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In Vitro Propagation of Higo Chrysanthemum
(*Dendranthema* × *grandiflorum* (Ramat.) Kitam.)

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Summary

Higo chrysanthemum (Dendranthema ×grandiflorum (Ramat.) Kitam.) is a unique group of chrysanthemums that have been raised and cultivated in the Kumamoto area for more than two hundred years. These cultivars are valuable not only because of their importance to Japanese cultural heritage but also because they are potential genetic resources for the improvement of modern chrysanthemums. Cultivar degeneration and contamination are common problems encountered in plant propagation and preservation aside from the high running costs. To develop a safe and economical method of propagation through tissue culture, different culture conditions were investigated in the 29 cultivars.

All of the apical and nodal segments were available for in vitro propagation of Higo chrysanthemum. Elongation of axillary shoot is possible regardless of the length of explant and the presence or absence of leaf blades. During subcultures, root formation in long nodal segments was more rapid than in short nodal segments and also in segments with leaf blades than without leaf blades. However, among these segments, no significant difference was observed in the number of expanded leaves on axillary shoot. Regardless of the positions of nodes, all the nodal segments were potential propagules for subculture. Therefore, efficient in vitro propagation was achieved when the nodal segments were cultured in a hormone-free MS medium at 25 °C and when segments from the elongated shoots underwent a series of subcultures. In vitro rooting was not essential for the transfer of regenerated shoots to the nursery bed, as vigorous growth and rapid root formation was demonstrated by cuttings from the regenerated shoots. Moreover, no somaclonal variation was detected in the regenerated plants.

Introduction

Higo chrysanthemum (*Dendranthema* × *grandiflorum* (Ramat.) Kitam.) is a unique group of chrysanthemums that have been continuously cultivated in Kumamoto area for more than two hundred years. Even now, these peculiar group of chrysanthemums are characterized by the unique techniques of cultivation and arrangement, based on Confucianism, originally established by the Hideshima School during the latter part of the 17th century[1]. These cultivars are valuable not only because of their importance to Japanese cultural heritage but also because they are potential genetic resources for the improvement of modern chrysanthemums.

Higo chrysanthemum cultivars are usually propagated by cutting and maintained in the field or pots. However, plants vegetatively propagated continuously are more likely to be infected with diseases. In addition, most of the cultivars often set seeds and when the seeds fall and germinate seedling contamination is possible to occur on the stock of cultivars. In vitro propagation may prevent the cultivars from the infection of disease, and seedling cultivar contamination. It may even save running costs for space and labor.

The establishment of aseptic culture system is a fundamental step for in vitro propagation of Higo chrysanthemum cultivars. For tissue culture in chrysanthemums different plant organs have been used: shoot tip[2,3,4], stem segment[5,6,7,8,9], leaf[10,11,12,13], petal segment and petal epidermis[14], anther[15], achene[16], young capitula[17], and floret [18]. In these studies, severe somaclonal variations were demonstrated in the regenerated plants via callus formations. Moreover, somaclonal variations were observed to occur even from direct shoot formation.

It is the aim of this study to develop a method of in vitro propagation that would minimize somaclonal variation and maintain the original characteristics of each cultivar in the regenerated plants of Higo chrysanthemum.

Materials and Methods

Establishment of aseptic cultures

This experiment was done twice in August 1991 and May 1992. Actively growing young stems were excised from rooted cuttings in the greenhouse under natural light and temperature conditions. The leaf blades were removed prior to sterilization.

Sterilization with 0.1 % $HgCl_2$ with a drop of Tween 20 was done for 5 min followed by 2 times rinsing of sterilized water for 2 and 3 min, respectively. On cultivars that turned brown using $HgCl_2$, other disinfectants in various concentrations were used, namely, 5 % and 10 % H_2O_2 for 5 and 10 min; 0.01 % benzalconium chloride for 10 min; 70 % C_2H_5OH for 15 sec; 70 % C_2H_5OH for 15 sec and then 2 % NaOCl for 5 and 10 min; 70 % C_2H_5OH for 15 sec and then CAE White-7 (Yuai-Kasei) for 5 and 10 min.

