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Studies on Culture of Cells and Tissues of Crop Plants

I. Survey on enzymatic isolation and culture of rice leaf sheath protoplasts

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Abstract : To establish a method for isolation and culture of rice protoplasts, enzyme solution compositions, culture conditions, and the selection of cultivars and tissues as materials were examined. The results suggested that the 1st and 2nd leaf sheath of 6-day old seeldings, which were cultured in the medium containing only minerals and vitamines of MS medium under 23°C, 16.2 W/m², and light length 14 h, were found suitable as materials. The materials were treated without shaking for 4 h in an enzyme solution (pH 5.6) containing 0.25% macerozyme R-10, 1% cellulase "Onozuka" R-10 and 1% BSA in a MS medium which contained MS-minerals, MS-vitamines, 1.0 mg/*l* 2, 4-D, 2.5 mg/*l* kinetin and 10 g/*l* sucrose. The isolated leaf sheath protoplasts were cultured in a MS medium (0.42 M) containing 1% BSA. All protoplasts from 11 cultivars kept their cell activity through the culture for 2 weeks or more. The addition of BSA in both the enzyme solution and culture medium was especially effective for the maintenance of protoplast viability. The vacuoles developed in the protoplasts during the culture period ; after 5 days in the culture the protoplasts enlarged to a diameter 1.75 times greater compared with that just after the isolation. The chloroplasts in protoplasts began to dedifferentiate during the culture, and both chloroplast size and chlorophyll content decreased. Protoplast division was not observed in this medium throughout the culture process.
Key words : BSA, Cell activity, Cellulase "Onozuka" R-10, Protoplasts, MS-medium, Rice leaf sheath.

作物の細胞組織培養に関する研究　第1報　イネの葉鞘プロトプラストの単離と培養に関する基礎的研究：大島雅子・遠山　益（お茶の水女子大学理学部）
要　旨：イネのプロトプラストの単離法と培養法を確立するために，酵素液の組成，培養の諸条件，材料として用いる組織と品種の選択などを詳細に試験した．その結果，播種6日後の幼植物の第1及び第2葉鞘が適当な材料であった．MS 培養液の中に 0.25%マセロザイム R-10，1%セルラーゼ "オノヅカ" R-10，及び 1% BSA を含む酵素液（pH 5.6）中で，材料を 4 h 静置処理をした．MS 培養液中には標準 MS 培養液の無機塩類及びビタミン類，1 mg/*l* 2,4-D，2.5 mg/*l* カイネチン及び 10 g/*l* ショ糖を含んでいる．単離した葉鞘プロトプラストは 1% BSA を含む MS 培養液（0.42 M）で培養された．11 品種から単離したすべてのプロトプラストが，2 週間以上にわたって細胞活性を保持した．酵素液と培養液にそれぞれ 1% BSA を添加することは，プロトプラストの活性持続に特に有効であった．培養中にプロトプラスト内では液胞が発達するので，単離直後のものに比べて，5 日培養後のプロトプラストは 1.75 倍にも大きくなる．また，培養中にプロトプラスト内の葉緑体は脱分化を始め，クロロフィル含量と葉緑体の大きさが減少した．この培養液中ではプロトプラストの分裂増殖は観察されなかった．
キーワード：イネの葉鞘，MS 培養液，細胞活性，セルラーゼ "オノヅカ" R-10，BSA，プロトプラスト．

Establishment of methods for the isolation and culture of protoplasts is indispensable for the fundamental and applied studies of plant technology ; cell multiproduction, somatic cell hybridization, incorporation of organelles and genetic substances into the cells and their exclusion from the cells, etc. In dicotyledons such as tabacco, carrot and petunia etc, the methods was practically established, and the protoplasts are capable of the cell wall formation, cell division and even the regeneration of entire plants[18]. In contrast, in monocotyledons the isolation and culture of protoplasts was difficult. In recent years, monocotyledonous protoplasts were isolated from the leaf sheaths and leaves of very young rice and wheat[4,14,20]. These methods, however, should be further examined. Since 1986, rice plants have been regenerated from protoplasts taken from the calli of embryo and anther [7,15,16,19,22,23], because of the difficulty in isolating rice mesophyll protoplasts. The present stydy on the

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cells and tissues culture of crop plants began with a detailed examination of the factors and conditions in relation to the isolation and culture of the rice leaf sheath protoplasts.

