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Plant Regeneration and Morphology during in-Vitro Organogenesis from *Heloniopsis orientalis* (Thunb.) C. Tanaka

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***Heloniopsis orientalis* (Liliaceae) is an important horticultural crop native to Korea. Under natural conditions, germination is poor and plant growth is delayed. Therefore, we have developed a vegetative propagation method to produce plants with vigorous growth characteristics via tissue culture. Leaf tissues were cultured on MS basal media supplemented with growth regulators 2,4-D, TDZ, BA, or zeatin. The regenerated shoots were then initiated directly from leaf explants on an MS medium containing either 0.1 to 1.0 mg/L 2,4-D or 0.1 to 3.0 mg/L BA. Healthy plantlets with adventitious roots were formed on the medium supplemented with 0.1 mg/L BA. TDZ triggered callus initiation without caulogenesis or rhizogenesis, and callus formation was better on the half-strength MS medium than on the full-strength medium. After the plants were acclimatized for one month at 4°C, they were successfully transferred to soil. In addition, we used LM and SEM to investigate shoot morphogenesis at various stages of differentiation.**

Keywords: *Heloniopsis orientalis*, regeneration

Heloniopsis orientalis (Liliaceae), a perennial evergreen herb, grows under closed canopies in wet soils and rich organic matter (Lee, 1982; Min, 2000a). Destruction of the forest and an increase in the annual mean air temperature have decreased the size of this species' habitat. In the natural population, *H. orientalis* normally produces 3,000 seeds per year, but only a few new plants are recruited in this manner (Min, 2000b).

Tissue culture techniques have been successful for rapid propagation of some members of the genus *Lilium*, including *L. longiflorum* (Nhut, 1998), *L. rubellum* (Nimii et al., 1997), *L. lancifolium* (Marinangeli and Curvetto, 1997), *L. auratum* (Takayama and Misawa, 1979, 1980, 1983), and *L. testaceum* (Wozniewski et al., 1991). However, micropropagation of *H. orientalis* has been reported only by Yun et al. (2000), who used young leaves originating from regenerants through in-vitro culture.

The time of year in which the explant is taken may also influence the results of a micropropagation program (Torres, 1989). Changes in temperature, daylength, light intensity, and water availability throughout the year may modify the levels of carbohydrates, proteins, and growth substances in the stock plant, thus affecting the response of the explant in-vitro.

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Morphology during in-vitro organogenesis has been described for leaf explants from roses (Ibrahim and Debergh, 2001) and lavender (Dronne et al., 1999), and from the petioles of grape (Martinelli et al., 2001). Those studies showed that calli developed from the cut edges of the leaf, and were compact and yellowish-white with a few green portions. To examine the patterns of shoot development into plants of *H. orientalis*, we observed the morphological and histological characteristics of differentiation during the culture of leaf explants. Here, we describe the optimal conditions for in-vitro multiplication, which may be helpful in the domestication of *H. orientalis*.

MATERIALS AND METHODS

Plant Material

Fresh leaves of *H. orientalis* were collected from Namhansanseong, Korea, during December of 2000, and were stored in an incubator at 25°C. Later, the leaves were washed thoroughly under running tap water. Following treatment for 10 s with 70% ethanol, they were surface-sterilized with an aqueous solution of sodium hypochlorite (approx. 2% active chlorine) for 15 min, and rinsed three times in sterile distilled water.

Callus and Shoot Induction

We tried to induce shoot formation from calli originating from leaves, but caulogenesis was not observed when culturing with IAA and NAA. Therefore, we cultured the leaf tissues on an MS (Murashige and Skoog, 1962) basal medium supplemented with growth regulators 2,4-D, TDZ (thidiazuron), and BA. All media were adjusted to pH 5.7 before being dispensed and autoclaved at 121°C for 15 min. The Petri dishes were sealed with Parafilm M. Each experiment was then pretreated in continuous darkness for 10 d. Afterward, the cultures were kept for eight weeks at 25°C ± 1°C and under a 16-h/8-h day/night photoperiod. All experiments were performed twice, with 20 replicates. At the end of the culture period, we recorded the percentage of callus or shoot induction.

