

Plant Regeneration from Suspension Cultures of Onion (*Allium cepa* L.)

Tsuyoshi TANIKAWA*, Masatoshi TAKAGI** and Masahiko ICHII*

* Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-07, Japan

** Kagawa Prefectural Takamatsu Kougei High School, Takamatsu, Kagawa 760, Japan

(Received December 12, 1995)

(Accepted June 6, 1996)

An efficient method for plant regeneration from suspension cultures of onion was studied. For callus induction, sterilized seeds were inoculated on solid MS medium supplemented with 50 μM 4-FPA, 1 μM 2 iP, 1 g/l casein hydrolysate and 0.1 M sucrose. For suspension culture, callus was mechanically disaggregated and transferred into liquid MS medium with 10 mM MES added, and placed on a rotary shaker. Suspension cultures were routinely subcultured. After pre-culture in liquid medium which increased sucrose concentration to 0.2 M, two types of cell clumps developed, a deep yellow, small type, and a light yellow, large type. The large type showed higher regenerative ability than the small type. Frequent regeneration of plantlets occurred on solid MS medium supplemented with 1-5 μM 2 iP. Regenerated plantlets developed to whole plants.

Introduction

In many crops, establishment of efficient techniques for plant regeneration through cell suspension cultures are important for future breeding based on somaclonal variation, cell selection, cell fusion and DNA transfer.

In the genus *Allium*, including many important vegetable crops, there are several reports of plant regeneration from suspension cultures of many important vegetable crops, such as garlic (*A. sativum*)^{1,2}, Chinese chives (*A. tuberosum*)³, Welsh onion (*A. fistulosum*)^{4,5}, and *A. fistulosum* \times *A. cepa* interspecific hybrid⁵. Furthermore, Buiteveld and Creemers-Molenaar⁶, and Schum *et al.*⁷ reported plant regeneration from protoplasts isolated from suspension cultures of leek (*A. porrum*).

However, in onion (*A. cepa*), there has been no report of successful whole plant regeneration from suspension cultures. Fridborg⁸, Nandi *et al.*⁹, and Nakashima¹⁰ reported the formation of only roots, whereas Song and Peffly⁵ reported the formation of only shoots from suspension cultures. Likewise, Hansen *et al.*¹¹ also reported shoot formation from suspension culture-derived protoplasts as well as suspension cultures; in no case have whole plants been recovered.

In the present study, we report whole plant regeneration from suspension cultures of onion.

Materials and Methods

Mature seeds of onion (*Allium cepa* L.) c. v. 'Momiji' (Shippo Seed Co., Ltd.) were used for callus induction. Seeds were surface sterilized in 70% ethanol for 10 min. followed by agitation in 3.5% sodium hypochlorite for 15 min. and rinsed 3 times in sterile distilled water. The seeds were inoculated on MS¹² medium supplemented with 50 μM 4-fluorophenoxyacetic acid (4-FPA), 1 μM N⁶(2-isopentenyl)adenine (2 iP), 0.1 M sucrose, 1 g/l casein hydrolysate and 0.2% Gellan Gum.

The hormone treatments in this medium were founded on preliminary experiments. The cultures were placed under continuous fluorescent light (below 1,000 lx) at 25°C.

After 2 months of culture, calli have formed and were cut into pieces. Using a stainless steel mesh (1 mm) placed on the top of a funnel, the callus pieces were strained by mashing with a spatula. Approximately 400-500 mg of strained callus were transferred to 100 ml Erlenmeyer flasks with 30 ml of liquid MS medium supplemented with 50 μ M 4-FPA, 1 μ M 2 iP, 0.1 M sucrose, 1 g/l casein hydrolysate and 10 mM 2-(*N*-morpholino)-ethane sulfonic acid (MES). MES was used for the pH stability¹³). The cultures were placed on a rotary shaker at 100 rpm under the same conditions as before. The suspension cultures were routinely strained and subcultured every 20 days. After 1-3 subcultures, cell clumps were transferred to 200 ml Erlenmeyer flasks containing 80 ml of liquid pre-culture medium, with the sucrose concentration increased to 0.2 M. The cultures were again placed on a rotary shaker under the same conditions.