Materials and Methods

Sixteen cultivars of *Oryza sativa* L. (Table 4) were used. Seeds were sterilized in 70% ethanol for 30 s and immersed in 0.5% sodium hypochlorite for 3—4 h. The seeds were germinated in the medium containing only minerals and vitamines of MS medium under 23°C, 16.2 W/m^2 and light length 14 h. The following tissues were used for protoplast isolation. Tissues a and b are small buds of 3-day old and 5-day old plants after sowing respectively. Tissues c and d located 3 cm from the base of the 1st leaf sheath of 7-day old and 10-day old plants respectively. Tissues e and f located 2.5 cm from the base and 5 cm from 2.5 cm respectively in the 2nd leaf sheath of a 15-day old plant. Small pieces (about 1 mm^2) of tissues mentioned above were treated in an enzyme solution (pH 5.6) containing 0.25% macerozyme R-10, 1% cellulase "Onozuka" R-10 and 1% bovine serum albumin (BSA) in a MS medium containing MS-minerals, MS-vitamines, 1.0 mg/*l* 2, 4-D, 2.5 mg/*l* kinetin and 10 g/*l* sucrose without aggitation for 4 h.

The protoplasts were separated by filtration through 200 meshes of nylon, washed two times in a mannitol solution of the same osmotic pressure as the enzyme solution and then centrifuged at 100× g for 15 min in the culture medium. The protoplasts were cultured at a density of 1—2×10^5 cells/m*l* without shaking. The medium was composed of MS medium containing 1.0 mg/*l* 2, 4-D, 2.5 mg/*l* kinetin and 10 g/*l* sucrose. The osmotic pressure was adjusted 0.42 M with mannitol. The culture was performed at 23°C in the dark. On the other hand, to prolong the cell activity, polyamines (L-arginine, putrescine, spermine and spermidine) and BSA were added in the enzyme solution and also in the culture medium. The assay of cell viability was examined using the fluorescene diacetate (FDA) method by Widholm [24]. Fluorescence was observed by means of an Olympus fluorescence microscope BH-RFL with exciting filter BG 12 and absorving filter Y495. The RNase activity in the protoplasts was assayed by the method of Altman et al.[1]. Protein content was measured using the method of Lowry et al.[12].

Results

Protoplasts from the 1st leaf sheath of a cultivar Honenwase were isolated by treatment in an enzyme solution finally adjusted 0.42 M with mannitol for 2.5 h. These protoplasts were 21.4 μm in average diameter and became 37.5 μm after 5-day culture. The vacuoles in protoplasts developed conspicuously during the culture. After 3-day culture budding of protoplasts, dedifferentiation of chloroplasts and aggregation of organelles in the protoplasts were frequently observed (Fig. 1). On the other hand, there were many protoplasts collapsed even after only a day culture. It is therefore necessary to establish a most suitable method for isolation and culture of rice protoplasts. The changes of protoplast viability and chloroplast size during the culture are shown in Fig. 2.

The effects of two kinds of enzyme solution, i.e. with or without 0.05% pectolyase Y-23, on the isolation and culture of rice leaf sheath protoplasts were examined. The treatment time for the protoplast isolation from the 1st leaf sheath took 2.5 h in the former and 4 h in

Fig. 1. Protoplasts isolated from the 2nd leaf sheath (tissue-e) of Honenwase and cultured for 3 days, during which a conspicuous aggregation of organelles was observed. Length of bar is 20 μm.

