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

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Summary

In order to establish a method for the preservation of Higo chrysanthemum cultivars (*Dendranthema* × *grandiflorum* (Ramat.) Kitam.), studies were conducted on the influences of the type of explant, temperature, light and other conditions on the survival and growth of cultures in vitro.

Cultures at 5 °C, 15 °C, 25 °C and 25°-15°-5 °C had maximum rates of 100%, 60%, 53% and 100% of survival, respectively, for a period of 12 months. Cultures at 15 °C and 25 °C showed a comparatively active growth and advanced senescence or withering after 12 months of incubation. On the other hand, cultures at 5 °C and 25°-15°-5 °C showed growth retardation and delayed senescence which had maximum rates of 100% and 75% survival, respectively after 24 months. Dark condition increased the survival rate of cultures at 5 °C although the plant height was comparatively smaller than that under light. Nodal segments showed higher survival rate than the apical buds, and the removal of the expanded leaf on the explant showed advantages upon incubation. One ml of MS medium without growth regulators in a 12×75 mm test tube was enough to ensure the survival of a culture at 5 °C for a period of 24 months without transferring to a new medium or without supplementing the medium.

Based on the above results, a practically safe, economical and useful system for the in vitro preservation of Higo chrysanthemum has been established. This is presently applied to the 21 cultivars.

Introduction

Higo chrysanthemum (*Dendranthema* × *grandiflorum* (Ramat.) Kitam.), Higo-giku in Japanese, is a unique group of chrysanthemum cultivars which has been exclusively cultivated in Higo (the old name of Kumamoto Prefecture) of Kyushu island for more than 2 centuries. This group of cultivars is unique in the sense that inflorescences which comprise different shapes (flat, tubular, spoon-like), sizes (small, medium, large) and colors (red, yellow, white) are still arranged in the strictly prescribed manner, originally established by the Hideshima school during the latter part of the 17th century (Murayama, 1974). Besides being a part of Japanese traditional horticulture, these cultivars are potential genetic resources for the improvement of modern chrysanthemums. Since these cultivars are continuously propagated in the field,

there is a high possibility that they may vanish due to their susceptibility to disease or may set seeds that would lead to cultivar contamination by the seedlings. It is worthwhile and necessary to establish a safe and convenient method for the preservation of these valuable cultivars.

Considerable interest has been generated in exploring the storage methods of the chrysanthemums through cryopreservation (Bannier and Steponkus, 1972; Fukai, 1990, 1992; Fukai et al., 1991), low temperature preservation (Fukai et al., 1988; Fukai and Oe, 1989), and room temperature preservation (Hosoki, 1989). In cryopreservation, the risk of genetic changes due to the callus formation or destruction of chimera structure prior to regeneration of shoots are to be considered. In the case of low and room temperature preservations, culture conditions such as appropriate temperature and light conditions, explant, medium, growth regulator, volume of medium, vessel, control of contamination and protection from desiccation still

remain to be improved.

The objectives of the present study were to investigate the influences of (1) temperature, (2) light, and (3) type of explant on the survival rate and growth of cultures *in vitro*.

Materials and Methods

Hormone-free MS medium (Murashige and Skoog, 1962) containing 2% sucrose and 0.8% agar was used in this study. The pH of the media was adjusted to 5.7 before autoclaving at 121 °C for 15 min and was distributed in polystyrene test tubes (12×75 mm) at 1 ml per tube. Apical buds and nodal segments (node Nos. 3 and 5, the node count starting from the first expanded leaf blade of the plant), with and without leaf blades, were excised from a 3-month old *in vitro* grown plantlets of Higo chrysanthemum, cv. Horen and were plated onto the media. The test tubes were placed in test tube racks, packed in a transparent plastic container (Sanplatec 2258AO, 270 × 205 × 96 mm) sealed with insulation tape (Fig. 1). Cultures were placed under light and dark at 5 °C, 15 °C, 25 °C and 25°-15°-5 °C. At the last temperature condition, the cultures were placed at 25 °C and 15 °C for 10 days each and then at 5 °C. Cultures

under light were exposed to 16 hr light (fluorescent light, 2500 lx measured at the center of the container) and 8 hr darkness, while cultures under dark conditions were wrapped with aluminum foil. Evaluation of survival rate and growth were made 6, 12 and 24 months after inoculation. For the evaluation of growth, only alive cultures were considered in plant height, number of leaves, percentage of cultures that rooted and formed axillary shoots.

Results

Temperature

On the 6th month, cultures at 5 °C (*) showed the highest survival rate among the four temperature conditions used (Fig. 2). There were many cultures that remained alive, but no visible signs of growth, e.g. leaf expansion or internode elongation, were observed at 5 °C and 25°-15°-5 °C under dark conditions. Cultures at 25 °C were observed to have the highest plant height and number of unfolded leaves among the four temperature conditions (Figs. 3 and 4). Very short internodes were observed in a considerable number of cultures at 15 °C under light (Fig. 3). Almost all the cultures at 15 °C and 25 °C formed roots

Fig. 1. Cultures of Higo chrysanthemum cv. Horen incubated on 1 ml medium in a 12×75 mm test tube and packed in a plastic container sealed with an insulation tape.

