



Title	STUDIES ON TISSUE CULTURE IN ASPARAGUS OFFICINALIS L. : Influences of growth regulators, L-glutamine, cell density and varietal difference on colony formation in cell culture of <i>Asparagus officinalis</i> L
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## STUDIES ON TISSUE CULTURE IN *ASPARAGUS OFFICINALIS* L.

### XI Influences of growth regulators, L-glutamine, cell density and varietal difference on colony formation in cell culture of *Asparagus officinalis* L.

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#### Introduction

Establishment of plantlet regeneration system through free cell culture is effective for reseaches on new variety breeding and a study on the physiological mechanisms in *Asparagus officinalis* L. . Co<sup>1</sup>man et al.<sup>1)</sup> and Peel<sup>2)</sup> worked on the biomechanism of photosynthesis. Jullien et al.<sup>3,4)</sup> attempted to select cells resistant to some strains of *Fusarium* sp. . Bui-Dang-Ha et al.<sup>5,6,7)</sup> succeeded in readily isolating protoplasts from cladophyll cells and in regenerating plants. The studies on the single cell culture of asparagus have been carried out by Jullien<sup>8,9,10)</sup>, but only one variety, 'Marché de Malines', has been used in their experiment.

*In vitro* culture conditions of growth regulators, L-glutamine, cell density and varietal differences require detailed examinations to enhance cell division, colony formation and plantlet regeneration. In the present experiment, the authors intend to clarify those culture conditions by culturing free cells which are easily isolated from a cladophyll.

#### Materials and Methods

*Plant materials.* Free cells isolated from cladophylls of *Asparagus officinalis* L. were cultured *in vitro*. Five varieties including 'Goldshatz', 'Eden', 'Zuiyo', 'Mary Washington 500W' and 'l'Argenteuil' that were grown in Experimental Farm of Hokkaido University (Sapporo, Japan) were used in an experiment to test the influence of varietal differences on cell division and colony formation. Influences of growth regulators (NAA, BA), L-glutamine and cell density were examined using 'Goldshatz'.

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*Isolation of cladophyll cells.* Cladophylls, borne on the nodes of juvenile branches on surface of a fern, were collected in early June. The cladophylls were surface-sterilized with 70% ethanol for 30 sec and with sodium hypochlorite solution (available chlorine 1%) for 15 min, followed by rinsing two times with sterile water.

Sterilized cladophylls were homogenized in a small volume of a liquid medium (containing MS medium used as basal medium uniformly in all experiments) in a glass homogenizer. The crude cell suspension was filtrated through a 31- $\mu$ m nylon mesh, and the filtrate was centrifuged at 100 $\times$ g for 2 min. The precipitate was collected with a pistol pipette and rinsed three times in a solution containing MS medium alone.

*Culture condition.* The media used contained MS medium, 20 g/l glucose and 6 g/l agar, and the pH was adjusted to 5.8. To examine the effects of growth regulators on cell division and colony formation, cladophyll cells were cultured in 9 media with combinations of 0 to 1 mg/l NAA and 0 to 1 mg/l BA. All cells were cultured through being enclosed in a thin layer of agar medium in a petri dish at densities of  $3 \times 10^3$  to  $3 \times 10^5$  cells/ml. The petri dish were sealed with plastic film, and placed under conditions of controlled humidity, constant temperature (25°C) and darkness. After 60 days of culture, the number of colonies per petri dish and a plating efficiency was measured.

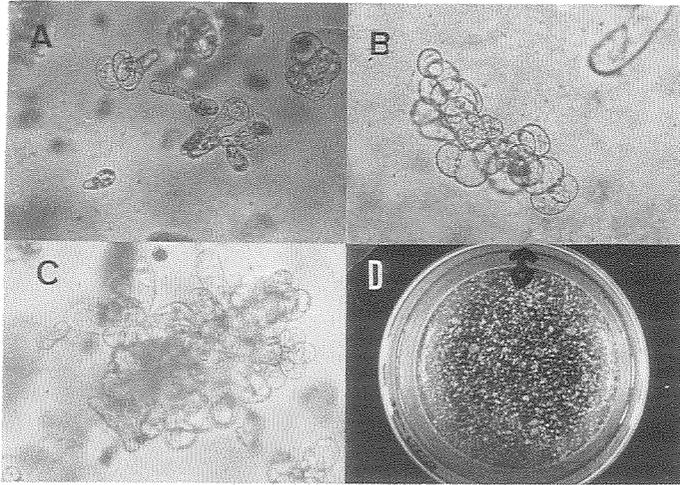
*Plantlet regeneration.* Induced colonies approximately 2 mm in diameter were selected and transferred onto a medium with 0.01 to 1 mg/l NAA, 0.3 mg/l 2, 4-D, 10 mg/l BA, 20 g/l glucose, 6 g/l agar and pH 5.8.

