Establishment of Culture Medium for Protoplasts and Plant Regeneration in Japanese Bunching Onion (Allium fistulosum L.)

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Summary

For the purpose of establishing protoplast culture systems in Japanese bunching onion (Allium fistulosum L.), based on BDS inorganic salts (Dunstan and Short, 1977), nutrient media suitable for protoplast culture were investigated. The optimum cell division was achieved with 5 mM potassium nitrate and a combination of 2 μM 2,4–dichlorophenoxy acetic acid (2,4–D) and 0.2 or 1 μM 6–bromylaminopurine (BAP). The suitable osmolality for protoplast culture was around 0.60 osmol·kg−1, whereas a combination of 0.2 M sucrose and 0.2 M glucose accelerated cell division.

The protoplasts developed into colonies by gradual reduction of sugar concentration. After 45 days of culture, numerous micro–calli formed which were subsequently transferred to a callus formation medium. Plantlets were regenerated by transferring protoplast–derived calli of ca. 2 mm in diameter to a modified Murashige–Skoog medium. Of the 75 calli inoculated, 24 (32%) gave rise to green shoots on a regeneration medium 2 to 3 months after inoculation. These shoots rooted when cultured on a hormone–free medium. These plantlets were successfully acclimatized and grew normally in a greenhouse.

Key Words: Allium fistulosum L., culture medium, Japanese bunching onion, plant regeneration, protoplast.

Introduction

Japanese bunching onion or Welsh onion (Allium fistulosum L.) is historically the main Allium vegetable in Japan and China (Brewster, 1994). Many local cultivars have been selected by open pollination, but a disease resistant variety has not yet been bred, mainly because of the Allium’s limited genetic resources. Recently, a male sterile line of Japanese bunching onion was developed by continuous back crossing, using Allium galanthum Kar. et Kir. as a cytoplasm donor (Yamashita et al., 1999). The introduction of traits from related species should be possible by somatic hybridization or genetic engineering (Karim and Adachi, 1996). To develop techniques of this type for Japanese bunching onion, a reproducible means of regenerating plants from protoplasts must first be achieved.

In the Allium species, several attempts over the last two decades have been made to isolate protoplasts (Otsuki and Takebe, 1969; Bawa and Torrey, 1971; Schnabl, 1980; Tashiro et al., 1984; Oosawa and Takayanagi, 1984) and to culture them (Bawa and Torrey, 1971; Oosawa and Takayanagi, 1984). However, Allium protoplasts are recalcitrant due to biological peculiarity, e.g. no cell division and low colony formation (Karim and Adachi 1997; Buiteweld and Suo, 1998). Previously, the establishment of a regenerable procedure was reported in leek (Buiteweld and Creemers–Molenaar, 1994), onion (Wang et al., 1986; Hansen et al., 1995), and garlic (Ayabe et al., 1995). But to date, no information is available on plant regeneration from protoplasts in Japanese bunching onion.

In this paper, we describe the optimum conditions in relation to inorganic salts, growth regulators, sugar sources and vitamins for protoplast culture, and plant regeneration of Japanese bunching onion.

Materials and Methods

Callus induction and cell suspension cultures

Seeds of the Japanese bunching onion (Allium fistulosum L.), ‘Kairyou–Hakushu 5 Gou’ selected by Tottori Horticultural Experiment Station, were surface sterilized in sodium hypochlorite solution (available chloride 5.0%) for an hour with occasional stirring, and then rinsed three times with sterile distilled water.
Subsequently, these seeds were transferred onto a modified B5 (Gamorg et al., 1968) medium (BDS, Dunstan and Short, 1977) supplemented with 0.2 M sucrose, 4.5 μM 2,4'-dichlorophenoxyacetic acid (2,4-D), 0.44 μM 6-benzylaminopurine (BAP), and 1.0 g·liter⁻¹ casamino acids (DIFCO Lab., USA). The medium was previously autoclaved at 121 °C for 15 min. Two weeks after incubation in the dark at 25 °C, basal portions of stems (5 mm height) were excised from aseptic seedlings and transferred to the identical medium. After a two-month incubation period, compact calli grown on the medium were transferred to 60 ml of callus-inducing liquid medium in a 200 ml Erlenmeyer flask containing half-strength growth regulators and 20 mM 2(N-morpholino)ethanesulfonic acid (MES). Cell suspension cultures were kept on an orbital shaker (120 rpm) at 25 °C in the dark. The compact yellowish cell clumps were selected and squashed on stainless steel sieves of 1 mm mesh, and then filtered. Fine cells (0.5 ml) were collected by pipette and subcultured in fresh liquid medium every two weeks.