*When only temperature is specified it includes cultures both under light and dark conditions.

Fig. 2. Survival rate of cultures of Higo chrysanthemum cv. Horen 6 (upper) and 12 months (lower) after inoculation. Apical buds (1) and nodal segments (3, 5) with (W) and without (O) expanded leaf blades were cultured under light (L) and dark (D) conditions at 5 °C, 15 °C, 25 °C and 25°-15°-5 °C.

while a considerable number of cultures at 5 °C and 25°-15°-5 °C were found to develop no root (Fig. 4). Axillary shoot formations were only

observed in a limited number of cultures at 15 °C and 25 °C under light.

After 12 months of culture, survival rate drasti-

Fig. 3. Cultures of Higo chrysanthemum cv. Horen incubated at 25 °C, 15 °C, 5 °C and 25°-15°-5 °C under light (first 4 test tubes from the left) and dark (last 4 test tubes in the same sequence) conditions 6 months after inoculation.

cally decreased in the cultures at 15 °C under light and 25 °C, while a slight decrease in the rate was observed in cultures at 5 °C, 15 °C under dark conditions and 25°-15°-5 °C (Fig. 2). Plant height of cultures at 15 °C under dark condition showed considerable increase, while a slight increase was observed in those at 5 °C and 25°-15°-5 °C (Figs. 4 and 5). On the other hand, it was difficult to detect the exact details of plant growth at 15 °C under light and 25 °C because they rapidly outgrew the culture vessel and many did not survive for a year. An enhanced root formation was clearly observed on cultures at 5 °C under light condition. Some cultures at 15 °C and 25 °C showed axillary shoot formation, while none was observed at 5 °C and 25°-15°-5 °C.

After 24 months of incubation, survival rate fairly decreased at both 5 °C and 25°-15°-5 °C (Fig. 6). The plant height of the cultures considerably increased at 5 °C and 25°-15°-5 °C under light while a moderate increase was observed in the cultures at 5 °C and 25°-15°-5 °C under dark conditions (Fig. 7). The accurate plant height under light in both temperatures could not be determined since the upper portion of some plantlets tended to wind 3~4 times upon reaching the top of the test tube. There was no obvious difference in root formation under both temperature condi-

tions between the 12th and 24th months of observations. Consequently, no considerable difference was observed in the survival rates and growth between cultures at 5 °C and 25°-15°-5 °C.

Light

Cultures under light showed green leaves and yellow-green stems, while etiolated stems and scale-like leaves were observed in the cultures under dark conditions (Figs. 3, 5 and 7). The etiolated cultures maintained the capability of chlorophyll synthesis for 2 years when transferred to standard proliferation conditions (25 °C under light). In the case of cultures at 5 °C under dark conditions, the survival rate was higher than that under light after 6, 12, and 24 months of incubation (Figs. 2 and 6). Cultures at 5 °C and 25°-15°-5 °C both under light showed higher plant height than cultures under dark conditions. On the 12th month, however, cultures at 15 °C under dark conditions showed significantly higher survival rate and plant height than those at 15 °C under light. Root formation was more frequent in the cultures under light than those under dark conditions in all the temperatures studied (Fig. 4).

Apical bud vs. nodal segments

Apical buds generally showed lower survival

Fig. 4. Plant height (upper), number of leaves (middle), and root formation (lower) of cultures of Higo chrysanthemum cv. Horen 6 months after inoculation. Symbols used are the same as in Fig. 2.

Fig. 5. Cultures of *Higo chrysanthemum* cv. Horen incubated at 25 °C, 15 °C, 5 °C and 25°-15°-5 °C under light (first 4 test tubes from the left) and dark (last 4 test tubes in the same sequence) conditions 12 months after inoculation.

Fig. 6. Survival rate of cultures of *Higo chrysanthemum* cv. Horen 24 months after inoculation. Symbols used are the same as in Fig. 2.

rate than nodal segments. However, in the cultures at 5 °C during 6 and 12 months of observations no significant differences were seen among the apical buds and nodal segments Nos. 3 and 5 (Figs. 2 and 6).

Expanded leaf blades

Presence or absence of expanded leaf on the ex-

Fig. 7. Cultures (with and without expanded leaf blade) of *Higo chrysanthemum* cv. Horen incubated under light at 5 °C (first 2 test tubes from the left) and dark (last two test tubes in the same sequence) conditions 24 months after inoculation.

plants showed no influence on the survival rate, plant height, and root formation (Figs. 2, 4 and 6). However, when expanded leaf blade touched the wall of the test tube, the explant was lifted and was forced to get into the medium or turned

upside-down which prevented the normal contact with the medium (data not shown).

Other observation

A volume of 1 ml MS medium without growth regulators in a 12×75 mm test tube ensured the survival of a culture without transferring to a new medium or supplementing the medium for a period of 24 months in cultures at 5 °C and 25°-15°-5 °C and 12 months in cultures at 15 °C and 25 °C. The size of the test tube was too small for the explant with expanded leaf blade but it was enough for the explant without expanded leaf blade. A slight desiccation was observed in cultures at 15 °C and 25 °C only under light on the 6th month and severe desiccation was observed in those at 15 °C and 25 °C on the 12th month of observation.

