REDIFFERENTIATION FROM TISSUE CULTURE AND ISOLATION OF VIABLE PROTOPLASTS IN *PANAX GINSENG* C.A. MEYER

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ABSTRACT

Ginseng cotyledon calli were cultured on ½MS media supplemented with combination of various growth regulators to induce more embryoids and plantlets in a short period. And tissues of ginseng root and calli were also incubated under various factors or conditions to establish methods for the isolation of viable protoplasts in *Panax ginseng* C.A. Meyer.

The calli derived from cotyledon produced numerous embryoids in ½MS media containing 0.5mg/ℓ 2,4-D and 0.5mg/ℓ kinetin after 2 months' culture. But only shoot formation was less frequent. Further development of these embryoids occurred on ½ MS medium supplemented with the same concentration of BA and GA.

Viable protoplasts were isolated from the root tissue and callus of ginseng. The specific conditions for the isolation of viable protoplasts were required of ginseng materials, root tissue and callus, being processed. For the production of viable protoplasts from 1-year old ginseng root tissue, an enzyme mixture of 2% cellulase "Onozuka" and 0.5% macerozyme, an enzyme solution pH of 5.2 to 5.8, a 7- to 8- hour incubation period at 28 ± 1°C, and 0.9M mannitol as osmoticum in the cell enzyme mixture were optimum, while the treatments with an enzyme mixture of 2% cellulase "Onozuka", 2% macerozyme and 1% driselase, and 25-hour incubation period at 28 ± 1°C were more efficient for the production of viable protoplasts from ginseng callus.

INTRODUCTION

Ginseng (*Panax ginseng* C.A. Meyer) is a perennial herbaceous plant which grows very slowly. It takes about 3 to 4 years from seeding to collecting the ripe seeds and the ginseng propagation is very difficult. In addition, development of viable seeds in most interspecific crosses of ginseng has been prevented because of specific inhibition or elimination of key steps in pollination, pollen tube growth, fertilization, and embryo or endosperm development. Thus techniques for large scale propagation via organogenesis on explants and for isolation, fusion, culture, and plant regeneration from protoplasts can be used as new tools in studies on the genetic manipulation and breeding of ginseng.

The formation of organs from ginseng calli has been reported by Chang and Hsing[4] and Choi *et al*[5,6]. However, it took a long time, 4 to 8 months, to induce embryoid and shoot from ginseng calli. And the induced shoots were transferred to the media prepared for induction of plantlets, but no signs of development into normal plantlet similar to ginseng seedling were observed, yet. Viable protoplasts have already been isolated from numerous plant species and various plant parts 7-29). A number of factors or conditions prescribed for the isolation of proto-

plasts from each plant or plant part have been sufficiently different to suggest that specific conditions were required of each plant or plant material being processed. This research has attempted to clarify the effects of growth regulators on the organogenesis of cotyledon calli and to establish methods for the isolation of viable protoplasts in *Panax ginseng* C.A. Meyer.

MATERIALS AND METHODS

1. Induction of callus and embryoid

Cotyledons used were produced by the aseptic culture of embryo. For callus induction, the segments of cotyledon were cultured on the basal medium with 0.1 to 8.0mg/l of 2,4-D in the dark for 3 months at 25°C. The basal medium used was a modified formulation from Murashige and Skoog, and hereafter designated as MS medium. The modifications were: 100mg/l myo-inositol, 0.5mg/l nicotinic acid, 0.5mg/l pyridoxine HCl, 0.1mg/l thiamine HCl, 2g/l glycine, 5g/l yeast extract, 30g/l sucrose, 2g/l casein hydrolysate, and 10g/l agar. The pH was adjusted to 5.8 before autoclaving. For organ formation from callus, a basal medium containing one half of Murashige and Skoog salts (½MS) was used and plant growth regulators, such as kinetin, 2,4-D, BA and GA, were added in various concentrations.

