Callus Formation and Plant Regeneration from Petiole Protoplast of Sweet Potato, *Ipomoea batatas* (L.) LAM.

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Protoplasts were isolated from young petioles of sweet potato (var. Chugoku No. 25). The enzyme solution for maceration contained 0.1% Pectolyase Y-23, 2% Cellulase Onozuka RS, 5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.5 M mannitol. The crude protoplast suspension was filtered through a Miracloth and protoplasts were isolated by discontinuous density gradient centrifugation. As the initial plating medium for cell division, modified MURASHIGE and SKOOG (MS) medium was used. It contained 0.1 mg/l kinetin (KIN), 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 M mannitol and 10 g/l sucrose with modified concentrations of NH_4NO_3 and KNO_3 . After one month, these media were replaced with new ones with a concentration of mannitol reduced to 0.25 M. Further, after one more month, these media were replaced to those without any mannitol. The most efficient colony formation from protoplasts occurred on a medium containing 200 mg/l NH_4NO_3 and 1,000 mg/l KNO_3 . After about three months, the cell colonies were transferred to agar plates of modified MS media (800 mg/l NH_4NO_3 , 1,400 mg/l KNO_3) supplemented with 20 g/l sucrose 6 g/l agar and various combinations of growth regulators (2,4-D, KIN, abscisic acid (ABA)). Callus formation occurred more efficiently on media containing KIN than on media without KIN. Furthermore, the presence of ABA did not inhibit callus growth. The micro-calli were then transferred individually to MS media supplemented with 30 g/l sucrose, 8 g/l agar, and various concentrations of KIN and BA for regeneration. Shoot formation was induced only from the callus on a medium supplemented with 1.0 mg/l KIN, and only when preceded by the culture on a medium containing 0.5 mg/l 2,4-D, 1.0 mg/l ABA and 0.5 mg/l KIN.

KEY WORDS : *Ipomoea batatas* (L.) LAM., sweet potato, petiole protoplasts, protoplast culture, callus formation, organogenesis, plant regeneration.

Introduction

Sweet potato varieties are divided into sixteen groups according to cross sterility such that they are not able to cross with other varieties of the same group. Moreover, it was very difficult to induce compatibility by bud pollination, old flower pollination or, further, by various physical and chemical treatment of stigma before pollination (FUJISE 1964). On the other hand, recent studies have shown that the natural barriers of sexual incompatibility of several crops can be overcome by somatic hybridization through protoplast fusion. If somatic hybridization among varieties of the same cross sterile group can be accomplished, it will offer new possibilities for advancement in sweet potato breeding.

Although there is only limited documentation of protoplast experiments with the sweet potato, as yet no shoot formation or embryogenesis has been reported. Wu and MA (1979) reported successful isolation of protoplasts from stem calli of the sweet potato. Protoplasts which were cultured on solid agar medium supplemented with

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growth regulators, developed into large calli. BIDNEY and SHEPARD (1980) cultured petiole protoplasts of the sweet potato and observed sporadic root development from protoplasts derived from calli, but neither embryogenesis nor shoot morphogenesis occurred.

Recently, we succeeded in the regeneration of a plant from petiole protoplasts of the sweet potato. In this paper, we present the procedure for the isolation, culture and production of callus, and organogenesis.

Materials and Methods

Plant materials : Sweet potato variety Chugoku No.25 was used in this experiment. Protoplasts were isolated from young petioles of sweet potato which were grown in a greenhouse.

Enzyme solution : The enzyme solution for maceration of petioles contained 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd.), 2% Cellulase Onozuka RS (Kinki Yakult Mfg. Co., Ltd.), 5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.5 M mannitol. The pH of this solution was adjusted to 5.8 with 0.1 N NaOH. The enzyme solution was sterilized by filtration through a 0.22 μm membrane filter.

Isolation procedure : Petioles of 3~5 cm long were excised from the plants. These were immediately sterilized in 70% ethanol for 15 seconds, then in 1% sodium hypochlorite solution for 10 minutes and rinsed with sterile distilled water. The sterilized petioles were then cut into thin pieces with a razor blade. Petiole pieces of about 1 gram in fresh weight were soaked in 10 ml of enzyme solution in a screw-capped 200 ml flask. The flask was evacuated for 7 minutes in a vacuum desiccator and it was shaken for about 4 hours at 28°C on a reciprocating shaker (20 strokes/minute).