Protoplast isolation

Protoplasts were isolated from calli of suspension cultures in the second week after subculture. About 500 mgFW of calli was transferred into 10 ml of an enzyme solution of 3% (w/v) cellulase “ONOZUKA” RS (Yakult Honsha Co., Japan), 0.5% (w/v) macerozyme R-200 (Yakult Honsha Co., Japan), 0.1% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Japan), 0.6 M mannitol, 5 mM MES, and protoplasts washing (CPW) salts (Frecarson et al., 1973), and 0.1% (w/v) potassium dextran sulfate at pH 5.8. The cells were digested for 4 hours in a reciprocal shaker (60 strokes·min⁻¹) in the dark at 28 °C. The cell mixture was filtered through a nylon sieve of 50 μm mesh and washed with a CPW salt solution containing 0.63 M mannitol and 5 mM MES adjusted to pH 5.8. After centrifugation at 60 x g for 3 min, the supernatant was discarded and aliquots of washing solution were added to the precipitate. Protoplasts were gently re-suspended in the washing solution with a Pasteur pipette, centrifuged and washed again. Finally, the protoplasts were re-suspended in aliquots of liquid medium and counted using a Fuchs–Rosenthal type hemocytometer (Erma Co. Ltd., Japan). Protoplast viability was assessed by staining with fluorescent diacetate (Larkin, 1976), and cell wall digestion was confirmed by Calcofluor staining (Nagata and Takebe, 1970).

Protoplast culture

Froshly isolated protoplasts were dispensed in 2.5 ml of medium at a density of 2.0 x 10⁵ protoplasts·ml⁻¹ and poured into a 60 x 15 mm plastic petri dish. For the growth regulator tests, 200 μl of protoplast suspension was pipetted into each well of a microplate (6 x 4 wells, Corning, USA).

Using BDS inorganic salts, protoplasts were exposed to 0, 5, 10, 15, 20 and 25 mM potassium nitrate (KNO₃), and 0.40, 0.45, 0.50, 0.55 and 0.60 M sucrose to seek the optimum osmotic pressure in the medium. The osmolarity of the medium was measured by osmometer (Vogel Co. Ltd., Germany). Various ratios of sucrose and glucose were also examined. In this experiment, the total osmolarity of each medium was adjusted to 0.61 osmol·kg⁻¹ by adding mannitol.

The optimum combination of an auxin, 2,4-D (0 – 5 μM) and a cytokinin, BAP (0 – 5 μM) as well as three kinds of vitamins were tested for culturing the protoplasts: B5 (Gamorg et al., 1968), TM-2 (Shahin, 1985) and 8p (Kao and Michayluk, 1975). All media were filter-sterilized. The initial cell division was evaluated by counting the number of dividing cells among the 200 – 300 viable cells after 10 days of culture at 25 °C in the dark.

