Original Papers Plant Tissue Culture Letters, **6**(3), 144–147 (1989)

Protoplast Culture and Plantlet Regeneration in Stock (*Matthiola incana* R. Br.)

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(Accepted May 31, 1989)

Protoplasts were isolated from callus derived from excised cotyledons of stock (*Matthiola incana* R. Br.), by using an enzyme mixture of 1.5% Meicelase and 0.05% Macerozyme R-10. Modified Murashige-Skoog major elements, Ringe and Nitsch minor elements and modified vitamins supplemented with 0.2 mg/*l* 2,4-D, 1 mg/*l* NAA, 1 mg/*l* BAP, 8.1% mannitol, 1% glucose and 1% sucrose supported protoplast division at 25℃. The frequency of colony formation 10 days after culture was the highest (17.9% of isolated protoplasts) at protoplast density of 0.5×10^5/ml. Colonies developed to the callus-stage 2 months after initial culture. When callus pieces were cultured in full strength MS medium supplemented with 1 mg/*l* BAP or 1 mg/*l* zeatin, adventitious buds were induced in 9%–16% of callus explants. The buds elongated with leaves open at 20℃ and rooted in medium supplemented with 0.1 mg/*l* or 5 mg/*l* IBA.

Stock (*Matthiola incana* R. Br.) is one of the most important ornamental annuals in Japan. The production of cut-flowers of stock is the 5th from the top behind rose and lily production according to statistics of horticultural crops in 1987.[1] This species belongs to Brassicaceae, but does not have enough cold hardiness (below 0℃) especially in non-branching type[2] or resistance to disease by *Sclerotina sclerotiorum*, *Botrytis cinerea* or turnip mosaic virus.[3] Previously, Melchers *et al.*[4] succeeded in obtaining somatic hybrid plants of potato and tomato to introduce cold hardiness from the former plant. Recently, introduction of disease resistance from sexually incompatible species to cultural plants is often attempted by the protoplast fusion technique.[5]

Meantime, no data on protoplast culture on stock have been reported. The objective of the present study is to establish protoplast culture and subsequent shoot and root regeneration in expectation of issue of somatic hybrid plant with the other species in Brassicaceae (for example, *Brassica napus*) which has cold hardiness.

Materials and Methods

Seeds of 'Senshouno-yuki' and 'Snow wonder', stock (*Matthiola incana* R. Br.) were aseptically sown in the test tubes (2 cm in diameter, 15 cm long) containing 15 ml of Murashige-Skoog[6] major elements and Fe EDTA, Ringe and Nitsch[7] vitamins and minor element, 2% sucrose and agar (0.8%) solidified medium at pH 5.6. The cultures were placed at 20℃ under 16 hr 4,000 lux illumination from cool white fluorescent lamps. Two to three weeks after culture, fully expanded cotyledons were vertically cut into 2–3 pieces to induce callus on the same medium supplemented with 1 mg/*l* NAA plus 0.1 mg/*l* BAP. Two to three weeks later, greenish yellow callus was induced from the edge of the cut section. The callus grew well and covered two third of the agar surface one month after initial culture. To obtain protoplasts from callus, about 2 g (fw) of actively growing cell-pieces were taken from the periphery on the callus mass. Then, they were put into 15 ml of enzyme solution (1.5% Meicelase, 0.05% Macerozyme R-10) containing 9.1% mannitol and 1% sucrose in modified MS major ele-

ments where the elements were reduced to half amount except NH_4NO_3 for which it was reduced to 200 mg/l.[8] Protoplast was isolated from callus at 28°C on a reciprocal shaker (45 rpm) for 6 hr in the dark. Longer incubation over 6 hr increased burst of protoprasts. The enzyme-protoplast mixture was then passed through a fine nylon sieve (62 μm pore size) and collected into two test tubes (1 cm in diameter, 10 cm long) with screw caps. The protoplasts were pelleted by centrifugation (600 rpm, 5 min) to remove enzyme solution and the pellet was re-suspended in 8 ml of 21% sucrose solution and collected into one test tube. Then, protoplasts were centrifuged at 830 rpm for 10 min so that only protoplasts floated at the surface and the other debris or damaged cells were separately sedimented.[9] The floated protoplasts were collected, re-suspended in 8 ml of the modified MS medium shown above and centrifuged at 600 rpm for 5 min. The pelleted protoplasts were re-suspended in 5 ml of protoplast culture medium to determine protoplast density for which a drop of the suspension was taken for counting the number with a hematocytometer. The protoplast culture medium consisted of MS modified medium, Fe EDTA (previously described), Ringe and Nitsch minor elements[7] and modified vitamins (0. 5 mg/l nicotinic acid and 0. 4 mg/l thiamin HCl) supplemented with 8. 1% mannitol, 1% glucose, 1% sucrose, 0. 2 mg/l 2, 4-D, 1 mg/l NAA and 1 mg/l BAP as growth regulators.[10] Usually, 0. 1–0. 3 ml of protoplast suspension was placed at a density (usually, 10^5/ml) in a petri dish (3. 5 cm in diameter) after solidifing protoplasts with 0. 6% agarose. Furthemore, 0. 5 ml of the same liquid culture medium was overlied on the protoplasts imbedded in the agarose to promote nutrient-uptake and to avoid desiccation. The petri dish was then wrapped with Parafilm and incubated at 25°C in the dark. Three days after culture, 0. 2 ml in the previously overlied liquid solution (0. 5 ml) was replaced with a new liquid medium (cell culture medium) in which 1% glucose and 2% sucrose were contained without mannitol, and 0. 1 mg/l 2, 4-D and 0. 2 mg/l BAP were supplemented without changing the other components. The frequency of divided cells (%) at 3 days and frequency of cell colony formation (%) at 10 days after culture were recorded. The further process is described in Results and Discussion.