The apical bud with the youngest expanded leaf (node No. 1), and nodal segments (node Nos. 3 and 5, the node count starting upward from the first expanded leaf blade of the plant) were excised and inoculated on a hormone-free MS medium[19] with 2 % sucrose and 0.8 % agar. The pH of the medium was adjusted to 5.7. The medium was distributed into the mayonnaise bottles at 40 ml per bottle and were autoclaved at 121 °C for 15 min. The inoculated explants (10 explants in each cultivar per segment) were maintained at 25°C with a photoperiod of 16 hr light and 8 hr darkness.

In vitro propagation

Explants that were successfully cultured and had reached the height of 6～7 cm underwent serial subcultures. During subcultures, the effect of the presence and absence of leaf blade and the effect of the length of nodal segment (long- 1.5 cm and short- 0.5 cm) were investigated using nodal segment No. 3 of 'Horen'. The number of expanded leaves

and root formation of axillary shoots were examined after 2, 4 and 8 weeks after inoculation.

The effect of node position on the growth of axillary bud was also examined using apical bud (node No. 1) and nodal segments (node Nos. 3, 5 and 7) of 'Horen', 'Kin Kujaku', 'Tennyo No Mai' and 'Shiranui'. The plant height, number of expanded leaves and root formation of the elongated shoots were examined 2, 4 and 8 weeks after inoculation.

Establishment of regenerated plants onto soil

After 8 successive subcultures, the regenerated plantlets were hardened for 1 week by loosening the cap of the bottle prior to transfer onto the nursery beds. Intact regenerated plantlets and cuttings (the bottom of the shoot and roots were cut-off) from the regenera-

Table 1 Survival rate of Higo chrysanthemum cultivars aseptically cultured after the sterilization with $HgCl_2$.

Cultivar name	Survival rate (%)					
	Summer 1991			Spring 1992		
	Node No.[a)]			Node No.[a)]		
	1	3	5	1	3	5
Chihiro No Umi	41.6	0	0	44.4	100	100
Chiyo No Kotobuki	50.0	0	0	50.0	0	0
Hatsu Shigure	100	20.0	26.7	0	40.0	10.0
Horen	88.9	90.0	100	30.0	100	100
Kin Kujaku	100	81.8	91.7	100	100	100
Kuni No Hikari	75.0	0	10.0	100	90.0	40.0
Tennyo No Mai	85.7	35.7	93.3	50.0	0	30.0
Akebono No Hiraki	100	86.7	80.0	10.0	80.0	90.0
Banshu	0	0	0	0	0	0
Kakurekimiko	100	20.0	35.7	60.0	100	90.0
Kinyo	100	85.7	85.7	100	100	100
Kojo No Tsuki	0	0	0	0	0	0
Mado No Yuki	41.7	30	33.3	0	0	40.0
Miyuki No Matsu	0	0	0	0	0	0
Shiranui	73.3	0	0	10.0	0	10.0
Tagoto No Tsuki	100	83.3	83.3	40.0	80.0	90.0
Takaragasa	0	0	0	0	0	0
Yumeji Guruman	0	0	0	0	0	0
Beni Kosode	0	0	0	0	0	0
Harugasumi	14.2	0	0	0	100	100
Kimi Ga Sode	81.8	100	90.9	50.0	30.0	80.0
Mikamimori	100	83.3	92.9	50.0	100	100
Mine No Matsu	0	0	30.0	0	0	10.0
Momiji Gari	11.1	0	0	0	0	0
Nogio	50.0	0	43.8	50.0	100	100
Nowake	30.0	0	0	66.7	0	0
Shuko	93.8	43.8	86.7	100	100	100
Yuki No Karasaki	33.3	0	0	80.0	0	0
Zuisei	0	0	0	0	0	0
Average	50.7±7.8	26.2±7.0	33.9±7.5	34.2±6.9	42.1±8.8	44.5±8.5

[a)]Node No. 1; Apical bud:node No. 3 and 5; the node count starting from the first expanded leaf of the plant.

