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Plant regeneration from protoplasts of hydroxyproline resistant cell line in *Onobrychis viciaefolia*

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ABSTRACT

An efficient protocol for plant regeneration from protoplasts of hydroxyproline(HYP)resistant cell line of *Onobrychis viciaefolia* was established. In SH medium supplemented with 1 mg /L 2, 4-dichlorophenoxy-acetic acid (2, 4-D), 0.5 mg/L kinetin (KT) and 0.2 mg/L naphthalene acetic acid (NAA), the division frequency of protoplast-derived cells reached up to over 60 %, and microcalli were obtained in 5-6 wk. Upon transferring them on agar solidified MS medium plus 2 mg /L indole-3-acetic acid (IAA), shoots were induced. After cultivating them on MS medium with or without IAA, roots were regenerated. Chromosome number of all protoplast-regenerated plants examined were normal (2n=28). The protoplast-derived calli and plants grew vigorously on the medium containing 10 mmol /L HYP.

Key words: *Sainfoin (Onobrychis viciaefolia), hydroxyproline resistant cell line, protoplast culture, plant regeneration.*

INTRODUCTION

Sainfoin (Onobrychis viciaefolia Scop.) is a forage legume growing in arid area. It combines drought resistant and higher palatability with valuable protein and non-bloating properties. Such species is an ideal partner for somatic hybridization with other forage legume such as alfalfa[1]. Although plant regeneration from sainfoin protoplast has been reported[2-3], no genetic markers in this species could be used

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for identification of somatic hybrid cells.

A stable HYP resistant cell line of sainfoin has been selected in our laboratory[4]. The calli have been subcultured for 16 months on MS medium without hydroxyproline. The present work described the conditions for protoplast isolation, culture and plant regeneration from protoplasts of this selected HYP-resistant cell line.

MATERIAL AND METHODS

Resistant cell line and its maintenance

A stable cell line of sainfoin (Onobrychis viciaefolia Scop) resistant to 10 m mol/L hydroxyproline (HYP)[4] was used as a initial material. Callus cultures of this cell line were subcultured at 3 - 4 week interval on MS medium[5] supplemented with 3% sucrose, 500 mg /L casein hydrolysate (CH), 1 mg /L 2, 4-D and 0.5 mg /L 6-BA.

Protoplast isolation

Protoplasts were isolated enzymatically from 9-10 d old calli of HYP resistant cell line. Usually 1.5 g callus was incubated in 10 ml enzyme solution on a shaker (50 rpm) at 25℃±1℃ for 6 h in dark. The enzyme solutions used are given in Tab 1. After digestion, the protoplast suspension was passed through 80μm nylon mesh, and 2X volume of 0.16 mol/L $CaCl_2.2H_20$ solution (PH 5.8 - 6.2) was added. Protoplasts were collected by centrifugation at 800 rpm for 10 min. The sedimented protoplasts were purified in 18 % sucrose solution by centrifugation for 5 min at 600 rpm. The floating protoplasts were sucked out with a pipette and placed to a new centrifuge tube. After a wash of protoplasts with protoplast culture medium (Tab 2) by centrifugation, they were used for culture.

Tab 1. The effects of different enzyme compositions on protoplast yield and viability*

No. of enzyme solution	Enzyme combination	Yield of protoplasts (cells / g .f.w)	Viability of protoplasts (%)
1	2% cellulase(Onozuka R-10) + 0.8% macerozyme R-10 + 1% hemicellulase(Sigma)	4.63×10^6	81.2
2	2% cellulase(Onozuka R -10) + 0.8% macerozyme R -10	3.52×10^6	75.9
3	1% cellulase(Onozuka R -10) + 0.5% pectinase(Serva)	5.14×10^6	79.7
4	1% cellulase(Onozuka R- 10) + 0.5% pectinase(Serva) + 0.2% hemicellulase(Sigma)	5.86×10^6	85.6

* All enzyme were prepared in the solution composed of 0.45 mol/ L mannitol and 0.015 mol / L CaCl2·2H2O, and pH was adjusted to 5.8-6.2.