Following enzyme treatment, the crude protoplast suspension was filtered through a Miracloth (Size 1, American Hoeschst Co., Ltd.) and the filtrate was centrifuged at $100\times g$ for 5 minutes. The precipitate then was washed three times in 0.5 M mannitol solution, and finally a 0.4 M sucrose and 0.1 M mannitol solution was added to the precipitate. This suspension was layered carefully over 5 ml of 0.5 M sucrose solution and then both layers were covered with 2 ml of 0.5 M mannitol solution. Afterwards, protoplasts were separated by centrifugation at $150\times g$ for 5 minutes, and they were extracted by pasteur pipette.

Protoplast culture : A modified MS medium (MURASHIGE and SKOOG 1962) was used as the initial plating medium. It contained 0.1 mg/l kinetin (KIN), 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 M mannitol and 10 g/l sucrose with concentrations of NH_4NO_3 and KNO_3 modified as shown in Table 1 (Medium A). All protoplasts were cultured in a thin layer of liquid medium at a density of $10^4 \sim 2.5 \times 10^4$ cells/ml. The culture dishes (60 mm) were sealed with Parafilm and placed in a humid plastic box at 28°C under 600 Lux continuous light conditions with white fluorescent lamps. One month after plating, these media were replaced by new ones with the concentration of mannitol reduced by 0.25 M. Further, after one more month, these media were again replaced, this time without any mannitol. Three months after plating, the cell colonies were transferred to agarplates of modified MS medium (800 mg/l NH_4NO_3 ,

1,400 mg/l KNO_3) containing 20 g/l sucrose, 6 g/l agar and various combinations of growth regulators (2,4-D, KIN and abscisic acid (ABA)) as shown in Table 2 (Medium B). One month after transferring Medium B, the calli were transferred individually to test tubes which contained MS medium supplemented with 30 g/l sucrose, 8 g/l agar and various concentrations of KIN and 6-benzylaminopurine (BA) as shown in Table 3 (Medium C). These test tubes were kept at 28°C with a 16-h day length at 2,500 lux.

Results

Freshly-isolated protoplasts were spherical with dense cellular content (Fig.1.A). The 25 combinations of concentrations in NH_4NO_3 and KNO_3 were tested for comparison on initial culture media (Table 1). The first cell division of protoplast occurred about 12 days after plating (Fig.1.B). Thereafter, cell clusters consisting of 10~20 cells were formed about 20 days after plating (Fig.1.C), and then visible cell clusters about 1 mm in diameter were formed for a period of one month after plating (Fig.1.D). However, when the number of cells did not increase within one month after plating, these media were replaced with new ones containing a concentration of mannitol reduced by 0.25 M. Further, after one more month, these media were again replaced, this time without any mannitol. About three months after plating, it was observed that cell colonies of the same size (2~3 mm in diameter) had formed only on Medium A which contained 200 mg/l NH_4NO_3 and 1,000 mg/l KNO_3 . These colonies were transferred

Table 1. Effects of NH_4NO_3 and KNO_3 concentration on cell division of protoplast

KNO_3 (mg/l)	NH_4NO_3 (mg/l)				
	0	200	400	800	1,200
0	+	++	+	+	++
200	+	+	++	++++	++
500	+	+	+	+	+
1,000	++	++++	++++	++++	+
1,400	—	++	++++	++++	+

— : no division

+: 2~5 cell divisions

++ : 6~10 cell divisions

+++ : cell cluster formation (about 1 mm in diameter)

++++ : cell colony formation (2~3 mm in diameter)

Cultured for 60 days

Table 2. The degree of callus formation on Medium B¹⁾

Concentration of growth regulator (mg/l)			Degree of callus formation ²⁾				Color of callus ³⁾
2,4-D	ABA	KIN	±	+	++	+++	
0.5	0.1		4	5	0	0	B
0.5	1.0		6	3	0	0	B
0.5	5.0		4	5	0	0	B
0.5	10.0		8	1	0	0	B
0.5	0.1	0.5	0	3	6	0	Y, W
0.5	1.0	0.5	0	0	8	1	G, W
0.5	5.0	0.5	0	0	10	0	Gr
0.5	10.0	0.5	0	1	9	0	Gr
1.0	0.1		3	6	0	0	B
1.0	1.0		5	4	0	0	B
1.0	5.0		6	3	0	0	B
1.0	10.0		5	4	0	0	B
1.0	0.1	0.5	0	8	1	0	Y, W
1.0	1.0	0.5	2	7	0	0	Y, G
1.0	5.0	0.5	0	0	9	0	G, W
1.0	10.0	0.5	0	0	9	0	Y, W

¹⁾ Modified MS medium (800mg/l NH_4NO_3 , 1,400 mg/l KNO_3) with addition of 20 g/l sucrose, 6 g/l agar

²⁾ ± : <3 mm, + : 3~5 mm, ++ : 6~9 mm, +++ : >10 mm

³⁾ B : brown, Y : yellow, W : white, G : gray, Gr : green

Fig.1. Plant regeneration from petiole protoplasts of sweet potato.