Fig. 1 Growth of apical buds (left) and nodal segments No. 3 (center) and No. 5 (right) of 'Harugasumi' 1 month after inoculation for the primary culture.

ted plantlets were planted onto sand and vermiculite. Roots of the hardened intact plantlets were first washed with running water to remove the medium and dipped in 0.8 % Captan (Nissan Kagaku) for 3 min. All the plantlets and cuttings were covered with transparent plastic sheet and newspaper on top to give a period of humidity and light acclimatization. The plastic sheet was partially lifted 1 week later for air circulation and later removed for 5-6 hr daily to allow adaptation to the outside environment. The plastic sheet was completely removed from the plantlets 2 weeks after transplanting when vigorous growth was observed. The plants were transplanted onto soil in plastic pots 1 month after transplanting onto sand or vermiculite and were finally transplanted in the field 1 month after transplanting onto soil in plastic pots.

The established plants were cultivated according to the standard way of cultivating Higo chrysanthemum cultivars[1]. From the growing stage until flowering, the observation of leaf and flower morphologies were conducted to ascertain the somaclonal variations.

Results

Establishment of aseptic cultures

Twenty-two and 21 cultivars were successfully cultured aseptically in summer 1991 and spring 1992, respectively (Table 1). The average rate of survival from the apical bud was higher than the lower nodes in summer while those from the lower nodes showed higher tendency of survival rate than the apical buds in spring. In some cultivars, apical buds turned brown but node Nos. 3 and 5 elongated (Fig. 1) and in other cultivars, the other way round. Survived cultures elongated axillary shoots and it was also observed that the height and number of nodes of the shoots from nodal segments were higher than those of the apical bud. However, the explants from the remaining 7 cultivars underwent severe browning soon after sterilization with $HgCl_2$ in both years. Although other disinfectants

Table 2 Number of expanded leaves and root formation of cultures from long and short nodal segments with and without leaf blades during subculture.

Explant	No. of expanded leaves	Root formation (%)		
	Weeks	Weeks		
	8	2	4	8
Long nodal segment with leaf blade	6.2±0.2	90.0	100	100
Long nodal segment without leaf blade	7.4±1.5	0	10.0	100
Short nodal segment with leaf blade	7.4±0.7	20.0	50.0	100
Short nodal segment without leaf blade	7.4±0.4	0	0	80.0

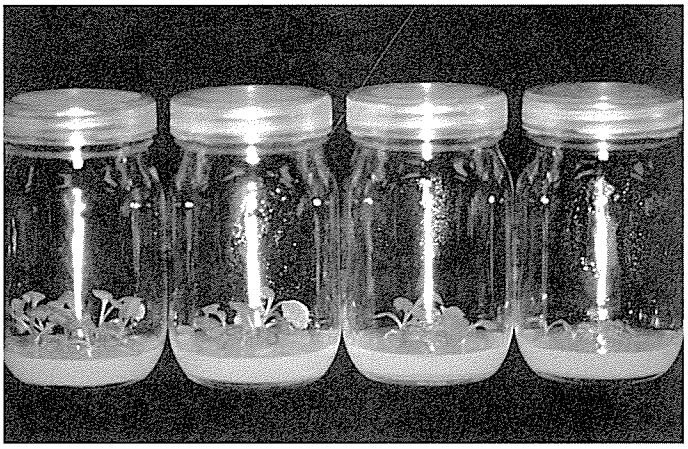

Fig. 2 Growth of long nodal segments with leaf blades (extreme left), long nodal segments without leaf blades (second from left),short nodal segments without leaf blades (second from right) and short nodal segments without leaf blades (extreme right) of 'Shiranui' 2 weeks after transplanting for subculture.

were used browning still existed, and in the case where there was slight browning, explants were highly infected with microorganisms.