Protoplast culture and callus formation

The collected protoplast pellets were resuspended in a defined volume of culture medium (Tab 2). The protoplast density was adjusted to about 5×10^5/ml. Viability of protoplasts was examined by staining with 0.1% phenosafranin solution(in 0.45 mol/L mannitol). 2 ml protoplast suspension solution was dispersed to a Petri dish (6 cm diameter) . The dishes were kept for 48 h at 4℃ , then moved to the culture chamber at 25℃ ± 1℃ in dark.

The division frequency of protoplasts was calculated after 8 d in culture. The plating efficiency was counted at 20 d of culture.

In order to stimulate sustained divisions and colony formation, freshly prepared medium with reduced osmoticum concentration was added after 10 and 20 d in culture. The visible colonies (1-2 mm in size) derived from protoplasts were transferred onto MS medium (Tab 2) to form callus.

Tab 2. Media for protoplast culture and callus formation

Supplements in media	Basal media		
	SH[6]	V-KM[7]	MS[5]
sucrose(g/L)	20	20	30
glucose(g/L)	81(0.45 mol/L)	20	0
CH(mg/L)	200	200	500
glutamine(mg/L)	500	500	0
HYP(mg/L)	200	200	0
2, 4-D(mg/L)	1	1	1
6-BA(mg/L)	0	0.5	0.5
zeatin(mg/L)	0	0.2	0
NAA(mg/L)	0.2	0.2	0
KT(mg/L)	0.5	0	0
agar(%)	0	0	0.7

Plant regeneration

Proliferating calli (ca. 4 mm in size) were transferred on MS medium with 3% sucrose, 500 mg/L CH, 2 mg/L 6-BA in combination with 0.2 mg/L NAA or IAA to induce shoot differentiation. All cultures for differentiation were incubated at 26℃/20℃ day/night) under illumination of 2000 lux fluorescent source 12 h per day). When shoots grew up to 4-5 cm high, they were cut and transplanted on MS medium with or without 1 mg /L IAA to induce roots.

Chromosome count

Root tips of protoplast-regenerated plants were cut and pretreated for 2 h in saturated p-dichlorobenzene solution, then washed thoroughly with distilled water for 4-5 times. The pretreated root tips were fixed in a mixture of ethanol and acetic acid (3:1, v/v) for 1-24 h, and hydrolyzed in 1 N HCl at 60℃ for 10-15 min. After rinsing with distilled water, the root tips were stained in 0.5% haematoxylin solution for 6 h at room temperature. After squashed, the chromosome numbers were counted under microscope.

Tests of resistance to HYP and sodium chloride (NaCl)

The protoplast derived calli were transferred on MS medium containing HYP or NaCl, and cultured for one month to observe their tolerance to HYP or NaC1. 20 regenerated plants were transferred on MS medium containing 10 m mol/L HYP or 1.0% NaCl to test their resistance.

RESULTS AND DISCUSSION

Isolation and purification of protoplasts

For protoplast isolation, different enzyme solutions were tested. As shown in Tab 1, a large number of protoplasts could be released from all enzyme solutions used. However, the yields of protoplasts were obviously higher in solution No 3 and No 4 than that in No 1 and No 2. Combinations of cellulase R-10(Yakult co. ltd) and pectinase, such as macerozyme R-10 (Yacult co. ltd) and pectinase(Serva) were suitable to the isolation of O. viciaefolia protoplasts, but pectinase(Serva) was much more effective. Hemicellulase(Sigma) could increase protoplast yield obviously. After purification with 18% sucrose solution, protoplast yield in solution No 4 was 5.86 x 10^6 per g.f.w. The rate of viable protoplasts isolated from enzyme No 4 was also relatively higher (over 85%). Besides, protoplast yields and viability strongly depeneded upon the states of source callus used for protoplast preparation. Only those calli which were in fast growing, i.e., 9 - 10 day old cultures after subculture on fresh medium, could give rise to higher yield and vigorous protoplasts.

The freshly isolated protoplasts were spherical with various size ranged from 15-25 μ m in diameter (Fig 1).