2. Isolation of protoplasts

The experiments of protoplast isolation were conducted with cells of root and callus of *Panax ginseng*. One year old ginseng was used as a source of root explant. Detached roots were surface sterilized with 2% NaOCl solution for 20 min. and then rinsed 4 times in sterile distilled water. Root pieces were cut transversely into 1.0mm segments, and protoplasts isolated using an enzyme mixture of cellulase and macerozyme. For a callus induction from ginseng roots, root segments were plated on Murashige and Skoog solid medium(MS) containing 5mg/l 2,4-D and 1mg/l kinetin and cultured at a constant 25±1°C. Light yellow callus developed at the cut edge of root segments and was of sufficient size for subculture within 5 to 7 weeks. Actively dividing and friable callus for protoplast isolation was maintained by subculturing the initial callus on solid MS medium. Callus for protoplast isolation was weighed and gently separated by passing through a coarse sieve (1mm) with the aid of spatula and several rinses of a cell protoplast washing(CPW) solution containing 13% mannitol. The cell slurry was transferred to screw-capped test tubes, and the cells were pelleted by centrifugation (100 x g; 5 min.). The supernatant was replaced by the protoplast release enzyme solution, a mixture of cellulase, macerozyme, driselase and mannitol. The cells suspended in the enzyme solution (ca. 2.0g/10ml) were transferred to petri dishes, which were wrapped with parafilm and placed on a reciprocal shaker (35 rpm) at 28 ± 1°C.

After enzymatic treatment, the enzyme solution containing the protoplasts was filtered through a nylon mesh to remove undigested tissue, cell clumps, and cell wall debris. The filtrate containing mainly protoplasts was collected in centrifuge tubes.

Intact protoplasts containing little or no particular contaminants were gently pelleted by centrifugation at 100 x g for 3 min. Residual enzyme was removed by three successive washes with CPW solution containing 0.7M mannitol, 10 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KNO_3, 1 uM KI, 200 uM KH_2PO_4, and 0.1 uM $CuSO_4$. 4 ml of protoplasts were added to each dish, which were then wrapped with parafilm and incubated at 25 ± 1°C. To clarify the optimal conditions for the isolation of viable protoplasts from callus and root cells of *Panax ginseng*, the most commonly used enzymes for the isolation of protoplasts, cellulase Onozuka R-10, macerozyme R-10, and driselase, were tested in combination and in a range of 0 to 3%, while incubation periods, mannitol concentrations, and enzyme solution pH were tested in a range of 1 to 25 hr, 0.3 to 1.1. M, and 4.0 to 6.4, respectively.

Protoplast yield was determined by counting in a haema - cytometer. Viability was determined

by staining with fluorescein diacetate(FDA).

RESULTS AND DISCUSSION

1. Induction of callus and embryoid

The segments of cotyledon were cultured on the Murashige and Skoog's basic medium(MS medium) supplemented with different concentrations (0.1 - 8 mg/l) of 2,4-D for induction of callus. Formation of somatic callus started comparatively late. About 3 weeks after the inoculation of explants, calluses were visible arising at the uninjured cambial cells of the cut surface of cotyledon. Regardless of the parts they arose, callus tended to be formed more profusely on the media supplemented with 5 mg/l 2,4-D. 2,4-D free medium produced a poor result.

In cotyledon, the cells of the lower epid ermis as well as mesophyll cells near the vascular bundless proliferated to form embryogenic callus. Only the compact, generally white, opaque, organized callus showed the capacity to form somatic embryos. The soft, friable, translucent, generally unorganized callus commonly formed from mature tissue explants did not form embryos. These phenomena were also observed in the leaves of a number of plants[30]. The embryogenic callus was composed for small, richly cytoplasmic, thin-walled cells which lacked conspicuous vacuolation but which contained many prominent starch grains.

Cotyledon calli were cultured on ½ MS media supplemented with combinations of 2,4-D + kinetin for embryoid formation. Embryoids were formed 2 months after culture in cotyledon calli. Besides normal embryoids, various malformed embryoids were observed (Fig. 1). Initially appearing as white small globular masses, the embryoids passed through successive developmental stages, heart- and torpedo-shaped stages, and ultimately gave rise to plantlets (Fig. 2). The pattern of their formation from cotyledon calli and their changing shapes were quite similar to those reported by Chang and Hsing[4] and Choi *et al.*[5,6] The somatic embryos formed *in vitro* were structurally similar to zygotic embryos.

Fig. 1. Various stages of embryoids induced from ginseng cotyledon callus.