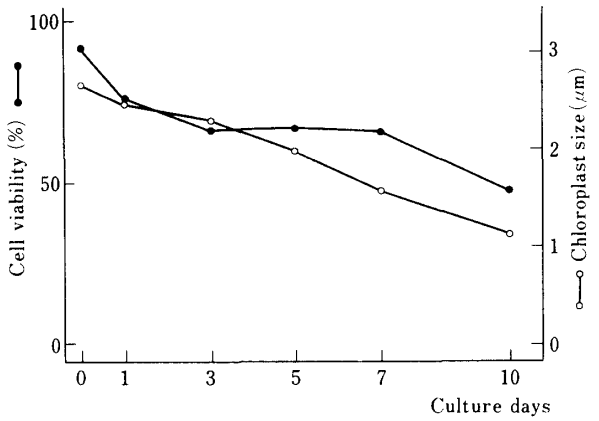

Fig. 2. Changes of the cell viability and chloroplast size of the protoplasts from tissue-d of Honenwase during the culture.

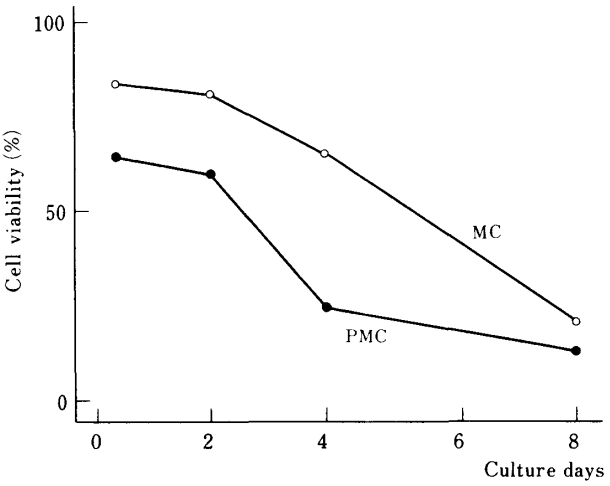

Fig. 3. Changes of the cell viability isolated with the treatment of different enzyme solutions from the tissue-c of Honenwase and followed by the culture in the MS medium containing 1% BSA. MC: the solution containing 0.25% macerozyme R-10 and 1% celluase "Onozuka" R-10, and PMC: 0.05% pectolyase Y-23, 0.2% macerozyme R-10 and 1% celluase "Onozuka" R-10.

the latter. Protoplast yield was higher in the former; however, the level of cell activity during the culture was higher in the latter (Fig. 3). The effect of osmotic pressure on the protoplast viability was compared between 0.42 M and 0.6 M of culture medium. In the former, 25% of protoplasts retained the viability even after 9-day culture, but in the latter 90% of protoplasts lost the activity after only

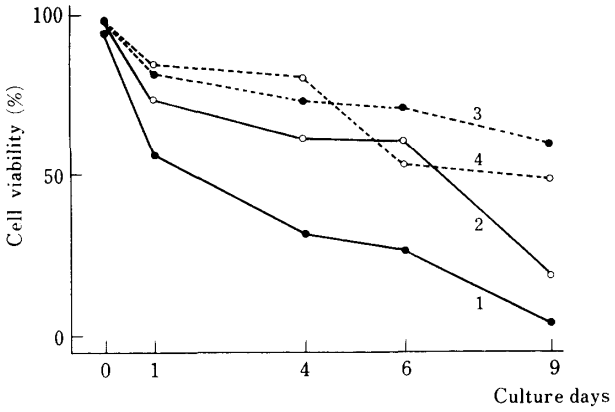

Fig. 4. Changes of the cell viability isolated with the treatment of enzyme solutions containing different osmotica from the tissue-c of Honenwase and followed by the culture in the MS medium containing 1% BSA. 1) : adjusted the osmotic pressure of enzyme solution with only mannitol, 2) : with mannitol, MS-minerals and MS-vitamines, 3) : with 2)-components, 2, 4-D and kinetin, 4) : with 3)-components and sucrose.