Results and Discussion

Protoplast isolation was almost complete 6 hr after enzyme incubation (**Fig. 1**). The isolated protoplasts (0. 2–2. 2%) had started cell division by 3 days with a maximum at 10^5/ml protoplast density, and 10 days after culture, about 6. 5–18% of the isolated protoplasts formed cell colony (8–16 cell stage) with a maximum at the 0.5×10^5 density (**Table 1**). Thus, active cell division occurred between 3 and 10 days. About one month after culture, the colony developed into 1–2 mm microcalli. The petri dishes were then transferred under dim light (1, 000 lux) of 16 hr illumination from cool white fluorescent lamps. Each microcallus was transferred onto a fresh cell culture medium (described previously) but solidified with 0. 6% agarose. The microcalli grew to 3–4 mm in size and showed yellow or greenish yellow color (**Fig. 2**). Another one month after culture, callus pieces (0. 6–1 cm in diameter) were transferred to test tubes (2 cm in diameter, 15 cm long) containing 15 ml of full strength MS medium supplemented with NAA (0, 0. 1, 1 mg/l) in combination with zeatin (1, 5 mg/l) or BAP (1, 5 mg/l). Six to nine callus pieces were alloted for each treatment (**Fig. 3**). Light intensity was raised

Table 1. Effect of protoplast density on cell division (3 days after culture) and cell colony formation (10 days after culture).

Protoplast density (per ml)	Percent of divided cells in isolated protoplasts (mean±SD)*	Percent of cell colonies in isolated protoplasts (mean±SD)*
0.25×10^5	0. 5±1. 6	6. 5±2. 0
0.50×10^5	1. 7±2. 8	17. 9±1. 8
10^5	2. 2±2. 8	9. 4±1. 8

* Ten microscopic areas were observed and means±standard deviation (SD) were calculated.

to 4, 000 lux without changing the other culture conditions. Two months after culture, two adventitious buds were induced in the calli in both 'Senshouno-yuki' and 'Snow wonder' (one bud from 1 mg/BAP and another bud from 1 mg/zeatin in both cultivars). In the other treatments, most of calli continued to grow without regeneration and some calli turned yellow. Growth of regenerated bud was arrested at 26°C. Therefore, temperature was lowered to 20°C. The buds elongated and opened several leaves (**Fig. 4**). The shoots of 'Senshouno-yuki' are then transferred to medium supplemented with 5 mg/IBA as reported by Fukuzumi.[11] Several roots emerged from the base one and half month after culture. The shoots of 'Snow wonder' rooted in medium supplemented with 0. 1 mg/*l* IBA.

In a preliminary experiment for protoplast culture, cotyledons or mature leaves grown in the greenhouse were used. Protoplast isolation was very successful in both cultivars using our enzyme solution,

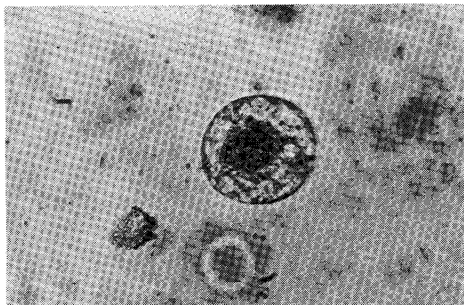

Fig. 1. Isolated protoplast from leaf callus in 1.5% Meicelase and 0.05% Macerozyme R-10 solution (6 hr after incubation at 28°C on a reciprocal shaker, 45 rpm).

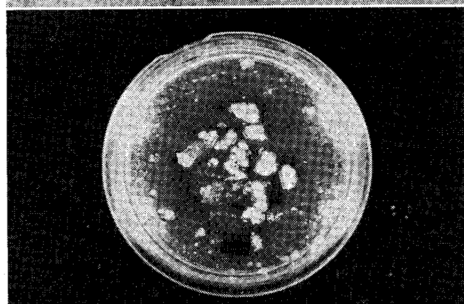

Fig. 2. Microcalli on modified MS medium supplemented with 1 mg/*l* NAA, 0. 1 mg/*l* 2, 4-D and 0. 2 mg/*l* BA.