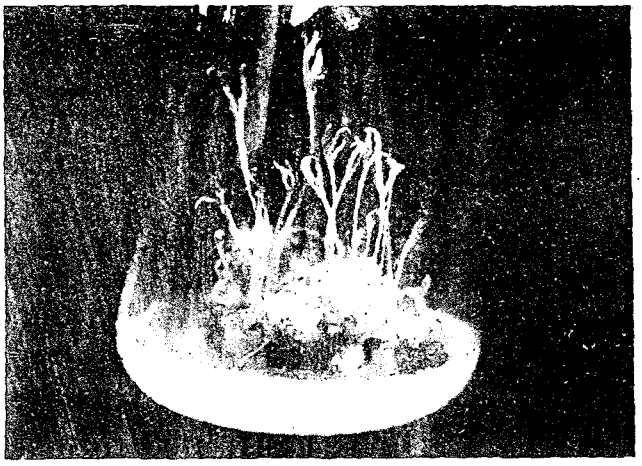

Fig. 2. Plantlets induced from ginseng cotyledon callus.

In previous studies[5], we reported that kinetin was not effective for embryoid formation from the segments and calli of ginseng root. However, we found, in these experiments, that low concentration of kinetin was effective for embryoid formation from the calli of ginseng cotyledon. These results suggest that the effects of growth regulators on the embryoid formation from the calli can be varied with different explant materials used for callus induction, cotyledon or root.

In order to determine the optimum levels of kinetin for embryoid formation from the calli of ginseng cotyledon, various concentrations of kinetin were incorporated into ½ MS medium with 0.5 mg/l 2,4-D, and the cotyledon calli

Table 1. Number of embryos induced from cotyledons callus of *Panax ginseng* cultured on different media

Concentration*		No. of embryos at various stage					Total	%
2,4-D	Kinetin	Globular-shaped	Heart-shaped	Torpedo-shaped	Normal	Deformed		
0	0	1	0	0	0	0	0	0.7
0.5	0	13	6	7	0	1	27	19.3
0.5	0.5	41	8	6	6	6	67	49.9
0.5	1.0	9	8	6	6	6	67	14.3
0.5	1.5	8	2	0	5	4	19	13.6
0 5	2.0	3	2	0	0	1	6	4.3

Table 2. Effects of BA and GA on the shoot differentiation of cotyledon callus

Concentration*		No. of test tube			No. of	Total	%
BA	GA	Cultured	With shoot	With root and shoot	Embryo		
0	0	10	2	0	0	2	20.0
0.1	0	10	1	0	2	2	20.0
0.1	0.1	10	2	0	1	3	30.0
0.1	1.0	10	2	0	1	3	30.0
1.0	0	10	0	0	0	0	0
1.0	0.1	10	1	2	1	4	40.0
1.0	1.0	12	8	2	0	10	83.4
1.0	2.0	10	4	0	0	4	40.0
5.0	0	9	0	0	0	0	0
5.0	0.1	9	1	0	2	2	22.2
5.0	1.0	10	2	0	1	3	30.0
5.0	5.0	10	6	1	0	7	70.0
5.0	10.0	8	1	0	3	4	50.0

* : mg/l

were cultured on these media for three months. Table 1 shows the number of embryos induced from cotyledon callus of *Panax ginseng*. The calli cultured on the medium without 2,4-D and kinetin did not exhibit embryoid formation. The frequency of calli exhibiting embryoid formation was increased when the level of kinetin in the medium was either reduced or totally eliminated. High percentage of embryoid formation occurred in the medium supplemented with the combination of 0.5 mg/l 2,4-D and 0.5 mg/l kinetin (Table 1). But only shoot formation was less frequent. Further development of these embryoids occurred on the medium supplemented with the combination of 1 mg/l BA and 1 mg/l GA, which was developed by Chang and Hsing[4] and Choi *et al.*[5] for the growth of embryoids induced from callus tissue of ginseng root.

The requirement for BA and GA in shoot formation from cotyledon calli was tested in the presence of different concentrations of BA and GA. Table 2 shows the number and percentage of calli exhibiting the formation of shoot and both shoot and root. Shoots were observed at a frequency of 83.4% in the presence of 1mg/l BA and 1mg/l GA, and at a frequency of 70% in the

presence of 5mg/l BA and 5mg/l GA. The high frequency of shoot formation might be due to the same concentrations of BA and GA which is favourable to this process. These results suggest that the interaction between BA and GA is critical to inducing shoots from cotyledon calli of ginseng.

In these experiments, we have found that the cotyledon calli are more effective than the root calli for more embryoid and shoot formation in a shorter period.

2. Isolation of viable protoplasts

1) Protoplast isolation

For the isolation of protoplasts, most of the early research was conducted with the mesophyll tissue obtained from mature leaves of plants. With the improvement of techniques it has become possible to isolate protoplasts from a wide range of plant genera and tissues[2,7,8,18,20,22,25,28].