A : Isolated protoplasts from petiole ($\times 200$). B : First cell division ($\times 200$).
 C : Cell cluster ($\times 200$). D : Cell colony ($\times 100$). E : Callus formation from colonies
 F : Shoot formation from callus. G : A plant induced by protoplast culture

Table 3. Effect of growth regulators on organ formation¹⁾ and callus growth²⁾

Medium for callus formation (Medium B)			Medium for regeneration (Medium C)								
Concentration of growth regulator (mg/l)			KIN (mg/l)					BA (mg/l)			
2,4-D	ABA	KIN	0	0.5	1.0	2.0	5.0	0.5	1.0	2.0	5.0
0.5	0.1		+	+	+	+	±	+	+	±	+
0.5	1.0		±	±	±	+	±	±	±	±	±
0.5	5.0		+	±	±	±	+	±	±	±	+
0.5	10.0		±	±	±	+	±	±	±	+	+
0.5	0.1	0.5	+++ (R)	+++ (R)	++	+++	+++	+++ (R)	+++	+++	+++
0.5	1.0	0.5	++ (R)	+++	+++ (S, R)	+++	+++ (R)	+++ (R)	+++	+++ (R)	+++ (R)
0.5	5.0	0.5	+++ (R)	++ (R)	+++ (R)	+++	+++	+++ (R)	+++	+++ (R)	+++
0.5	10.0	0.5	++	+++	+++	+++ (R)	+++	+++ (R)	+++	+++	+++
1.0	0.1		±	±	+	+	±	±	±	±	+
1.0	1.0		±	±	±	+	+	±	±	±	±
1.0	5.0		±	+	±	±	±	±	±	±	+
1.0	10.0		±	+	+	±	±	±	±	+	±
1.0	0.1	0.5	++ (R)	+	++ (R)	+++	++ (R)	++	++	++	++
1.0	1.0	0.5	+	±	+	+	+	+	±	±	+
1.0	5.0	0.5	+++ (R)	+++	+++ (R)	++	++	+++	+++	+++ (R)	+++
1.0	10.0	0.5	+++ (R)	+++	+++	+++	+++ (R)	+++ (R)	+++	+++	+++

¹⁾ (S) Shoot bud, (R) Root ²⁾ ± : <3 mm, + : 3~5 mm, ++ : 6~9 mm, +++ : >10 mm

to secondary media (Medium B) to continue proliferation of the calli. As shown in Table 2, callus formation on the media containing KIN occurred more efficiently than on media without KIN, and the presence of ABA did not inhibit callus growth (Fig. 1. E). The color of calli cultured on media containing KIN was yellow, white, gray or green, but it was brown for calli on media lacking KIN.

One month after transferring Medium B, for regeneration, the micro-calli (6~10 mm in diameter) were transferred individually to MS medium containing 30 g/l sucrose and various concentrations of KIN and BA as shown in Table 3. The medium for callus formation (Medium B) influenced callus growth, root and shoot formation on Medium C. Shoot formation was induced only from the callus on the Medium C supplemented with 1.0 mg/l KIN, and only when preceded by the culture on the Medium B containing 0.5 mg/l 2,4-D, 1.0 mg/l ABA and 0.5 mg/l KIN (Fig. 1. F). Root formation was observed on media with various concentrations of KIN and BA as shown in Table 3. Regenerated plant was then transplanted to soil and grown to maturity (Fig. 1. G).

Discussion

Generally, the salts of MS (MURASHIGE and SKOOG 1962) and B5 (GAMBORG *et al.* 1968) medium and their modifications have been used for protoplast culture media.

It was proposed that concentrations of iron, zinc and ammonium ion in these culture media may be too high for protoplast culture (VON ARNOLD and ERIKSSON 1977). Ammonium ion, especially, has a deleterious effect on protoplast culture for some plant species (UPADHYA 1975, OKUMURA *et al.* 1984). With the sweet potato, BIDNEY and SHEPARD (1980) cultured petiole protoplast on ammonium-free medium by using the cell layer-reservoir system. However, NISHIMAKI and NOZUE (1985) reported that protoplasts from callus of sweet potato were cultured in the presence of ammonium salt (200 mg/l $(\text{NH}_4)_2\text{SO}_4$ in PRL-4-C basal medium), and the reduction or deficiency of this salt did not largely affect the cell division of protoplasts. On the other hand, KLIMASZEWSKA and KELLER (1985) reported that shoot organogenesis in thin cell layers of *B. napus* was strongly effected by the $\text{NO}_3^-/\text{NH}_4^+$ ratio.