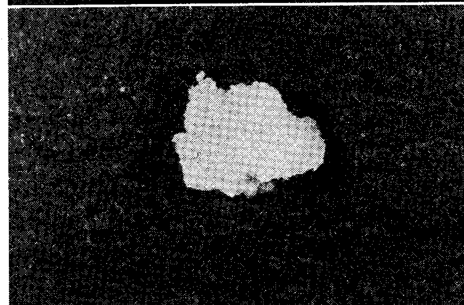

Fig. 3. Callus on MS medium supplemented with 1 mg/*l* zeatin and 0. 1 mg/*l* NAA.

Fig. 4. Adventitious shoot induced from callus in MS medium supplemented with 1 mg/*l* zeatin.

but high frequency of bacterial contamination was a serious problem. Therefore, cotyledons of seedlings aseptically sown in the test tubes were used, but cell division did not occur in our medium or even in KMP 8 medium.[12] Next, we have attempted protoplast culture using callus from excised cotyledons. Protoplast isolation, cell division and colony formation were repeatable more than 5 times in this callus system. However, plant regeneration rate from callus was not so high (9-16% of callus explants). Fukuzumi[13] reported that high frequency (about 80%) of adventitious bud formation was obsereved when excised cotyledons of 'Senshouno-yuki' were directly cultured (not protoplast) in MS medium supplemented with 1 mg/*l* BAP. In tomato (*Lycoperisicon esculentum*), such a reduction of regeneration capacity from protoplast in contrast with high regeneration capacity from excised leaf tissue has also been reported.[14] In our protoplast culture, the bud forming capacity might have been reduced during long time culture at the callus stage.

Although more work is necessary to enhance regeneration frequency and to establish plants in the soil, we could demonstrate protoplast culture and subsequent plantlet regeneration of stock in this report for the first time.

References

1) Matsuyama, K., 1987. In "Horticultural Statistics" (ed. by Department of Statistics of Agriculture), p. 192-193, Min. of Agr. Forest. and Fish., Tokyo (in Japanese).
2) Fujita, M., 1984. In "Encyclopedia of Ornamental Plants" (ed. by Tsukamoto, Y.), p. 432-484, Yokendo, Tokyo (in Japanese).
3) Uezumi, S., J. Nishimura, 1974. Diseases and Pests of Ornamental Plants, p. 189-192, Nobunkyo Press, Tokyo. (in Japanese).
4) Melchers, G., M. D. Sacristan, A. A. Holder, 1979. Carlsberg Res. Commun., **43** : 203-218.
5) Helgeson, J. P., T. Geraldine, J. P. Haberlach, S. Austin, 1988. Plant Cell Organ Cult., **12** : 185-192.
6) Murashige, T., F. Skoog, 1962. Physiol. Plant., **15** : 437-497.
7) Ringe, F., J. P. Nitsch, 1968. Plant Cell Physiol., **9** : 639-652.
8) Nishio, T., M. Hirai, T. Satoh, K. Watanabe, 1987. Abstr. Jpn. Soc. Hort. Sci. Autumn Meet. 1987, p. 232-233 (in Japanese).
9) Power, J., E. Frearson, P. Evans, D. George, S. Berry, C. Hayward, E. C. Cocking, 1976. Plant Sci. Lett., **7** : 51-56.
10) Kao, K. N., 1977. Mol. Gen. Genet., **150** : 225-230.
11) Fukuzumi, H., 1988. Abstr. Jpn. Soc. Hort. Sci. Autumn Meet. 1988, p. 520-521 (in Japanese).
12) Kao, K. N., M. R. Michayluk, 1975. Planta, **126** : 105-110.
13) Fukuzumi, H., 1986. Abstr. Jpn. Soc. Hort. Sci. Autumn Meet. 1986, p. 332-333 (in Japanese).
14) Stephen, A. Y., T. L. Barsby, 1988. Sci. Hort., **37** : 179-200.

≪和文要約≫

ストック（*Matthiola incana* R. Br.）の葉片カルスからのプロトプラスト培養と植物体再生

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ストックの葉片カルスからのプロトプラスト培養と植物体再生について検討した．子葉切片由来のカルスから，1.5% メイセラーゼと 0.05% のマセロザイム R-10 を含む酵素液中でプロトプラストを単離した．プロトプラストは，修正 MS 主要塩類，Ringe･Nitsch の微量要素と修正ビタミン溶液に 0.2 mg/*l* 2, 4-D, 1 mg/*l* NAA, 1 mg/*l* BA, 8.1% マンニトール，1% ぶどう糖，および 1% しょ糖を含む培地で培養（25℃）すると分裂が見られた．培養 10 日後のコロニー形成率は，プロトプラスト密度が 0.5×10^5/m*l* の場合でもっとも高かった．コロニーは，培養 2 カ月後小カルスに発達し，1 mg/*l* の BA または 1 mg/*l* zeatin 添加培地で培養することで不定芽が生じた（9～16% の頻度）．これらを 20℃ に移すと芽が伸長し，発根は 0.1 mg/*l* または 5 mg/*l* の IBA 添加培地で見られた．