Plant regeneration

Calli derived from protoplasts were cultured for about two weeks on the callus induction medium in the dark at 25 °C. Calli, more than 2 mm in diameter, were then transferred to a regeneration medium in a plastic jar (IWAKI, Japan). Plant regeneration was conducted on a modified MS (Murashige and Skoog, 1962) medium, in which inorganic nitrogen sources were reduced to half strength (1/2·N MS), containing 0.5 μM α-naphthaleneacetic acid, 1 μM BAP, 1.0 g·liter⁻¹ casamino acids (w/v), 10 mM MES, 0.22% Gellan gum (Wako, Japan) and 0.15 M sucrose. After a two-month culture or an additional subculture, plantlets with 1 – 2 cm shoots were transferred to a hormone-free 1/2·N MS medium solidified with 0.2% Gellan gum. All plant regeneration experiments were conducted under a 14-hr photoperiod using cool white fluorescent light with 20 μmol·m⁻²·sec⁻¹ in photosynthetic photon flux at 25 °C.

Results and Discussion

Callus induction and cell suspension culture

Calli proliferated mainly on the surface of the basal portions of stems after one month of culture. After subculture for a month, two types of calli 2 – 6 mm in diameter emerged; one type was a pale yellow callus with dense cytoplasm, and the other was white with a vacuolated cytoplasm. Both types of calli were compact and rarely exported mucilaginous substances into the medium. On the surface of the yellow calli, green spots, which appeared sporadically, occasionally grew into shoots in the subsequent culture. When yellowish calli were transferred into a liquid medium, they did not spontaneously disperse into small cell clumps. Therefore, it was necessary to squash them into small fragments and pass them through stainless steel sieves to achieve a successful suspension culture. After several selective subcultures at two-week intervals, during
which the rate of cell proliferation increased approximately 10-fold each time, homogeneous suspension cultures were established.

For the isolation and culture of protoplasts, efforts have been made to develop suspension cultures in *Allium fistulosum* (Song and Peffley, 1994), *A. cepa* (Song and Peffley, 1994; Tanikawa et al., 1996), *A. ampeloprasum* (Buiteveld et al., 1994) and *A. tuberosum* (Matuda and Adachi, 1996). They succeeded in establishing a cell suspension culture and obtaining plantlets in their respective *Allium* species. Except for leek, their callus induction was performed from aseptically germinated plantlets, which was applicable to our study. It is very easy to produce explants, and culturing is not restricted to a particular season (Matuda and Adachi, 1996). As for the cell proliferation rate in culture, our value is quite high compared to 2.5 fold/20-day rate for onion (Tanikawa et al., 1996). Thus successful techniques of suspension culture for the isolation and culture of protoplasts in Japanese bunching onion was achieved in this trial.

In our preliminary experiments, no cell division had occurred when mesophyll protoplasts were cultured. We postulate that mature leaf mesophyll protoplasts are no longer meristematic. Similarly, there is no evidence that protoplasts, isolated from leaves and shoots of grass species, can sustain cell division (Vasil and Vasil, 1992).

**Protoplast isolation and culture**

Our suspension cultures yielded $9.5 \times 10^6$ protoplasts

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**Fig. 1.** Effects of potassium nitrate (KNO$_3$) on cell division in protoplast culture of Japanese bunching onion. The cell division rate was evaluated by counting the number of dividing cells among the 200–300 viable cells 10 days after incubation; $n=3$. Different letters indicate significant differences at the 5% level by Duncan’s multiple range test.

**Fig. 2.** Effects of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) at different concentrations on cell division in protoplast culture of Japanese bunching onion; $n=2$.

**Sucrose concentration (M)**

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>Osmolality (osmol/kg)</th>
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<tbody>
<tr>
<td>0.40 (0.54)</td>
<td></td>
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<tr>
<td>0.45 (0.63)</td>
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<tr>
<td>0.50 (0.73)</td>
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<tr>
<td>0.55 (0.81)</td>
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<tr>
<td>0.60 (0.90)</td>
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**Fig. 3.** Effect of osmolality on cell division in protoplast culture of Japanese bunching onion 10 days after incubation; $n=3$. Different letters indicate significant differences at the 5% level by Duncan’s multiple range test.