After 30 days of pre-culture, cell clumps with 1-3 subcultures were transferred to test tubes containing MS regeneration medium, which was supplemented with 1 μ M 2 iP, 0.1 M sucrose, 1 g/l casein hydrolysate, 10 mM MES and 0.2% Gellan Gum. To investigate the effects of cytokinins on plantlet regeneration, various concentrations of cytokinins [2 iP, 6-benzyladenine (BA) or 6-furfurylamino purine (kinetin)] were added to hormone free MS medium supplemented 0.1 M sucrose, 1 g/l casein hydrolysate, 10 mM MES and 0.2% Gellan Gum, then cell clumps with one subculture were transferred onto those regeneration media. They were cultured under continuous fluorescent light (3,000 lx) at 25°C. After 12 weeks of culture on regeneration media, the number of cell clumps with regenerated plantlets was counted. Frequency of plantlet regeneration was defined as the percentage of cell clumps with plantlets per total number of cell clumps counted.

The regenerated plantlets were transferred to 500 ml culture bottles containing hormone free solid MS medium supplemented with 0.1 M sucrose. After 3-5 weeks, well developed regenerants were transferred to pots containing vermiculite. The plants were covered with polyethylene bags to keep the humidity high. After 4-5 days, the bags were partly opened and 2 weeks later the bags were removed. The acclimatized plants were then transferred to a greenhouse.

All media were adjusted to pH 5.8 prior to autoclaving (121°C; 15 min.). Cytokinins were added prior to sterilization, 4-FPA was filter sterilized and added to the media after autoclaving.

Results

Callus formation was initiated around the radicle after seed germination on MS dedifferentiation medium. The frequency of callus formation was 56% (No. of calli induced per No. of seeds inoculated). The callus induced was light to moderate yellow in color (**Fig. 1-A**), and not friable enough for liquid culture. In a preliminary experiment, it was found that in liquid culture calli did not disintegrate to form free cells or small cell clumps. Similar reactions of callus in liquid culture of onion were reported by Fridborg⁸) and Nandi *et al.*⁹) To get small clumps, callus had to be mechanically disaggregated by straining (**Fig. 1-B**).

After 20 days of suspension culture, the increase in cell volume was approximately 2.5 fold. After 30 days of pre-culture, two types of cell clumps had developed, a deep yellow and small (about 2 mm diameter) type (DS type), and a light yellow and large (about 5 mm diameter) type (LL type) (**Fig. 1-C**). Both types of clumps were transferred onto regeneration medium (the LL type clumps were then broken into 2-4 pieces, and one piece was transferred to each test tube).

Table 1 shows how the clump type and the duration of subculture affects plantlet regeneration. Plantlets were regenerated directly from both types of clumps on the regeneration medium. The

