)	Max. leaf length (cm)	Shoots (no.)	Max. leaf length (cm)
0	1.6±0.4[z]	1.9±0.3	1.4±0.2	1.5±0.3
1	3.0±0.6	1.2±0.2	4.8±0.6	1.0±0.1
5	5.2±0.5	1.0±0.1	7.0±0.6	1.1±0.1
10	4.3±0.7	1.1±0.2	12.5±2.1	1.2±0.1
20	4.8±0.8	0.8±0.1	8.6±0.1	1.1±0.1

[z] Each value is the mean±standard error.

Table 4. Shoot proliferation when shoots were cultured in complete submerged condition.

Species	Shoots (no.)	Max. leaf length (cm)
Cryptocoryne pontederiifolia	1.4±0.2[z]	2.5±0.4
Cryptocoryne wendtii 'Tropica'	21.4±2.8	1.7±0.1

[z] Each value is the mean±standard error.

markably decreased, while that of *C. wendtii* was promoted (Table 4). These differences of growth responses in two species may reflect the differences of growth condition in their original habitat. *Cryptocoryne wendtii* grows in merged, submerged, and aerial states, while *C. pontederiifolia* grows in merged and aerial state.

In this paper, we reported the efficient propagation protocol of two *Cryptocoryne* species. However, double-layer culture method was not suitable for large-scale propagation. Therefore, it is necessary to develop the large-scale production systems using liquid culture. We consider that aeration or oxygen supply to the culture medium could be an important factor for the development of an efficient liquid culture system.

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