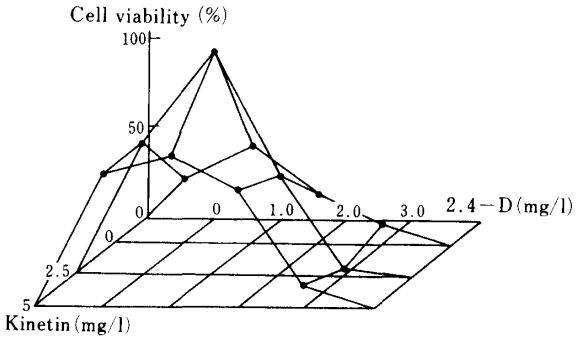

Fig. 5. Cell viability after 9 day culture of the protoplasts isolated from the tissue-b of Honenwase in the media combinated with the different concentration of 2, 4-D (0—4.0 mg/*l*) and kinetin (0—5.0 mg/*l*).

3-day culture. When the osmotic pressure of the enzyme solution was adjusted with the mixture of four kinds of osmotica; mannitol, minerals, vitamines and sucrore, the isolated protoplast viability was durable for longer period than that adjusted with only mannitol through the subsequent culture (Fig. 4).

The concentration of hormones added in the enzyme solution and culture media was examined. The most effective combination of 2, 4-D (0—3.0 mg/*l*) and kinetin (0—5.0 mg/*l*) concentrations for the durability of cell

Table 1. Effects of polyamines on the viability of protoplasts isolated from different leaf sheaths (tissues a～f) of 4 cultivars during the culture.

Cultivar	Tissue	L-arginine	Putrescine		Spermine	
		10 mM	2 mM	10 mM	2 mM	10 mM
Honenwase[1)]	a	31.7	100.1	82.4	0	0
〃	c	177.3	—	14.0	—	—
〃	d	117.0	39.8	0	—	—
〃	e	79.1	20.9	0	0	0
〃	f	47.8	85.2	48.7	0	0
Sasanishiki[2)]	d	125.0	—	0	—	—
Hatsukoshiji[2)]	d	112.8	—	10.0	—	—
Hastunemochi[2)]	d	77.3	—	52.0	—	—

Each number shows the relative value when the viabilty of protoplasts (FDA activity) cultured for 5 days without polyamines was regarded as 100 of value. [1)] : after 3-day culture, [2)] : after 10-day culture.

Table 2. Effects of L-arginine on the protein content in the protoplasts from different tissues of Honenwase after 3-day culture.

Tissue	Medium	0-day		3-day	
		Av	Rv	Av	Rv
1st leaf sheath (tissue-d)	MS medium	0.552	100	0.347	100
	MS medium+10 mM L-arginine	0.626	113	0.388	112
2nd leaf sheath (tissue-e)	MS medium	0.350	100	0.298	100
	MS medium+10 mM L-arginine	0.445	127	0.304	102

Av : absolute value, Rv : relative value.

activity was investigated. After 9 days, the highest cell activity was observed in the culture solution containing both 1.0 mg/*l* 2, 4-D and 2.5 mg/*l* kinetin (Fig. 5). Therefore, all subsequent experiments were performed with this combination of hormone concentrations.

As for the effect of four polyamines on the protoplasts viability, it was found that an addition of L-arginine to the enzyme solution and culture media within the range of 0 to 20 mM was effective in prolonging the activity of protoplasts taken from several cultivars. The effect, however, differed with the kind of cultivar, the aging of tissues and the period of protoplast culture, etc. (Table 1). Other polyamines proved to be useless in culturing rice protoplasts. The effects of L-arginine on the protein content were measured using the protoplasts from 1st and 2nd leaf sheaths of Honenwase. It had little effect on them both just after the isolation and after 3-day culture (Table 2).