We also tested quarter-, half-, full-, and double-strength MS media for their ability to initiate shoots from the leaf tissue. These media were supplemented with 0.5 mg/L BA and 30 g/L sucrose, at a pH of 5.8. The percentage of callus or shoot induction was also recorded after seven weeks of culture on an MS medium without growth regulators. This cycle was repeated twice.

The regenerated shoots from our explants were excised and cultured in Magenta boxes with 1/4 MS salts, solidified with 0.4% gelrite, and supplemented with 0.5 mg/L BA. Lighting and temperature conditions were identical to those used during the shoot initiation process. Rooted plantlets were transplanted to 6-cm pots filled with a 1:3 (v:v) mixture of vermiculite and commercial compost. They were then held in an isolation room to maintain a high relative humidity before being placed in a growth chamber at 4°C or 24°C under a 16-h photoperiod. The plants were acclimatized by decreasing the relative humidity to ambient conditions over a one-month period.

Morphological Observations

We used both a light microscope (LM) and a scanning electron microscope (SEM) to record the events leading to shoot formation from our explants. During this eight-week process, we kept the tissues in an MS medium with 0.5 mg/L BA, and subcultured them every two weeks. For LM, we fixed the shoot-forming tissue overnight in FAA (1:1:18 v/v formalin:acetic acid: alcohol) solution, then embedded and mounted it as described by Clark (1981). The 10-µm sections were deparaffinized and stained for 1 h in 10 g/L Safranin O, rinsed in water, and incubated in 1 g/L Fast-green for 3 min. Sections were then washed, dried, and mounted in clove oil (Sigma: St. Louis, MO, USA). For the SEM study, we slightly modified the method of Martinelli et al. (2001), fixing the shoot-forming calli for 12 h in 2.5% paraformaldehyde-glutaraldehyde in a phosphate buffer (pH 7.2), then dehydrating them through a graded alcohol series. The specimens were dried to the critical point, fixed on holders with silver adhesive, coated with gold in a vacuum spatter coater, and observed using a Hitachi S-2300 SEM with a tungsten filament at 25 kV.

RESULTS

Influence of Plant Growth Regulators on Callus and Shoot Induction

Leaf explants cultured after the dark pretreatment showed a high rate of shoot induction in both the

Table 1. Effects of growth regulators on plant regeneration from leaf explants of *H. orientalis*. Explants were maintained in darkness for 10 d before culture under light/dark conditions, and were maintained for eight weeks on MS media.

Treatments (mg/L)		Callus induction (%)	Shoot induction (%)	No. of shoots/ explant	Root induction (%)	Plantlet formation (%)
2,4-D	0.1	2.5 ± 2.5	51.0 ± 11.0	1.3 ± 0.3	60.5 ± 20.5	36.0 ± 16.0
	0.5	10.0 ± 10.0	32.5 ± 12.5	0.7 ± 0.2	65.0 ± 10.0	22.5 ± 12.5
	1.0	19.5 ± 10.5	22.0 ± 13.0	0.4 ± 0.2	28.5 ± 1.5	5.0 ± 5.0
	3.0	0	0	0	0	0
TDZ	0.1	85.5 ± 1.5	32.5 ± 0.5	0.5 ± 0.1	8.0 ± 8.0	2.0 ± 2.0
	0.5	92.5 ± 2.5	20.0 ± 10.0	0.5 ± 0.2	7.5 ± 2.5	0
	1.0	85.0 ± 15.0	15.0 ± 0.0	0.3 ± 0.1	2.5 ± 2.5	0
	3.0	78.0 ± 3.0	3.0 ± 3.0	0.1 ± 0.0	3.0 ± 3.0	0
BA	0.1	0	40.0 ± 13.0	0.5 ± 0.0	38.0 ± 15.0	27.0 ± 13.0
	0.5	0	37.5 ± 7.5	0.5 ± 0.2	70.0 ± 0.0	36.0 ± 5.0
	1.0	8.0 ± 8.0	34.0 ± 6.0	0.6 ± 0.1	36.0 ± 4.0	24.0 ± 4.0
	3.0	42.5 ± 12.5	27.5 ± 7.5	0.4 ± 0.1	55.0 ± 10.0	17.5 ± 12.5