In vitro propagation

While buds were visible in the axils of the long nodal segment 1 week after inoculation, those of the short nodal segments just started to swell regardless of the presence or absence of leaf blades. Leaves from long nodal segments expanded ahead and the leaf blades were bigger than those of short nodal segments regardless of the presence or absence of the leaf blades (Fig. 2). However, 8 weeks after inoculation no significant difference was observed on the number of expanded leaves among the explants except for long nodal segment with leaf (Table 2). Regardless of the length of explants, root formation was observed on nodal segments with leaf blades while none was observed on those without leaf blades 2 weeks

Table 3 Plant height, expanded leaves and root formation on cultures from different node position in the cultivars of Higo chrysanthemum during subculture.

Cultivar name	Node No.	Plant height (mm)	Number of expanded leaves	Root formation (%)		
		Weeks	Weeks	Weeks		
		8	8	2	4	8
Horen	1	20.3±2.5	6.8±0.3	0	30.0	90.0
	3	20.3±2.8	5.3±0.4	0	70.0	70.0
	5	20.8±2.2	5.7±0.3	100	100	100
	7	26.0±2.5	7.3±0.3	100	100	100
Kin Kujaku	1	15.0±2.3	4.5±0.6	40.0	60.0	90.0
	3	29.5±2.0	5.2±0.2	100	100	100
	5	32.0±2.2	6.4±0.5	80.0	100	100
	7	44.9±3.3	6.9±0.3	90.0	90.0	100
Tennyo No Mai	1	15.2±2.4	6.3±0.7	30.0	90.0	100
	3	23.4±1.7	7.3±0.3	100	100	100
	5	17.4±1.5	7.4±0.5	100	100	100
	7	17.1±2.8	8.8±0.6	60.0	60.0	100
Shiranui	1	24.9±3.4	6.0±0.7	70.0	90.0	100
	3	29.0±1.4	6.7±0.4	60.0	100	100
	5	32.8±2.8	7.1±0.3	80.0	100	100
	7	34.8±2.2	7.7±0.5	40.0	100	100

Note: Data are presented with mean ± standard error.

Table 4 Root formation of intact regenerated plantlets and cuttings from regenerated plantlets transplanted onto sand and vermiculite 1 month after transplanting.

Material	Nursery medium	Number of roots			Length of roots		
		Old stem	Axillary shoot	Total	Old stem	Axillary shoot	Total
Intact	Sand	6.7±0.9	2.9±0.4	9.4±0.9	53.1±5.5	49.9±5.6	60.4±5.4
	Vermiculite	4.2±0.7	2.8±0.5	7.0±0.8	54.5±4.2	48.5±6.5	61.4±4.2
Cutting	Sand	−	7.6±1.3	7.6±1.3	−	58.1±2.7	58.1±2.7
	Vermiculite	−	5.8±0.6	5.8±0.6	−	69.2±5.9	69.2±5.9

after inoculation. After 4 weeks of inoculation, those from long and short nodal segments with leaf blades showed 100 % and 50 % root formation, respectively. It required 1-2 weeks for explants with leaf blades to form roots while 5-6 weeks were necessary for explants without leaf blades to form roots.

The height of axillary shoot from nodal segment No. 7 was higher than that of the apical shoot in 'Horen', 'Shiranui', and 'Kin Kujaku' except 'Tennyo No Mai' (Table 3). It was further observed that the lower nodes showed more number of leaves than the apical buds in 'Shiranui', 'Kin Kujaku' and 'Tennyo No Mai' except 'Horen'. Approximately 5-9 nodes with expanded leaves were produced for 2 months from 1 nodal segment. Root formation was earlier in nodal segments (node Nos. 3, 5, 7) than those of the apical bud.

Establishment of regenerated plants onto soil

One month after transplanting, all the intact plantlets and cuttings from regenerated

Table 5 Growth of intact regenerated plantlets and cuttings from regenerated plantlets firstly transplanted onto sand and vermiculite and then onto potted soil.

Explant	Nursery medium	Plant height (cm)	No. of expanded leaves
Intact	Sand	23.3±1.3	18.8±0.7
	Vermiculite	22.3±1.2	20.2±0.7
Cutting	Sand	24.2±0.8	15.7±0.5
	Vermiculite	21.5±1.3	17.5±0.6

Note: Data were taken 1 month after transplanting onto potted soil.