The effects of BSA and L-arginine on the protoplast activity during culture were examined (Table 3). The application of 1% BSA was fully effective on the durability of protoplast activity in all cultivars used (Table 3). The protoplasts from the 2nd leaf sheath of Nihonbare and the 1st leaf sheath of Minaminishiki were collapsed more than 90% after 3-day culture without 1% BSA. The addition of BSA, however, made it possible to prolong the level of protoplast viability by more than 10 days. One percent of BSA was the most suitable concentration in the rice protoplast culture solution. The increase of RNase activity was inhibited by the addition of BSA in culture medium after 3-day culture of protoplasts from the 1st leaf sheaths of Hatsunemochi and Yamadanishiki (Table 3). On the other hand, L-arginine did not repress the RNase activity at all.

First and 2nd leaf sheaths of 16 cultivars (Table 4) were used to isolate the protoplasts. The protoplasts from 5 cultivars, namely, Honenwase, Yamabiko, Hatsukoshiji, Hatsunemochi and Yamadanishiki, kept the cell activity during 10 days or more culture after

Table 3. Effects of BSA and L-arginine on the RNase activity in the protoplasts isolated from 1st leaf sheaths (tissue-d) of 3 cultivars after 3-day culture.

Cultivar	Medium	RNase activity after 3-day culture	
		Av	Rv
Hatsunemochi	MS medium	0.300	100
	MS medium+1% BSA	0.011	4
Yamadanishiki	MS medium	0.734	100
	MS medium+1% BSA	0.072	10
	MS medium+3% BSA	0.275	37
Honenwase	MS medium	0.414	100
	MS medium+10 mM L-arginine	0.446	108

Av : absolute value, Rv : relative value.

Table 4. Cultivars and tissues used for protoplast isolation and effect of BSA on cell activity (esterase) during isolation and culture of protoplasts.

Enzymes	MC	MC+1% BSA
Treatment time	4 h	4 h
Culture medium	MS medium	MS medium+1% BSA
Cultivars		
Honenwase	+(c, d, e, f)	
Koshihikari	±(e)	+(d, e)
Gohyakumangoku	+(d)	+(d)
Nihongare	−(d, e)	+(e)
Yamabiko	+(d) ±(e)	+(e)
Minaminhishiki	−(d)	+(d)
Todorokiwase		+(d)
Reiho		+(d)
Sasanishiki	±(d)	
Hatsukoshiji	+(d) ±(e)	+(e)
Kuju		+(d)
Hatsunemochi	+(d) −(e)	+(e)
Yamadanishiki	+(d)	
Koshijiwase	±(d)	
Shimokita		+(d)
Kinmaze	±(d)	

MC : Macerozyme and cellulase "Onozuka" R-10. + : 30% of isolated protoplasts or more keeps their cell activity after 5-day culture and there are some protoplasts having the activity even after 10-day culture. − : About 5% of protoplasts keeps their activity after 5-day culture. ± : Intermediate of the above both. The alphabet in parentheses represents the plant tissues used.

the isolation. On the contrary, the protoplasts from Nihonbare and Minaminishiki were 5% and less active after 5-day culture, and their cell activity, however, was maintained for 10 days or more by adding 1% BSA. As shown in Table 4, without using BSA, the activity of isolated protoplasts was variable according to cultivars and tissues, and application of BSA made little differences of cell activity between cultivars or tissues of all 11 cultivars assayed. The development of vacuoles, aggregation of organelles and structural dedifferentiation of chloroplasts were observed in the protoplasts from all cultivars used during the culture.

Discussion

The isolation of *Gramineae* mesophyll protoplasts was very difficult. Sethi and Maeda isolated wheat mesophyll protoplasts through treatment with 0.1% pectoryase and 4%

cellulase[21]. The application of pectoryase in the case described in the present study was not suitable, although it shortened the treatment time necessary to isolate the protoplasts, because most of the isolated protoplasts became inactive. This problem should be carefully reexamined.

The osmotic pressure of a culture medium is an important factor in a protoplast culture. Pearce and Cocking[17] have observed that regeneration of the cell wall was possible after several day culture in the medium containing 12% sucrose ; however, it took several weeks in the medium containing 24% sucrose. The culture medium of 0.6 M mannitol, which is hypertonic to the protoplasts from Honenwase, caused the protoplasts to collapse during the culture.