2,4-D and the BA treatments, with the highest frequencies arising from 0.1 mg/L 2,4-D (51%) and 0.1 mg/L BA (40%) (Table 1). Treatment with TDZ produced lower induction frequencies. Rates of root formation were 70% with BA and 65% with 2,4-D.

Callus induction was high (78 to 92%) from tissue treated with TDZ, whereas the other growth regulators promoted lower frequencies, i.e., 2,4-D (0- to 19%) and BA (0- to 42%). Rates of plantlet formation were high from treatments with 2,4-D (36%) or BA (36%). The optimal BA concentration for inducing plantlet formation was 0.1- to 0.5 mg/L.

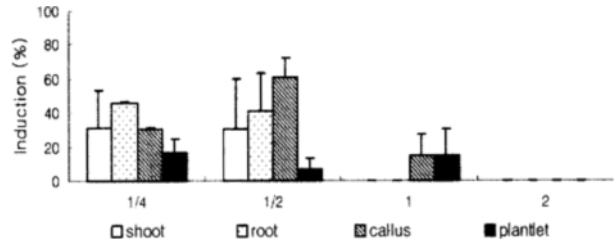

Figure 1. Effect of MS-medium strength on shoot, root, callus, and plantlet induction from leaf explants of *H. orientalis*. Explants were maintained in darkness for 10 d, then cultured under light/dark conditions for seven weeks.

Influence of MS-Medium Strength on Organogenesis

Based on our previous results, we determined that 0.5 mg/L BA was most effective for regenerating plantlets from leaf explants. Therefore, to investigate the effects of medium strength on organogenesis, we cultured the leaf explants on quarter-, half-, full-, and double-strength MS media that were supplemented with 0.5 mg/L BA. Quarter-strength media produced better efficiencies than did the half- or full-strength media; no shoots were induced from the double-strength trials. Callus induction was >60% on the half-strength medium (Fig. 1).

Plantlet Acclimatization

Pretreating the plantlets in a growth chamber, and incrementally decreasing the ambient relative humidity over a one-month period at 4°C, led to an average survival rate of 60%. In contrast, no plantlets survived after treatment at 24°C. This positive response under cooler conditions may have resulted from a reduction in hyperhydricity caused by water vapor condensing on top of the soil (see also Ibrahim and Debergh, 2001).

Morphology of Shoot Development

We noted striking differences in both cell size and morphology between the adaxial and abaxial faces of the leaves. When the adaxial surface was cultured face-down in the medium, cell sizes were gradually reduced from the lower to the upper adaxial region. In addition, an increase in cytoplasm was observed, as