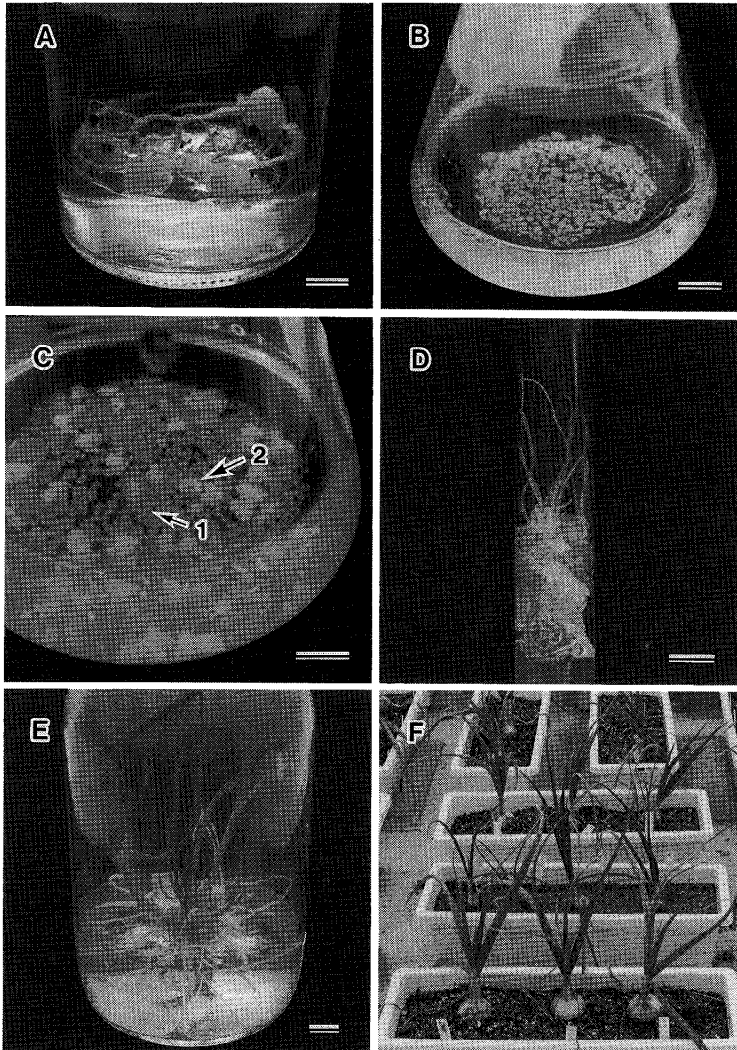


Fig. 1 A : Callus formation after 2 months of culture.
 B : Suspension culture initiation.
 C : Two types of cell clumps developed after 30 days of pre-culture(1: DS type clumps; 2: LL type clumps).
 D : Plantlets regenerated from suspension cultures.
 E : Well developed regenerants in the 500 ml culture bottle.
 F : Acclimatized plants growing in the green house.
 Bars in photographs indicate 1 cm.

regeneration frequencies of LL type clumps were higher than that of DS type clumps, and the most frequent plantlet regeneration (73.3%) was observed in the LL type subcultured only once. In DS type clumps, there was no significant difference in regeneration frequency during 1-3 subcultures. However, in LL type clumps, successive subculturing lowered the regeneration frequency (28.0% for 3 subculturing).

Table 2 shows how cytokinins affected plantlet regeneration from suspension cultures(LL type clumps with one subculture were used in this investigation). For plantlet regeneration, 2 iP was superior to BA and kinetin, and the highest regeneration(75%) was observed at 1-5 μ M 2 iP.

When the clumps with regenerated plantlets(**Fig. 1-D**) were transferred to hormone free MS medium in 500 ml culture bottles, regenerants were well developed(**Fig. 1-E**). A total of 117 well-

Table 1. Effects of cell clump type and frequency of subculture on plantlet regeneration from suspension cultures.

Number of subcultures* ¹	Frequency of plantlet regeneration (%) * ²	
	DS* ³	LL* ³
1	9. 3a* ⁴	73. 3a
2	17. 3a	53. 3ab
3	12. 0a	28. 0b

Each treatment consisted of 3 replicates, and each replicate consisted of 25 test tubes containing one cell clump.

*¹ Cell clumps were subcultured every 20 days.

*² (Number of cell clumps with plantlets/total number of cell clumps) × 100.

*³ DS: deep yellow and small type clumps.

LL: light yellow and large type clumps.

*⁴ Values within column followed by different letters are significantly different at 5% level by Duncan's multiple range test.

Table 2. Effects of cytokinins at variable concentration on the regeneration of plantlets from suspension cultures.

Cytokinins	Concentration (μ M)	Frequency of plantlet regeneration (%) * ¹
Hormone free	0	50. 0bc* ²
2iP	1	75. 0a
	5	75. 0a
	10	60. 0abc
	10	60. 0abc
BA	1	66. 6ab
	5	58. 3ab
	10	38. 3c
Kinetin	1	43. 3c
	5	48. 3bc
	10	55. 0abc

The LL type clumps with one subculture were transferred to each regeneration medium.

Each treatment consisted of 3 replicates, and each replicate consisted of 20 test tubes containing one cell clump.

*¹ (Number of cell clumps with plantlets/total number of cell clumps) × 100.

*² Values within column followed by different letters are significantly different at 5% level by Duncan's multiple range test.

developed regenerated plants derived from 50 cell clumps were transferred to pots in the culture room for acclimatization. Ninety-one plants were successfully transferred to a greenhouse, and these regenerants showed normal growth and appearance (**Fig. 1-F**). After 4-6 months, the bulbs of regenerants were harvested.

Discussion

Fridborg⁸), Nandi *et al.*⁹), Nakashima¹⁰), Ping and Peffley⁵), and Hansen *et al.*¹¹) used 2, 4-dichlorophenoxyacetic acid (2, 4-D) as the auxin source in onion tissue culture, but they did not obtain regenerated whole plants through liquid culture. On the other hand, Phillips and Luteyn¹⁴) reported that picloram was superior to 2, 4-D for callus culture and subsequent plant regeneration of onion.

In preliminary experiments, we also used picloram for callus induction and suspension culture. However, the frequency of plantlet regeneration from suspension cultures was low (data not shown). In these experiments, 4-FPA was used for callus induction and suspension culture of onion, which led to a high frequency of plantlet regeneration from suspension cultures. Thus, 4-FPA is considered to be the auxin of choice for onion tissue culture.