## Results

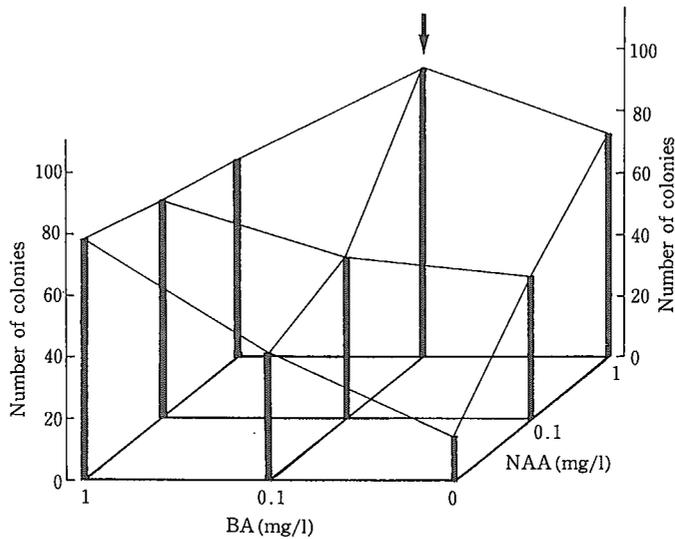
Cell division began after 10 days of culture, and the divided cells developed into clumps, clusters and colonies in order (Fig. 1). Number of the colonies increased with the increase of NAA concentration (Fig. 2). The largest number of induced colonies was obtained in the medium with 1 mg/l NAA and 0.1 mg/l BA. On the concentration of L-glutamine, numerous cell divisions were observed in the medium with 0 to 100 mg/l. Little difference in cell division was recognized among these concentrations. No cell division, however, occurred at 1000 mg/l of L-glutamine (Table 1).

Cladophyll cells divided and developed into colonies at  $3 \times 10^5$  cells/ml, while no cell division was observed at densities less than  $3 \times 10^4$  cells/ml (Table 2).

Differences in colony formation existed among varieties used after 120 days of culture. In 'Goldshatz' and 'Eden', a rapid colony formation was recognized from 30 th day through 60 th day, and the number of the colonies gradually increased after 60 days of culture. In other three varieties, 'Mary Washington 500 W', 'Zuiyo' and 'l'Argenteuil', the onset of colony formation was later than that of the two varieties mentioned above. On the 120 th day in the culture, in 'Mary



**Fig. 1.** Cell division and colony formation in cladophyll cell culture of *Asparagus officinalis* L..  
 A : Initial division after 10 days of culture.  
 B : Cell clusters after 30 days of culture.  
 C : Clumps after 45 days of culture.  
 D : Colonies after 60 days of culture.



**Fig. 2.** Effect of NAA and BA on colony formation in cladophyll cell culture of *Asparagus officinalis* L..  
 Arrow shows the largest number of colonies.

TABLE 1. Effect of L-glutamine on colony formation in cell culture of *Asparagus officinalis* L. ('Goldshatz').

Concentration of L-glutamine (mg/l)	Number of colonies	Plating efficiency (%) <sup>2</sup>
0	344	0.11
1	—	—
10	505	0.16
100	307	0.10
1000	0	0

<sup>2</sup>Plating efficiency = (total number of colonies/total number of cultured cells) × 100.

TABLE 2. Influence of cell density on colony formation in cell culture of *Asparagus officinalis* L. ('Goldshatz').

Cell density (cells/ml)	Number of colonies	Plating efficiency (%) <sup>2</sup>
$3 \times 10^3$	0	0
$3 \times 10^4$	0	0
$3 \times 10^5$	344	0.11

<sup>2</sup>Plating efficiency = (total number of colonies/total number of cultured cells) × 100.

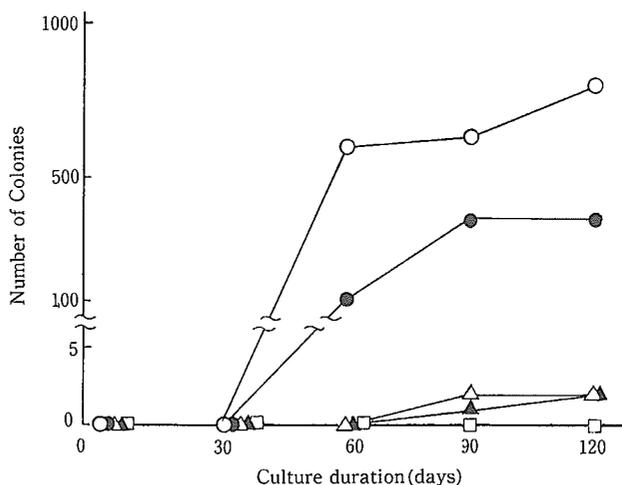
Washington 500 W' and 'Zuiyo', the number of induced colonies was very small, and no colony was formed in 'l'Argenteuil' (Fig. 3, Fig. 4).