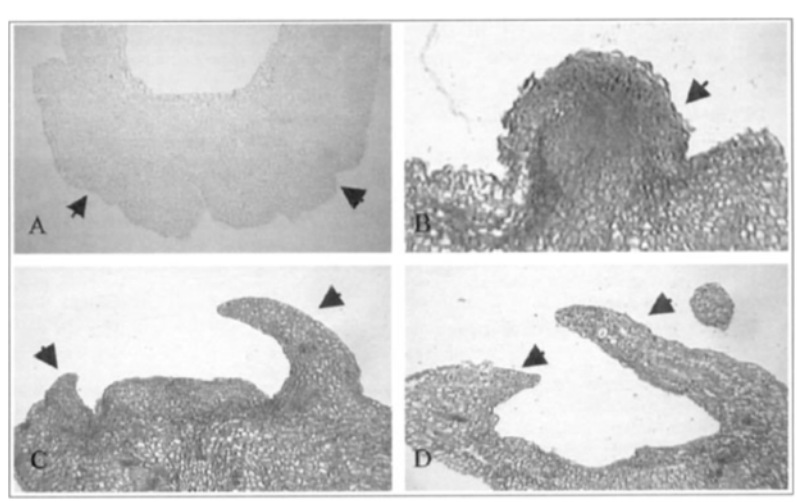

Figure 2. Histological patterns of shoot formation with leaf primordia by light microscope from the callus of *H. orientalis*. **A,** Callus initiation from leaf epidermis; **B,** Region of meristemoid; **C and D,** Development of shoot primordia. (Arrowheads in 'A' indicate region of callus induction; a future leaf primordia-forming region in a round protuberance in 'B'; two leaf primordia in 'C' and 'D').

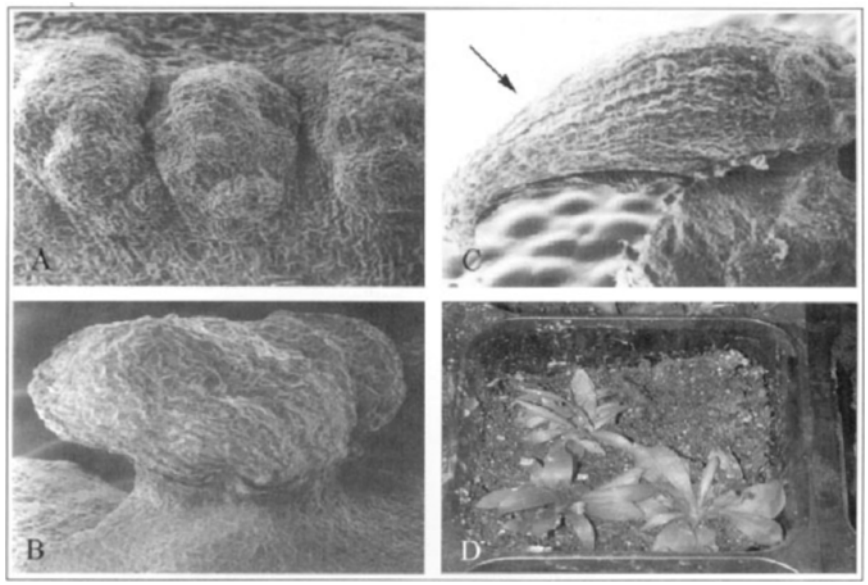

Figure 3. Development of shoot primordia induced from callus of *H. orientalis*. SEM pictures at developmental stages (**A,** Early; **B,** Middle; **C,** Late). **D,** Photograph of regenerants (arrows indicate the region of shoot apex).

evidenced by the dense staining response. The upper external layer, where the cell size was much reduced and cytoplasm became dense, showed the features of merismatic cells. These cells exhibited active proliferation and initiated clusters progressively, forming numerous huge protruding masses (Figs. 2 and 3).

DISCUSSION

When we attempted to induce regeneration from leaves after the plant had flowered, we were unable to obtain callus or organ formation. Therefore, we selected leaf explants before flowering had occurred. Torres (1989) has also shown that the stage of development, i.e., the age of the explant, is a critical factor in determining the success of micropropagation.

Various plant growth regulators influence callus development from cultured explants of Liliaceae (Nhut, 1998; Dronne et al., 1999; Yun et al., 2000; Wawrosch et al., 2001). We also examined their effects on shoot induction from leaf explants, and found that organogenesis depended on the specific type of plant growth regulator used in the MS media. Several studies have demonstrated that BA is a very effective promoter of shoot induction and multiplication from Liliaceae, roses, and cassava (Konan et al., 1997; Nikam and Shitole, 1997; Godo et al., 1998; Nhut, 1998; Ulrich et al., 1999; Han et al., 2001). Although plants treated with higher levels of BA produced more shoots, they were very short and compact, and some had abnormal leaves. In contrast, smaller concentrations may have little effect on shoot growth (Ulrich et al., 1999; Han et al., 2001).