It is known that polyamine compounds inhibit senescence of plant tissues, namely, increase of the activity of RNase and protease to promote the senescence[1,11], decrease of chlorophyll content, destruction of chloroplast ultrastructure[3], and the ethylen synthesis to expedite the senescence[5]. Altman *et al.*[1] reported that the application of polyamines to *Avena* protoplasts made their viability maintain and checked senescence progress. The use of polyamines in the present experiments, however, did not bring about significant results on the protoplasts culture, except for slight effects of L-arginine. This may be due to the plant species specificity and also the aging of tissues used.

Protoplast isolation from the rice blade is very difficult[14,20], except in the case of the leaf sheath of rice seedlings immediately after the germination. Chen cultured rice protoplasts from the leaf sheath in a P-2 medium containing 10% sucrose and observed dedifferentiation of protoplasts budding like "bursted ballons"[2]. As in the present study, budding protoplasts including the dedifferentiated chloroplasts were often observed during the culture. When protoplasts from the rice leaf sheath were cultured in a combination of three established media, Dek and Sen observed the first division after 5 days in culture and then observed callus formation. In the medium used in the present study, no protoplasts from the leaf sheath caused cell division. The difference on cell division may be attributed to the composition of culture medium and breeding cultivars and aging of rice plants[4].

For the disired redifferentiation of protoplasts and their regeneration of an entire plant, the selection of plant materials is also an important factor, as is the establishment of methods for the isolation and culture of protoplasts. Using the suspension culture cells of 25 rice cultivars, Yamada and Yo observed cell division in three cultivars, Akage, Norin-1 and Norin-22, and colony formation of two cultivars, Toyotama and Fujiminori. The division and redifferentiation of mesophyll protoplasts from the 5 cultivars mentioned above remain to be examined.

In recent years, instances of regeneration of rice protoplasts from the calli of an embryo and an anther, but not from mesophyll protoplasts, into an entire plant have been reported[7,15,16,19,22,23]. It is desirable that the plant is regenerated from the mesophyll protoplasts as in the case of tobacco and petunia, etc., because it takes for a long time for callus formation, during which mutation is liable to happen. In this experiment, culture of protoplasts from rice leaf sheath was possible over a three week period, but reproduction was not possible through cell division. At present, the redifferentiation of mesophyll protoplasts from expanded rice leaves is under examination.

The addition of BSA in both the enzyme solution and culture medium was very effective to keep the protoplasts from collapse. The medium containing BSA prolonged protoplast viability over a three week period. Yamada and Yo reported that the medium containing 0.8% BSA worked cell on the division of protoplasts from the rice callus[25]. Previous studies suggested that BSA is effective for the proliferation activity and serially transferred culture of animal cells[10]. It is generally accepted that albumin functions in the nutritional supply and in the protection of animal cells[9] ; maintenance of colloid osmotic pressure[13], supply of amino acids to peripheral system, and as a carrier of fatty acids[26], Ca^{2+}, tryptophan and glutathion etc.[6], although its primary function of albumin *in vivo* is not always clear. The satisfactory result from the experiments done using BSA in the present study may be attributed to : (1) The reduction in the rate of protoplast collapse will be dependent on the mechanical protection of

albumin which adheres to the outer surface of cell membrane. (2) The maintenance of protoplast viability seems attributed to the supply of nutrients into the cells by adding albumin, especially long-chained unsaturated fatty acids, oleic and linoleic acids, which are indispensable for the living cells. (3) BSA used in the present study, Cohn's fraction V, usually contains 1—4% of other serum proteins. Therefore the possibility can not be ingnored that favorable results from using BSA may be attributed to proteins other than albumin. (4) Since albumin has recently been identified as a carrier of the SH-radical[8], the relation between an albumin binding SH-radical and cell activity is attracting investigator's attention.

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