To obtain protoplasts, tissues of leaves, roots and calli of ginseng were treated with the enzyme mixtures consisting of 2% cellulase Onozuka R-10, 0.5% macerozyme R-10, and 0.7M mannitol for 5 hours. These enzyme solutions gave very poor yields of protoplasts from the tissues of root (Fig. 3). However, it was almost impossible to obtain protoplasts from the leaves and calluses of ginseng under the same conditions. Though the protoplasts can be isolated, their yield depends on a number of factors, the most important ones being the kind, concentration, and purity of enzyme. In addition, particular attention should be paid to the period of incubation, their pH, and specially the plasmolyticum as they immensely effect the viability of the protoplasts and their subsequent growth and division in cultures[1]. Therefore, the follow experiments were carried out to determine optimal conditions for portoplast isolation from the l-year old roots and calli of ginseng.

2) Effect of enzyme concentration on protoplast isolation

Although ginseng protoplasts were isolated

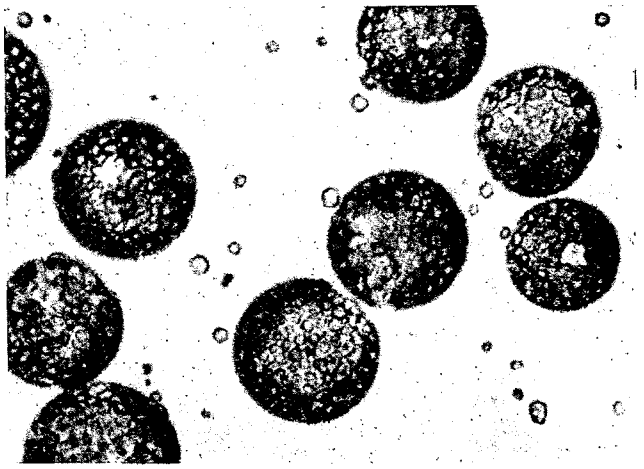

Fig. 3. Protoplasts isolated from the tissues of ginseng root.

in the presence of 2% cellulase Onozuka R-10, 0.5% macerozyme and 0.7M mannitol, it was not known whether this concentration or osmoticum was also suitable for isolation of protoplasts from ginseng roots. Therefore, various concentrations of cellulase Onozuka R-10 were tested in a range of 1.5 to 3.0% in the presence of 0.5% macerozyme R-10 and 0.7M mannitol (Table 3), and various concentrations of macerozyme R-10 were tested in a range of 0.1 to 1.1% in the presence of 2% cellulase Onozuka R-10 and 0.7M mannitol at pH 5.8 (Table 4).

When other parameters were optimal, freshly prepared root tissues were almost completely dissociated after 7 hours' incubation in a range of 1.5 to 3.0% cellulase Onozuka R-10 yielding large numbers of separated cells and approximately half as many isolated protoplasts. The effect of various concentrations of cellulase Onozuka R-10 on protoplasts production is shown in Table 3. Protoplast yields were increased with increasing the concentration of cellulase Onozuka R-10 by a further 7 hours, the time required for maximal protoplast isolation. At higher concentrations of cellulase Onozuka R-10, yields of protoplasts increased and were highest between 2.0 to 3.0% (w/v). A low concentration of cellulase Onozuka R-10 (< 1.5%) increases the treatment time. The optimum concentration of macerozyme R-10 was spread over a wide range after 7 hours' incubation (Table 4).

Table 3. Effect of concentration of cellulase "Onozuka R-10" and incubation time on isolation of protoplast from ginseng root cells

Concentration (%)	Incuhation time (hour)								
	1	2	3	4	5	6	7	8	9
1.5	–	–	–	10**	10	20	50	70	70
2.0	–	–	10	20	40	70	80	80	90
2.5	–	–	10	20	40	70	80	80	90
3.0	–	–	10	20	50	80	80	90	90

* : Not isolated.

** : Degree of isolation is represented by per cent.

Enzyme solution: 0.5% macerozyme and 0.7M mannitol at pH 5.8.

Table 4. Effect of concentration of macerozyme "R-10" and incubation time an isolation of protoplast from ginseng root cells

Concentration (%)	Incubation time (hour)								
	1	2	3	4	5	6	7	8	9
0.1	–*	–	10**	10	20	40	40	70	70
0.3	–	–	10	10	20	40	40	70	70
0.5	–	–	10	20	40	70	80	80	80
0.7	–	–	10	20	40	70	80	80	80
0.9	–	–	10	20	40	80	80	90	90
1.1	–	–	10	20	50	80	80	90	90

* : Not isolated.