Applications of low levels of TDZ can enhance meristem formation and shoot proliferation (Fellman et al., 1987), and may manifest results similar to those caused by cytokinins in many species (Reynolds, 1987). Likewise, Yun et al. (2000), studying the influence of 2,4-D, kinetin, and zeatin on the development of *Heloniopsis orientalis*, found that zeatin was most effective in promoting regeneration. However, in our preliminary experiments (data not shown), treatment with zeatin gave rise to the lowest frequency of shoot formation among the cytokinins. Although Yun et al. (2000) were able to induce callus production with 2,4-D, we were more successful with callus proliferation in media containing TDZ. Moreover, our highest frequency of shoot induction was in response to 0.1 or 0.5 mg/L BA, rather than 2,4-D or TDZ (Table 1).

These somewhat different responses of organogenesis to growth regulators may be attributed to the origin of the leaf explants. For example, Yun et al. (2000) used young leaves originating from regenerants through in-vitro culture, whereas we analyzed mature leaves collected before flowering occurred on field-grown plants.

Treatment with TDZ has resulted in a wide variety of responses during the propagation of cassava (Konan et al., 1997), roses (Ibrahim and Debergh, 2001), and

Spathiphyllum (Han et al., 2001). Those studies demonstrated that TDZ had a strong capacity for promoting shoot-formation. However, our experiments showed that TDZ caused very low frequencies and was less effective than either 2,4-D or BA. Overall, we propose that the addition of BA seems to be the more important parameter for successful caulogenesis.

In the present study, the shoot-forming capacity of leaf explants was significantly affected by the presence of light during the culturing period. Our preliminary experiments indicated a difference in response between tissues pretreated in the dark, followed by culture under illumination versus those not receiving the dark pretreatment. Incubation in the dark also increases shoot regeneration in species such as blueberry (Billings et al., 1988), pear (Chevreau et al., 1989), apple (Fasolo et al., 1989), quince (Baker and Bhatia, 1993), and rose (Ibrahim and Debergh, 2001). Therefore, we conclude that incubating the tissue for 10 d in the dark is more effective for promoting shoot regeneration in *H. orientalis*.

The importance of the concentration of the MS medium has proven to be important for callus induction and shoot regeneration from leaf tissue in the pear (Chevreau et al., 1989) and the lily (Nhut, 1998). Yun et al. (2000) reported that decreasing the strength of the medium led to an increase in both parameters. In our experiments, however, a lower-strength medium did not improve plantlet induction (i.e., rooted shoots), but did have a positive effect on callus and shoot induction (Fig. 1). Half- and quarter-strength media were more effective than full- or double-strength media, results that are consistent with those of Chevreau et al. (1989), Nhut (1998), and Yun et al. (2000).

To maximize survival after transplantation, it is common practice to acclimatize the plantlets in a humid environment. Roberts et al. (1990) found that placing the culture vessels on a cooled shelf could harden the plantlets. In the current study, we investigated the growth patterns of regenerants that were kept in growth chambers under a 16-h photoperiod at either 4°C or 24°C. Many of the regenerants grew successfully at 4°C, but died when held at 24°C. This demonstrates the positive effect of a cold treatment before transplantation of *H. orientalis* plantlets.

Using SEM and LM to examine the adaxial and abaxial surfaces of the leaf extracts, we observed that the upper external layer had much reduced cell sizes and denser cytoplasm, with features common to merismatic cells. Active proliferation and large, numerous protruding masses proved that callusing was occurring from the surface cells (Figs. 2 and 3). Usually, calli develop from the cut edges of the leaf, and are compact and yellowish-white, with a few green portions (see also Dronne et al., 1999; Ibrahim and Debergh, 2001).

This research demonstrates the excellent regeneration capacity for *H. orientalis*. Our success with in-vitro establishment clearly indicates that micropropagation is an effective and useful technique for increasing this species. Mass production via tissue culture may also become a desirable alternative to seed propagation, the latter method having inherently low germination rates and a slow growth process. It is hoped that procedures developed in this research will also be applicable to other *Heloniopsis*-related species and cultivars.

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