The frequency of plantlet regeneration varied remarkably depending on cell clump type and the frequency of subculture. The LL type clumps showed higher regenerative ability than DS type clumps. However, the ability of LL type clumps to regenerate plantlets decreased with successive subcultures. In many higher plants, it is generally true that the ability of cultured cells to differentiate decrease with increasing duration of subculture. However, Ozawa and Komamine¹⁵⁾ reported for certain rice (*Oryza sativa*) cultivars that the ability of cultured cells for differentiation was maintained by improvement of culture conditions. Therefore, it may be possible to maintain the regenerative ability of onion cells in tissue culture.

Cytokinin was not necessary for plant regeneration, but the addition of 1-5 μM 2 iP to the regeneration medium stimulated the regeneration of plantlets. In previous reports, Fridborg⁸⁾, and Dunstan and Short¹⁶⁾ obtained shoots regenerated from onion callus on the differentiation medium containing 2 iP. Thus, we suggest that 2 iP is an effective cytokinin for regeneration in onion.

In this paper, we demonstrate successful whole plant regeneration from suspension cultures of onion. This method of plant regeneration from suspension cultures may be useful for mutant selection. Furthermore, the suspension cultures may be a good starting material for the isolation of totipotent protoplasts.

Acknowledgement

The authors thank Shippo Seed Co., Ltd., Kagawa for providing the onion seeds.

References

- 1) Nagasawa, A., J. J. Finer, 1988. *Plant Cell Tissue and Organ Culture*, **15**: 183-187.
- 2) Cid, L. P. B., R. D. Illg, A. E. Piedrabuena, 1994. *In Vitro Cell. Dev. Biol.*, **30**: 150-155.
- 3) Matsuda, Y., T. Adachi, 1993. *Japan. J. Breed.*, **43** (Suppl. 2) : 133.
- 4) Shimonaka, M., H. Tabira, S. Omura, 1992. *Japan. J. Breed.*, **42** (Suppl. 1) : 38-39.
- 5) Song, P., E. B. Peffley, 1994. *Plant Science*, **98**: 63-68.
- 6) Buiteveld, J., J. Creemers-Molenaar, 1994. *Plant Science*, **100**: 203-210.
- 7) Schum, A., H. Junge, L. Matiesch, 1994. *Gartenbauwissenschaft*, **59**: 26-30.
- 8) Fridborg, G., 1971. *Physiol. Plant.*, **25**: 436-440.
- 9) Nandi, S., G. Fridborg, T. Eriksson, 1977. *Hereditas*, **85**: 57-62.
- 10) Nakashima, T., 1989. *Journal of Agricultural Science*, **44**: 117-121.
- 11) Hansen, E. E., J. F. Hubstenberger, G. C. Phillips, 1995. *Plant Cell Reports*, **15**: 8-11.
- 12) Murashige, T., F. Skoog, 1962. *Physiol. Plant.*, **15**: 473-497.
- 13) Tanimoto, S., H. Harada, 1991. *Plant Cell Technology*, **3**: 235-240.
- 14) Phillips, G. C., K. J. Luteyn, 1983. *J. Amer. Soc. Hort. Sci.*, **108**: 948-953.
- 15) Ozawa, K., A. Komamine, 1991. *Plant Tissue Culture Letters*, **8**: 147-151.
- 16) Dunstan, D. I., K. C. Short, 1978. *Scientia Horticulturae*, **9**: 99-110.

タマネギ懸濁培養細胞からの植物体再分化

谷川 毅*・高儀雅俊**・一井眞比古*

* 香川大学農学部

** 香川県立高松工芸高校

タマネギの懸濁培養細胞から植物体再分化を試みた。50 μM 4-fluorophenoxyacetic acid(4-FPA), 1 μM N^6 (2-isopentenyl) adenine(2 iP), 1 g/l カゼイン加水分解物及び 0.1 M ショ糖を添加した MS 脱分化培地に滅菌種子を播種した。2 ヶ月後、誘導されたカルスをステンレスメッシュ(1 mm)を用いて裏ごしし、それを 10 mM MES を加えた MS 液体培地とともに三角フラスコに移し、振とう培養した。1-3 回の継代培養後、ショ糖濃度を 2 倍にした培地で前処理培養したところ、濃黄色でかつ小さな細胞塊(DS 型)及び淡黄色でかつ大きな細胞塊(LL 型)が得られた。これらの細胞塊を 1 μM 2 iP とした MS 再分化培地に移した結果、LL 型細胞塊から高頻度で植物体が再分化した。また、1-5 μM 2 iP の再分化培地への添加は植物体再分化率を向上させた。順化させようとした再分化植物体の多くは鱗茎を形成し、それらの植物体のあいだには形態的変異は認められなかった。