** : Dregree of isolation is represented by per cent.

Enzyme solution: 2% cellulase and 0.7M manhitol at pH 5.8.

Equal yields of protoplasts, about 80% of the cells, were obtained when the cells were treated with macerozyme concentrations of 0.5, 0.7, 0.9 and 1.1% (w/v) for above 7 hours. These results suggest that a 7 to 9 hours' incubation period is necessary for reasonable yields of protoplasts from ginseng root tissues.

Unless the procedure produces viable entities, the isolation of protoplasts has little significance. The viability of protoplasts was, therefore, tested in the presence of different concentrations of cellulase Onozuka R-10 and macerozyme R-10. Figs. 4 and 5 show the number of released protoplasts as influenced by cellulase Onozuka R-10 and macerozyme R-10 and survival of the protoplasts during subsequent incubation *in vitro*. Yields of protoplasts were highest at 2% cellulase Onozuka R-10 and 0.5% macerozyme R-10 while lower or higher concentrations than these conditions have produced low yields of protoplasts (Figs. 4 and 5). The percentage of protoplasts that were alive immediately after isolation and their survival during subsequent incubation *in vitro* was not significantly different over the range of concentrations of cellulase Onozuka R-10 and macerozyme R-10. Therefore, yields of live protoplasts were also highest in the presence of 2% cellulase Onozuka R-10 and 0.5% macerozyme R-10 (Figs. 4 and 5).

Callus tissue 4 to 5 weeks after subculture was the most suitable material for protoplast production; older tissue produces progressively lower yields of protoplasts. Protoplasts are more readily released from cells which are just entering

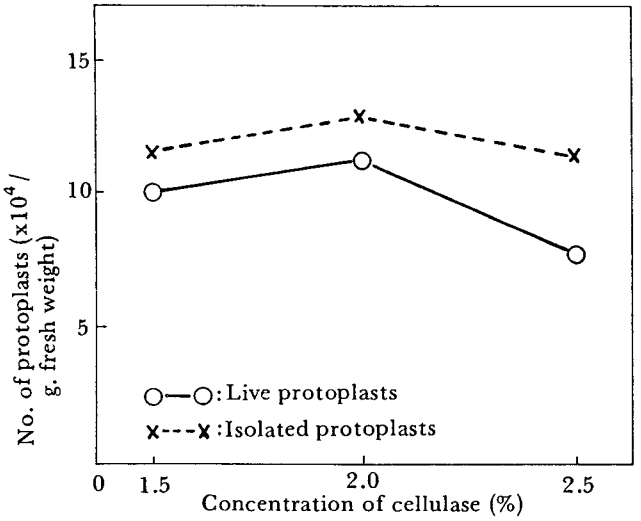

Fig. 4. Yields of isolated and live protoplasts as influenced by the concentration of cellulase "Onozuka R-10" in ginseng root tissue. 0.5% macerozyme "R-10" with 0.7M mannitol was added to enzyme solution and incubation period was 7 hours.

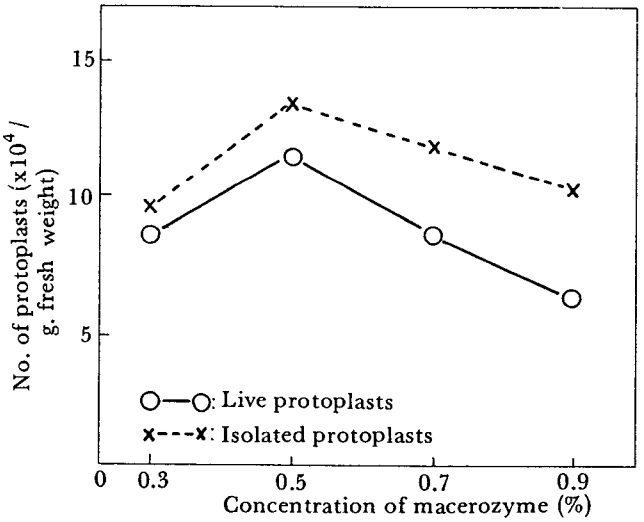

Fig. 5. Yields of isolated and live protoplasts as influenced by the concentration of macerozyme "R-10" in ginseng root tissue. 2% cellulase "Onozuka R-10" with 0.7M mannitol was added to enzyme solution and incubation period was 7 hours.