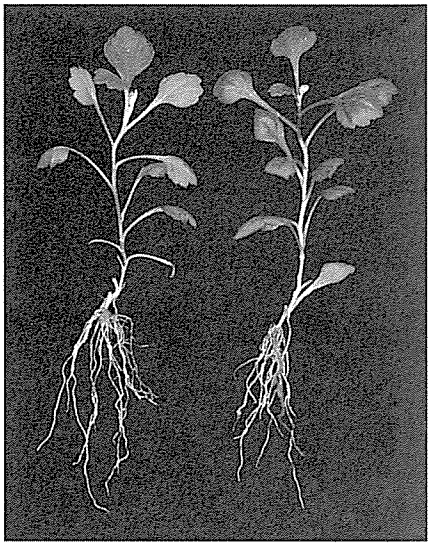

Fig. 3 Root formation of intact regenerated plantlets of 'Horen' transplanted onto sand (left) and vermiculite (right) 1 month after transplanting.

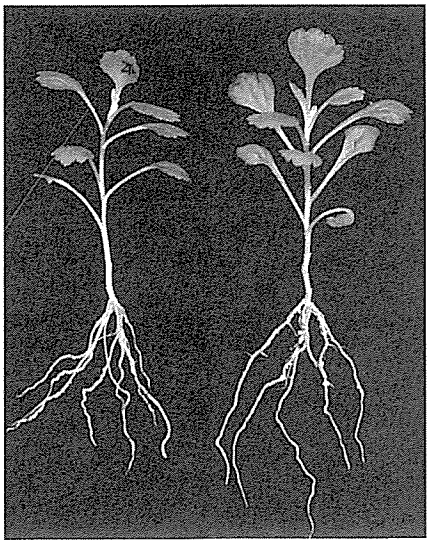

Fig. 4 Root formation of cuttings from regenerated plantlets of 'Horen' transplanted onto sand (left) and vermiculite (right) 1 month after transplanting.

plantlets showed 100 % of root formation and vigorous growth with 100 % rate of survival when transplanted onto sand and vermiculite as nursery media (Figs. 3, 4). Although root formation was observed to have originated in the axillary shoot of the intact plantlets, more roots were observed in the old stems. (Table 4). High activity of root formation was also observed in the cuttings. The cuttings showed higher numbers of roots in the axillary shoot than in intact plantlets. However, there was a tendency that the total numbers of roots from the intact plantlets were higher than those from the cuttings. The length of roots that originated from old and new stems showed no significant difference intact plantlets. The cuttings from regenerated plantlets had longer roots from axillary shoots than intact plantlets. Nevertheless, no significant difference in the length of total roots was observed between the intack plantlets and the cuttings. Roots of the cuttings were more or less longer when planted onto vermiculite than onto sand.

One month after transplanting onto potted soil, it was observed that there was no

Fig. 5 Inforescences of a regenerated plant of 'Horen' in the field

significant difference on the plant height between intact plantlets and cuttings although there was a tendency that the plant height was larger in the plants transplanted from sand than those from vermiculite (Table 5). Numbers of expanded leaves were larger in the intact plantlets than in the cuttings. Plants transplanted from vermiculite showed a tendency to have higher number of expanded leaves than those from sand.

The established plants finally planted in the field exhibited no difference in their morphological characters; growth habit, flower and leaf shape, flower color and height of the plants (Fig. 5).

Discussion

Aseptic cultures were established to the 22 cultivars of Higo chrysanthemum using apical bud and nodal segments sterilized with 0.1 % $HgCl_2$. However, the remaining 7 cultivars underwent severe browning and finally died regardless of the position of the nodal segment. In order to prevent from the browning other disinfectants were used, but the results could not be improved. These cultivars seem to have high contents of phenolic compounds whole year round. The oxidation products of phenolic compounds were known to inhibit enzyme activity, kill the explants, darken the tissues and culture media[20]. Therefore, it seems necessary to find the cultivation conditions of the mother plants for explants in order to minimize the contents of phenolic compounds. From 1 nodal segment, more or less 7 nodal segments can be produced after two months of subculture which means that the propagation rate is seven-fold every other two months.