In our experiment, modified MS media containing various concentrations of NH_4NO_3 and KNO_3 were used. As a result, the most rapid growth was achieved in the medium containing 200 mg/l NH_4NO_3 and 1,000 mg/l KNO_3 . These cell clusters were transferred to agar plates of modified MS medium containing various concentrations of ABA. ABA is generally regarded as being a plant growth inhibitor, perhaps because its activity is contrary to that of cytokinins and gibberellins. Several investigators have shown, though, that ABA may enhance the embryogenic potential of cultured plant tissue. YAMAGUCHI and NAKAJIMA (1972) and YAMAGUCHI (1978) concluded that ABA acted antagonistically on endogenous cytokinins in cultured tissue derived from the root tuber of sweet potato and promoted adventitious bud formation. Furthermore, SHEPARD (1980) reported that ABA enhanced shoot initiation in protoplast-derived calli of potato. INOUE and MAEDA (1981), on the other hand, suggested that the roles of ABA in the preculture medium and KIN in the last culture medium enhanced organogenesis.

As mentioned above, a shoot formation was induced from the callus on the medium supplemented with 1.0 mg/l KIN, when preceded by the culture medium containing 0.5 mg/l 2,4-D, 1.0 mg/l ABA and 0.5 mg/l KIN. The effect of ABA is not clear from the results of this experiment, but it would be interesting to investigate the relationship between ABA and the other growth regulators, for it may also prove useful to add ABA to the medium for promotion of shoot formation. It is necessary to examine a more effective culture medium for embryogenesis and shoot formation.

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サツマイモの葉柄プロトプラストからのカルス形成および植物体再生

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サツマイモには, 16 群に及ぶ交配不和合群が存在し, 同一群内では交配が不可能である。もし, 同一群内における体細胞雑種が可能になれば, サツマイモの育種に新たな展開をもたらすと思われる。そこで, 本実験において, サツマイモの葉柄プロトプラストの単離とその培養を試みた。

温室で育成したサツマイモ品種「中国 25 号」の葉柄を供試材料とした。採取した葉柄を殺菌した後細切し, 酵素液 (0.1% ペクトリナーゼ Y-23, 2% セルラーゼ・オノヅカ RS, 5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 M マニトール) で減圧処理後, 28°C, 20 回/分で約 4 時間振とうすることにより処理を行った。10 ml の酵素液に対し 1 g の葉柄を用いた。その後ミラクロスでろ過し, 3 回 0.5 M マニトール液で洗浄した。プロトプラストを純化するために, 遠心管に 0.5 M シュ糖液を入れ, その上に 0.4 M シュ糖液, 0.1 M マニトール液の混合液に懸濁したプロトプラストを静かに重ね, さらにその上に 0.5 M マニトール液をのせ, 遠心をかけた。プロトプラストは緑色の薄層に分離するので, これをパスツールピペットでとり培養に用いた。

プロトプラストの培養は, $10^4 \sim 2.5 \times 10^4$ 個/ml の密度で, MS 培地の NH_4NO_3 と KNO_3 を種々の濃度で組み合わせ, 0.1 mg/l KIN, 0.1 mg/l 2,4-D, 0.5 M マニトール, 10 g/l シュ糖を添加した液体培地 (Medium A) で, 弱光, 28°C 下で行った。培養期間中, 約 30 日間隔でマニトールの濃度を 0.25 M, 0, と順次低下させた新鮮な培地を追加し約 3 カ月間培養を行った。その結果, 200 mg/l NH_4NO_3 , 1,000 mg/l KNO_3 添加区でのみ, コロニーが形成された (Table 1)。

次にコロニーを, 修正 MS 培地 (800 mg/l NH_4NO_3 , 1,400 mg/l KNO_3) に 20 g/l シュ糖, 6 g/l 寒天, および種々の濃度の 2,4-D, ABA, KIN を組み合わせて添加した培地 (Medium B) に移植した。その結果, KIN の添加区でカルスの形成が良好であった (Table 2)。次に, これらのカルスを MS 培地に 30 g/l シュ糖, 8 g/l 寒天および種々の濃度の KIN, BA を単独で添加した培地 (Medium C) に移植した。このような培

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養法によっていくつかの区で不定根が形成されたが、不定芽は、0.5 mg/l 2,4-D, 1.0 mg/l ABA, 0.5 mg/l KIN 添加区で形成されたカルスを、さらに 1.0 mg/l KIN 添加区に移植した場合のみ 1 個体分化した (Table 3, Fig. 1 F, G).