the growth phase than from mature cells[24]. Cells from later in the growth phase release protoplasts no more easily than do mature cells, but they tend to survive better. It has been observed by other workers that cells from young, actively growing callus of many tissues most readily release protoplasts. Since driselase has been reported to be effective in the isolation of protoplasts from callus tissues of *Nicotiana tabacum*[12] it was decided to test its efficacy on protoplast

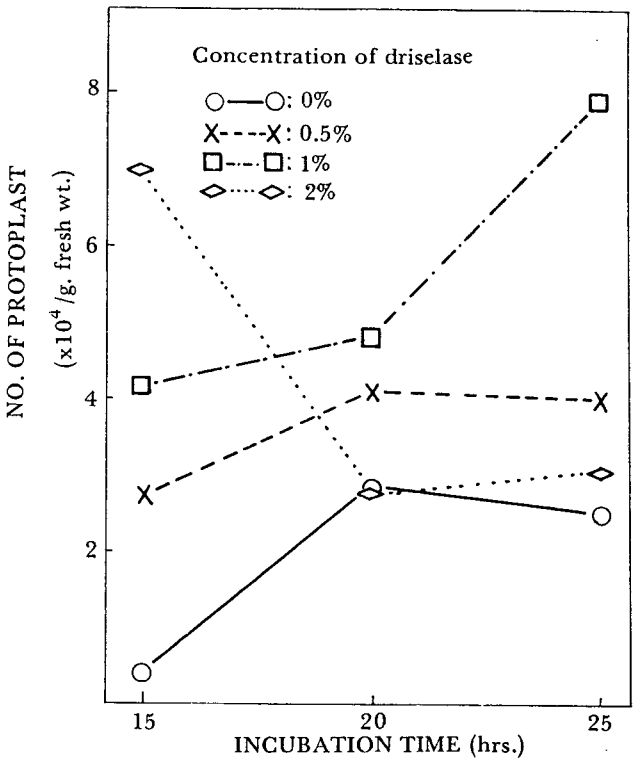

Fig. 6. Effect of concentration of driselase on yield of protoplasts from ginseng callus. The enzyme mixture contains 2% cellulase "Onozuka" and 2% macerozyme.

isolation. Therefore, various concentrations of driselase were tested in a range of 0 to 2.0% in the presence of 2% cellulase Onozuka R-10 and 2% macerozyme R-10. As shown in Fig. 6, the treatment with enzyme solution containing 1% driselase for 25 hours gave the highest yields of protoplasts from ginseng callus while the enzyme solutions containing low concentration (0 to 0.5%) of driselase gave poor yields. When the callus cells were treated with 2% driselase for 15 hours, protoplast yields were high but reduced with extending the incubation time. Especially, it was found that a number of protoplasts were bursted during 20 and 25 hours' incubation at 2% driselase. Fig. 7 shows the number of protoplasts that are alive immediately after 15, 20 and 25 hours' incubations at various concentrations of driselase. The percentage of viable protoplasts was not significantly different over the range of

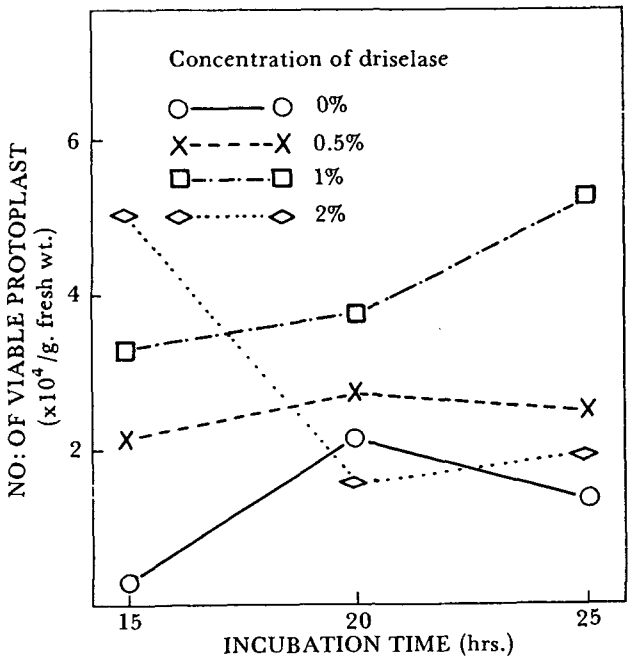

Fig. 7. Effect of concentration of driselase on yield of viable protoplasts from ginseng callus, The enzyme mixture contains 2% cellulase "Onozuka" and 2% macerozyme.