After 8 weeks of subculture, no significant difference was observed in the number of expanded leaves among the explants regardless of the length of stem segments and presence or absence of leaf blades. Thus, the short nodal segment without leaf blade can be used for in vitro propagation of Higo chrysanthemum cultivars. During subcultures, rapid root formation was observed in long nodal segment compared to short nodal segment. It seems that the under cut surface affected the connection between leaf trace and stem

vascular bundle, although anatomical observation was not conducted. In addition, nodal segments with leaf blades showed rapid root formation. This result suggests that leaf blade stimulates root formation.

All of the apical bud and nodal segments from aseptic cultures were available for in vitro propagation of Higo chrysanthemum cultivars. Axillary shoots from nodal segments Nos. 3, 5, and 7 exhibited more active growth than the shoots from apical buds. Endogenous hormones have possibly promoted the growth of the axillary shoot of the lower nodal segments or the lower nodes have possibly higher reserved nutrition than the apical buds. The use of exogenous hormones has always been inseparable from in vitro propagation of chrysanthemum. In the present study, active growth and high root formation were attained by the cultures without hormone. Besides, callus and adventitious shoot formations which are usually the root causes of somaclonal variation in the regenerated plants were prevented.

Cuttings from the regenerated plantlets transferred onto sand and vermiculite nursery bed showed rapid root formation and vigorous shoot growth with 100 % of survival. Kitto and Young[21] observed that root formation was achieved in the 44 % of the unrooted in vitro-proliferated shoots of *Carrizo* citrange after 7 weeks under mist. In the present study, nursery root formation in the cuttings from regenerated plantlets of Higo chrysanthemum cultivars was observed within 2 weeks after planting without mist. Therefore, the cuttings from regenerated plantlets possibly have or produce enough endogenous hormones to enhance the root formation without passing wilting. This indicates that root formation during in vitro culture is not essential for nursery establishment of regenerated plantlets of Higo chrysanthemum cultivars.

Regenerated plants finally planted in the field showed no variation in any character as observed from seedling up to flowering stages. Severe somaclonal variations were observed in the leaf and flower shapes, flower color and chromosome number in the regenerated plants from internodal segments[6,7,8,9,18]. Therefore, the present method of in vitro propagation using nodal segments without hormone is a superior method which prevents somaclonal variation. This method will also be useful for long term preservation of Higo chrysanthemum cultivars in vitro.

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肥後ギクの in vitro 増殖

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摘 要

肥後ギクは200年以上前から熊本地方で育成・保存されて来た品種群で，その独特で厳格な栽培法と共に重要な文化遺産であるのみならず，現代の栽培ギクの育種にとっても貴重な遺伝資源である。しかし，通常の肥後ギク品種の増殖および保存方法では品種の退化および混合が生じ易く，かなりの人手と場所を要する。そこで，本研究では組織培養法を応用した安全で経済的な増殖方法を開発するために，現存する肥後ギク品種の中から29品種を供試して種々の培養条件を検討した。

外植体として頂芽と節切片の両方が初代培養によるシュートの増殖に有効であったが，節切片の腋芽からのシュートの伸長が頂芽からのシュートの伸長より旺盛であった。継代培養用の切片としては長い節切片が短い節切片より，また，葉身をつけた節切片が葉身を切除した節切片より根の形成がよかったが，これらの節切片の腋芽から伸長したシュートの展開葉数には有意差が見られなかった。また，いずれの部位の節切片も継代培養用の切片として有効であった。したがって，節切片をホルモンを添加しないＭＳ培地に植え付け，25℃で培養し，腋芽から伸長したシュートをさらに節切片にして継代培養を繰り返すことにより，効率的にシュートを増殖することができた。増殖されたシュートは培養容器から取り出して育苗床に挿し木すれば，容易に発根・活着するので，培養中の発根は必ずしも必要ではないことが分かった。さらに，節切片培養によりシュートを増殖後，再生された植物体を圃場で栽培し，花の形態等を調査した結果，培養変異は全く検出されなかった。