Subsequently, the colonies were transferred onto the medium (described in methods) for plantlet regeneration, and some colonies developed into calli. After 100 days of subculture, several plantlets regenerated from the callus in 'Goldshatz' and 'Eden' (Fig. 5). The frequency of the plantlet regeneration was low after 220 days of culture.

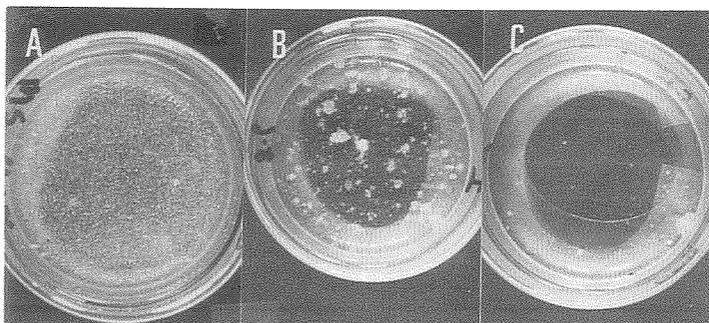
### Discussion

On plant growth regulators, a combination of 1 mg/l NAA and 0.1 mg/l BA in medium was found to be highly efficient for colony formation from cladophyll cells. This result agrees approximately with the report of Jullien<sup>8</sup>). From these facts, it was assumed that the combination of NAA and BA had an effect not only on cell division but also on colony formation.

In the present study, colony formation was obtained in the media with 0 to 100 mg/l L-glutamine, and showed no difference according to the concentrations, whereas no colony formation was observed at a high concentration (1000 mg/l) of L-glutamine. These results suggest that L-glutamine shows no promotive effect



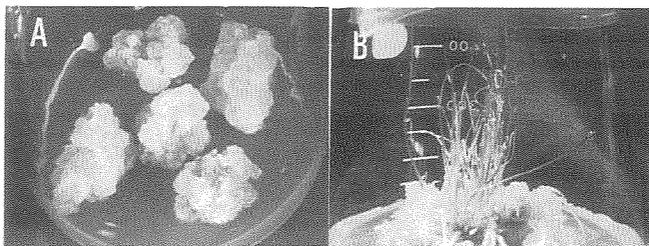
**Fig. 3.** Influence of varietal difference on colony formation in cladophyll cell culture of *Asparagus officinalis* L..  
 ○, 'Goldshatz'; ●, 'Eden'; △, 'Zuiyo'; ▲, 'Mary Washington 500W';  
 □, 'l'Argenteuil'.



**Fig. 4.** Colony formation after 60 days of cladophyll cell culture of *Asparagus officinalis* L..  
 A, 'Goldshatz'; B, 'Eden'; C, 'Mary Washington 500W'.

on cell division, and inhibits cell division at a high concentration of 1000 mg/l. Bui-Dang-Ha et al<sup>6)</sup> reported that an addition of 1000 mg/l L-glutamine resulted in the sustained division of protoplasts isolated from cladophylls. It is noted that a role of L-glutamine for colony formation in protoplast culture was different from that in free intact cell culture.

On cell density, in the various results reported previously, free cells were cultured at densities of more than  $10^5$  cells/ml, because the capacity of cell division was reduced at a low level of cell density. The present results agree with



**Fig. 5.** Callus formation and plantlet regeneration from isolated cladophyll cells of *Asparagus officinalis* L..  
 A: Proliferated callus after about 60 days of subculture.  
 B: Regenerated plantlet.

the results from the experiment of Jullien<sup>9</sup>). In asparagus (*Asparagus officinalis* L.), it is recognized that high cell-density culture at more than  $10^5$  cells/ml is necessary to obtain a frequent cell division and a vigorous colony formation.

A defined difference in the cell division and the colony formation were observed among the used varieties. Free cells of 'Goldshatz' and 'Eden' have characteristics suitable for a plant material used in cell culture. Further studies will be required to determine the culture conditions for various varieties.

### Summary

Cladophyll cell culture of *Asparagus officinalis* L. (var. 'Goldshatz', 'Eden', 'Mary Washington 500 W', 'Zuiyo' and 'l'Argenteuil') was carried out in relation to the effects of growth regulators, L-glutamine, cell density and varietal difference on colony formation. Basal medium used contained MS medium, 20 g/l glucose and 6 g/l agar, and cell-enclosing culture method was applied. The cells divided, and developed into colonies in a medium with 1 mg/l NAA, 0.1 mg/l BA and 100 mg/l L-glutamine. A high concentration (1000 mg/l) of L-glutamine inhibited cell division. Cell division occurred at cell density of  $3 \times 10^5$  and not at less than  $3 \times 10^4$  cells/ml. Differences in cell division and colony formation existed among the varieties: free cells developed into colonies in 'Goldshatz' and 'Eden'; a few colonies were induced in 'Mary Washington 500 W' and 'Zuiyo'.

### Acknowledgement

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