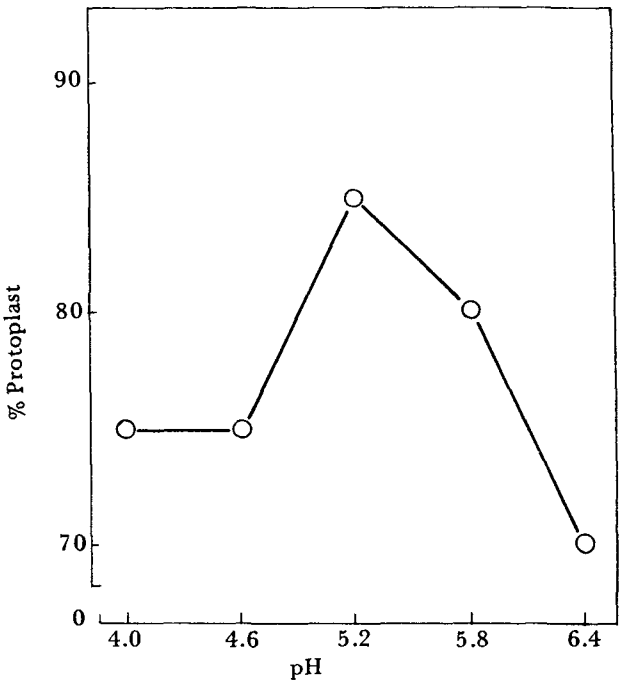

Fig. 8. Effect of pH of enzyme solution on isolation of protoplast from ginseng root cells treated with enzyme mixture containing 2% cellulase, 0.5% macerozyme and 0.9M mannitol for 7 hours.

concentrations of driselase and incubation periods. Thus yields of live protoplasts were highest using 1% driselase for 25 hours.

In general, several powerful enzymes are available for the isolation of protoplasts and the source of the cells or tissues used determines what enzyme is the best[1]. The most commonly used enzymes are cellulase Onozuka, driselase, and macerozyme. A low enzyme concentration increases the treatment time. Some cells can tolerate a long treatment period while others can not [10]. It is, therefore, necessary to determine the optimum concentration of the enzymes. Most previous investigators have included cellulase Onozuka ranging from 0.5[13] to 5.0%[20], macerozyme ranging from 0.02[9] to 1.0%[3], and driselase ranging from 0.5[31] to 2.0%[19] in the protoplast isolation mixture. In this investigation, it was found that a number of root cell protoplasts were obtained by an initial digestion with enzyme solution, containing 2% cellulase Onozuka R-10, 2% macerozyme R-10, and 1% driselase, for 25 hours.

3) Effect of incubation conditions during enzyme digestion on protoplast isolation

Experiments were conducted to establish optimal conditions of incubation during cell wall digestion. Stationary digestion of ginseng root tissues produced poorer yields of protoplasts and their formation occurred slowly. With agitation (35 rpm), the rate of protoplast release was accelerated. Higher agitation rates caused more protoplast lysis. Fig. 8 shows the effect of pH of enzyme solution, containing 2% cellulase Onozuka R-10, 0.5% macerozyme R-10, and 0.9M mannitol, on protoplast yield. Maximal rates of isolation of protoplasts were obtained over a pH range from 5.2 to 5.8. A pH below or above this range showed progressively and substantially lower yields of protoplasts. In general, protoplasts isolated at a lower pH were less viable than those obtained at a higher pH, according to ability to take up fluorescent vital stains[26]. For this reason, a pH of 5.2 to 5.8 was selected as being optimal.