**Sugar concentration**

<table>
<thead>
<tr>
<th>Sugar concentration</th>
<th>Rate of initial cell division (%)</th>
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<tbody>
<tr>
<td>0.4 Suc. (M)</td>
<td>0</td>
</tr>
<tr>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>0.0</td>
<td>25</td>
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**Fig. 4.** Effect of sucrose (Suc.) and glucose (Glu.) on initial cell division in protoplast culture of Japanese bunching onion. 10 days after incubation; $n=3$. The osmolality of each medium was adjusted to 0.61 osmol·kg$^{-1}$ with mannitol. Different letters indicate significant differences at the 5% level by Duncan’s multiple range test.
Fig. 5. Photographs of stages in protoplast cultures and plant regeneration in Japanese bunching onion. (A) Freshly isolated protoplasts from cell suspension culture, (B) First cell division after a week culture, (C) Actively dividing cell cluster after a 2-week culture, (D) Colony development after 30 days culture, (E) Micro-calli after 45 days culture, (F) Development of shoots from protoplast-derived calli, (G) Regenerated plantlets, (H) Acclimatized plant in a greenhouse. Scale bars indicate 50 μm in (A), (B) and (C), and 500 μm in (D).

g⁻¹ FW with a viability above 95%. The size of the protoplasts ranged from 15 to 45 μm (Fig. 5A).

Initially, less than 3% of the protoplasts derived from suspension cultures of Japanese bunching onion were meristematic; cell divisions rarely occurred by the use of MS medium (Wang et al., 1986).

Dunstan and Short (1977) obtained a significant increase in the callus growth rate of *A. cepa* after varying the ammonium, phosphate and nitrate levels of B5 medium. Hence, BDS inorganic salts (Dunstan and Short, 1977) in our basal medium. For the main inorganic nitrate source, cell division at 5 and 10 mM KNO₃ were higher than those of 0, 15, 20, and 25 mM. The rate of cell division was 10.4% at the concentration of 5 mM KNO₃ (Fig. 1.). At 25 mM, the original concentration of BDS salts, more than half of the protoplasts shrunk and died. Therefore, it was necessary to reduce the concentration from 25 to 5 mM for our protoplast culture.

As for hormones, the combination of 2 μM 2,4-D...
Table 1. Effect of three vitamin types on cell division in protoplast culture of Japanese bunching onion.

<table>
<thead>
<tr>
<th>Vitamin Type</th>
<th>Cell division rate ± Standard deviation (%)</th>
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<tr>
<td>B5</td>
<td>6.0 ± 3.2b</td>
</tr>
<tr>
<td>TM - 2</td>
<td>10.3 ± 1.7a</td>
</tr>
<tr>
<td>8P</td>
<td>4.3 ± 1.8b</td>
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1B5; Gamborg et al. (1968), TM - 2; Shahin (1985), 8P; Kao et al. (1975).
2The cell division rate was evaluated by counting the number of dividing cells among the 200 - 300 viable cells 10 days after incubation; n=5.
3Different letters indicate significant differences at the 5% level by Duncan's multiple range test.

and 0.2 or 1 μM BAP promoted rapid cell division (Fig. 2). Karim and Adachi (1997) achieved the best results with 4.5 μM 2,4-D, 4.4 μM BAP, and 5.4 μM NAA, but they failed to obtain plantlets. Comparatively, our hormonal concentrations were relatively low; our medium did not include NAA.

Buiteveld and Creemers-Molenaar (1994); Ayabe et al. (1995); Hansen et al. (1995); Karim and Adachi (1997) studied factors promoting protoplast division in Allium species but not osmolarities and sugar concentrations. Sucrose at 0.40 and 0.45 M are equivalent to 0.54 and 0.63 osmol·kg⁻¹, respectively (Fig. 3). Our results on cell division indicate that ca. 0.60 osmol·kg⁻¹ is suitable for protoplast culture. Because Japanese bunching onion contains sucrose, glucose, and fructose (Mizuno and Kinpyo, 1955), we found that at 0.2 M sucrose plus 0.2 M glucose or 0.4 M glucose, 19.5% and 15.4% of the protoplasts divided, respectively, a significant improvement over the three other treatments (Fig. 4). At 0.4 M sucrose, only 7.8% of the protoplasts divided. We speculate that the protoplasts utilize glucose preferentially over sucrose.