Discussion

Cultures at low temperatures such as 5 °C and 25°-15°-5 °C showed higher survival rate than those at high temperatures of 15 °C and 25 °C (Fig. 2). Cultures at low temperatures, especially under dark conditions, showed slow shoot elongation, less frequencies of root and axillary shoot formation which may indicate low metabolic activity. Therefore, it is with reason to say that low metabolic activity contributed to the higher survival rate of cultures at low temperature for longer period of in vitro culture.

In this study, it is demonstrated that the maximum survival rates were 100% at 5 °C and 71% at 25°-15°-5 °C, after 24 months of culture. Furthermore, cultures at 5 °C have advantages over those at 25°-15°-5 °C since the former does not require extra attention and additional facilities for the temperature control. Thus, preculture at 25 °C and 15 °C preceding the culture at 5 °C seems unnecessary in this system. In the cold storage of chrysanthemum shoot tips Fukai and Oe (1989) mentioned that preculture was effective when explants were excised during summer. In their study, explants were directly taken from greenhouse-grown plants, while in the present study explants were from aseptically cultured plantlets. In this way, necessity of preculture seems to be influenced by such state of the mother plants. To obtain a stabilized result of in vitro preservation it will be essential to establish the aseptic cultures prior to the inoculation.

Dark condition increased the survival rate of cultures at 5 °C although plant height was comparatively restricted than that under light which supports the results of Marino et al. (1985) in the in vitro storage of *Prunus*. It has not been confirmed yet if cultures under dark conditions for over 2 years can maintain the capability of chlorophyll synthesis. In the case of strawberry cold-stored for 6 years under dark, regenerated plantlets exhibited normality when transferred to sterilized soil (Mullin and Schlegel, 1976).

In the present system, nodal segments showed higher survival rate than the apical buds. Only one apical bud is available in a single shoot but several nodal segments can be supplied from the same shoot. At this point, the nodal segments seem to surpass the apical bud as explant. Attached expanded leaf blade touched the wall of the test tube and caused the culture to be elevated from the media or made the cultures turned upside-down and eventually caused death. Therefore, it is practical to excise the expanded leaf blade from the explant upon inoculation.

One ml of MS medium without growth regulators in a 12×75 mm test tube was enough to ensure the survival of a culture at 5 °C for a period of 2 years without transferring to a new medium or supplementing the medium. Other treatments such as growth retardants, high osmotic pressure, and low or high concentrations of sucrose which often causes abnormal growth are not necessary in the in vitro preservation of Higo chrysanthemums for a period of 2 years as shown in this study. Previous studies (Hosoki, 1989; Mullin and Schlegel, 1976) met the problems of contamination and desiccation when bare test tubes were used. In this study, all culture tubes were set in air-tight plastic containers. Even though the comparative data were not shown, the containers used in this study are supposed to actually prevent desiccation and contamination of cultures. Another valuable advantage of using these plastic containers is that only surface sterilization of the test tube is necessary at transplanting during sub-culture and micropropagation. The method of micropropagation, establishment of regenerated plantlets to soil and field test for somaclonal variation in Higo chrysanthemum cultivars will be reported separately.

It was demonstrated that in vitro culture was an interesting and convenient method for the pres-

ervation of the unique cultivars of Higo chrysanthemum. This system is presently applied to the 21 cultivars of Higo chrysanthemum based on the results of the present study. It can be adapted for the preservation of the other cultivars and strains of chrysanthemums and may facilitate germplasm preservation to any other plant species.

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肥後ギクの in vitro 保存

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摘 要

約 200 年前から熊本地方で育成・保存されて来た肥後ギク品種群を in vitro で保存する方法を開発するために、肥後ギク培養物の生存および生長に及ぼす温度、光およびその他の培養条件の影響を調査した。

培養温度が培養物の生存に及ぼす影響は顕著で、培養 1 年後の生存率の最大値は 5℃ 区で 100%，15℃ 区で 60%，25℃ 区で 53% および 25℃-15℃-5℃ 区 (25℃ で 10 日間および 15℃ で 10 日間前培養後、5℃ で培養) で 100% であった。15℃ 区および 25℃ 区では培養物の生長が比較的旺盛であり、培養 1 年後には老化、褐変あるいは枯死した培養物が多かったが、5℃ 区および 25℃-15℃-5℃ 区では生長が緩慢で、老

化・褐変が遅く、生存率の最大値は 2 年後でもそれぞれ 100%，71% であった。5℃ 区では暗黒下が照明下より草丈は低かったが、生存率は高かった。節切片は頂芽より生存率が高く、節に着生する展開葉の葉身を切除した方が培養に好都合であった。12×75 mm の試験管に入れた 1 ml のホルモンフリー MS 培地に節切片を植え付け、5℃ で培養すれば、2 年間は移植あるいは培地の追加をしなくても、培養物の生存が十分に保証された。

以上の結果から、簡便で安全な肥後ギクの in vitro 保存法を確立することができ、現在、21 品種を本法で保存中である。