The effect of mannitol as the osmoticum in the incubation medium was examined to determine optimal concentrations for the rapid isolation of protoplasts from ginseng root tissues. Highest numbers of live protoplasts were obtained

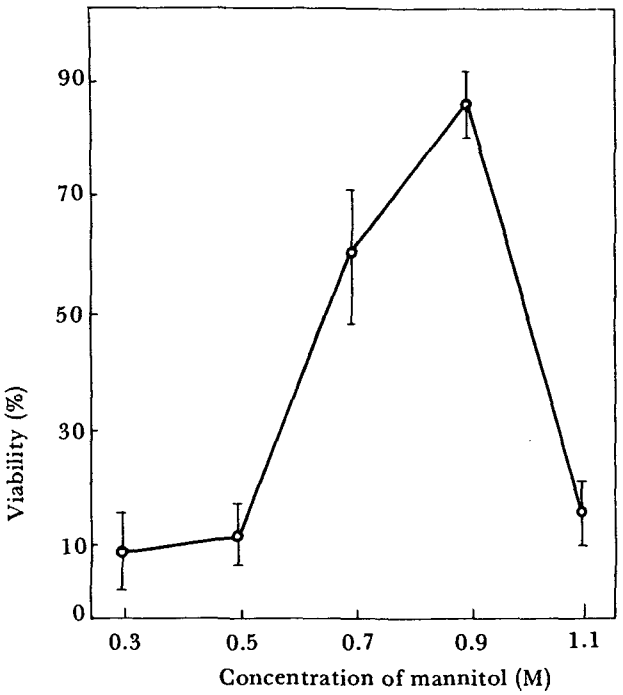

Fig. 9. Effect of mannitol concentration on the viability of protoplasts from ginseng root cells. Enzyme solution contained 0.5% macerozyme, 2.0% cellulase at pH 5.2 and the tissue was incubated for 8 hours in the dark.

when mannitol was present in the isolation medium at 0.9M, the live protoplasts representing then approximately 86.8% of those recovered (Fig. 9). Yields were reduced when the concentration of mannitol was less or greater than 0.9M. This reduction could be attributed to a progressive decline in both the proportion of protoplasts that were alive immediately after isolation and the total yields of protoplasts, the latter being associated with incomplete digestion of the tissue. In fact, optimum osmolalities have been demonstrated for the yields of protoplasts from several plant species[14-16,21,27], and indeed may vary with the conditions under which the plant material has been grown[15], the concentration of enzymes employed[17]. Up to date, either mannitol or sorbitol has been used as the osmoticum by many investigators. And the effects of mannitol and sorbitol are equally satisfactory[29]. Most investigators have included mannitol ranging from 0.3[23] to 0.9M[3] in the protoplast isolation mixture. This research with ginseng root cells disclosed that the 0.9M concentration of mannitol was retained as standard for the isolation of ginseng protoplasts.

Chang: Have you tried to culture the isolated protoplast?

Choi: Yes, we tried the isolation of protoplast and now we are carring out the culture and the specific protoplast fusion. I think further step for the elucidation of culture conditions for ginseng protoplast is the examination of cell wall regeneration, cell division, and regeneration from the protoplast callus.

고려인삼의 조직배양에 의한 기관형성과 원형질체배양에 관한 연구

최광태, 양덕춘, 김남원, 안인옥
한국인삼연초연구소

인삼의 대량증식 방법과 원형질체배양기술을 개발하기 위한 연구의 일환으로써 인삼의 자엽 callus 조직에서 많은 유식물체를 생산하는데에 미치는 식물생장조절물질의 영향을 구명하고, 또한 일반 식물과는 전혀 다른 인삼의 생존력이 있는 원형질체를 나출하여 배양할 수 있는 방법을 확립하고저 수행하였던 바 그 결과를 요약하면 다음과 같다.

(1) Murashige & Skoog(MS) 배양기조성 성분의 반량(½MS)에 2,4-D 0.5㎎/ℓ Kinetin 0.5㎎/ℓ 를 첨가한 배양기에서 가장 많은 양의 배상체가 유기되었다.

(2) 유기한 배상체를 동일배기에서 계속 배양할 경우에는 다시 탈분화되는 경향을 보였으나 ½MS 배양기에 BA와 GA를 같은 농도(BA : GA=1 : 1)로 첨가한 배양기에 옮겨서 배양하였던 바 배상체의 발육이 진전되어 유식물체로 발달하였다.

(3) 인삼은 1년근 세포에서 원형질체를 나출할 경우의 최적요건은 효소로서 cellulase 2%, macerozyme 0.5% 였으며, osmoticum으로서는 mannitol 0.9 M, pH 5.2였고, 처리기간은 28±1℃에서 7~8시간이었다.

(4) 인삼 callus조직세포에서 원형질체 나출할 시에는 cellulase 2%, macerozyme 2%, driselase 1%씩 첨가한 효소용액으로 28±1℃에서 25시간 이상 처리하는 것이 나출원형질체의 수와 생존율면에서 볼 때 가장 적합한 것으로 사료되었다.

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