Our data indicate that TM - 2 vitamin was considerably more effective than B5 or 8P (Table 1.), whereas for protoplast culture of leek (Buiteveld and Creemers-Molenaar, 1994), onion (Hansen et al., 1995) and garlic (Ayabe et al., 1995), the 8P vitamin was preferred. Because the results are inconsistent, we attribute our success to the agents contained in TM - 2. However, we could not determine which factor in TM - 2 improved its efficacy.

Most protoplasts form cell walls and become ellipsoidal after 3 to 5 days and divided after about 7 days (Fig. 5B). When the concentration of sugars in the culture medium was gradually reduced by adding the medium which contains half - strength sugars, the protoplasts successfully grew into colonies (Fig. 5C, D); no reduction of sugar concentration induced plasmolysis and cessation of cell development. After 45 days of culture, numerous small calli in petri dishes became visible to the naked eye (Fig. 5E). The plating efficiency (number of small calli / number of applied protoplasts) was around 0.02%.

Plant regeneration
In our previous experiments on the micropropagation of Japanese bunching onion 'Bouzu-shirazu', full strength of MS inorganic salts sometimes caused vitrification. Therefore, plant regeneration was performed by transferring protoplast - derived calli of ca. 2 mm in diameter to 1/2 - N MS medium. Among the 75 calli imbedded, 24 (32%) gave rise to green shoots 2 - 3 months later (Fig. 5F). When these shoots were excised and transferred to growth - regulator - free medium, a number of roots differentiated from the base of the shoots (Fig. 5G). The rooted plantlets in culture flasks, which appeared morphologically healthy and normal, were successfully acclimatized to soil in pots in a greenhouse (Fig. 5H).

In conclusion, we have demonstrated for the first time that plant regeneration from protoplasts in Japanese bunching onion is possible. Furthermore, the compositions of protoplast culture medium were reported. Our knowledge also enables the introduction of disease resistant or male sterile genes from related Allium species into Japanese bunching onion by means of cell fusion or electroporation.

Acknowledgements
The authors are grateful to Dr. Uchida, the former president of Tottori Horticultural Experiment Station, and the current president Mr. Inoue for encouragement.

Literature Cited


ネギ (*Allium fistulosum* L.) におけるプロトプラスト培養培地の確立および植物体の再生

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摘 要

ネギ (*Allium fistulosum* L.) のプロトプラスト培養系の確立を目的とし、BDS 無機塩類 (Dunstan and Short, 1977) を基礎としてプロトプラストの培養に好適な栄養条件を検討した。硝酸カリウムの濃度は 5 mM が適当であった。植物生長調節物質として、2 μM の 2,4-D と 0.2 または 1 μM のベンジルアデニンの組み合わせが細胞分裂を促進した。培地の浸透圧は 0.60 osmol·kg⁻¹ 前後に適した。0.2 M のスクロースと 0.2 M のグルコースを組み合わせると、細胞分裂が向上した。糖濃度を減減することによってプロトプラストはコロニーへと発達し、培養 45 日後には多くの小カルスが観察された。これらの小カルスをカルス形成培地に移植し、約 2 mm 大に発達したプロトプラスト由来カルスを用いて、MS 培地上で植物体の再生を図った。2-3か月後には、発芽した 75 個体のカルス中の 24 個体のカルス (32%) からジュートの形成が認められた。これらのジュートを植物ホルモンを含まない培地に移植して発根を促した。ガラス温室室内に移植した幼植物体は正常に育育した。