Protoplast culture and callus formation

Using liquid thin layer culture method and at given conditions as described above, the first division usually occured within 4-5 d of protoplast culture (Fig 2). The second to fourth divisions were followed after 10 d in culture (Fig 3). Meanwhile, both equal and unequal divisions were observed. Regular dilutions of protoplast cultures by adding fresh liquid medium with reduced osmotic pressure enhanced the growth of cell clusters and colony formation. When 0.5 ml fresh inedium with half stretlgth of osmoticum (0.225 mol/L glucose) was added to the cultures at 10th day and 20th day of culture, cell clusters and colonies successfully grew (Fig 4). Numerous colonies visible to naked eyes were obtained within one and half month since protoplast inoculation (Fig 5). It was beneficial to improve oxygen condition by shaking the culture dishes twice a day.

When colonies reached to 1-2 mm in size, they were transferred to agar-solidified MS medium with lmg/L 2, 4-D and 0.5 mg/L 6-BA (as shown in Tab 2) and subcultured twice at 3-week interval. This made the colonies proliferate and callus be formed (Fig 6),

The effects of several factors on protoplast culture were compared. Tab 3 indicated the influences of protoplast density and two kinds of basal media on cell division. It was observed that SH medium[6] was apparently preferable to V-KM medium[7] for culturing sainfoin protoplasts. The highest division frequency and plating efficiency were obtained when SH medium was used and the protoplast density was 4.0-5.0 x 10^5/ml. As to osmoticum, perfect results were obtained when 0.45 mol/L glucose in combination with 2% sucrose was used as osmotical regulator

(Tab 4). The results from Tab 4 suggested that no clear difference was observed in cell division frequency whatever glucose or mannitol was used Whereas, the plating efficiency was much higher when using glucose (5.2%).

Tab 3. Effects of basal medium and protoplast density on protoplast culture

Medium*	Protoplast density	Division frequency (%)	Plating efficiency (%)
SH[6]	1.0×10^5/ml	8.5	2.3
	2.0×10^5/ml	19.4	2.6
	4.0×10^5/ml	57.1	5.7
	5.0×10^5/ml	60.2	4.9
V-KM[7]	1.0×10^5/ml	3.3	
	2.0×10^5/ml	17.6	3.8
	4.0×10^5/ml	55.5	4.2
	5.0×10^5/ml	52.8	3.4

* All media were supplemented with 2% sucrose, 0.45 mol/L glucose, 200 mg/L CH, 500 mg/L glutamine, 1 mg/L 2, 4-D, 0.5 mg/L KT and 0.2 mg/L NAA.

Tab 4. Influence of glucose and mannitol as medium(SH) osmoticum to protoplast culture*

Osmoticum	Protoplast density	Frequency of cell division (%)	Plating efficiency (%)
glucose(0.45mol/L)	5.0×10^5/ml	56.9	5.2
mannitol(0.45mol/L)	5.0×10^5/ml	55.5	1.4

* The media were supplemented with the same amount of CH, glutamine, 2, 4-D, KT and NAA as shown in Tab 3. Plating efficiency was the percentage of formed microcalli in total protoplasts inoculated.

Some chemical compounds, such as CH, glutamine and HYP exhibited effective results for protoplast culture (Tab 5). It was observed that any of the three compounds used alone resulted in remarkable increase of division frequency and promoting colony formation. However, even higher frequency of division and efficiency of colony formation could be obtained when these three compounds were used in combination (Tab 5). The promotive effect of certain amount HYP to protoplast growth have been reported by other anthors[8].

Tab 5. Influence of CH, glutamine and HYP on sainfoin protoplast culture*

Compounds added	Concentration (mg/L)	Frequency of cell division (%)	Efficiency of colony formation(%)
control	0	20.6	1.7
CH	200	34.8	2.1
glutamine	500	52.2	3.4
HYP	200	66.2	2.4
CH + glutamine + HYP	200 + 500 + 200	60.3	4.9

*Medium used in this experiment was SH supplemented with 2% sucrose, 0.45 mol/L glucose, 1 mg/L 2,4-D, 0.5 mg/L KT and 0.2 mg/L NAA.

Plant regeneration

For shoot induction from protocalli, several constitutive media with various phytohormone combinations were tested. The most frequent bud differentiation was observed on MS medium with 3% sucrose, 500 mg /L CH, 2 mg/L 6-BA and 0.2 mg/ L NAA or IAA. With this medium almost all protocalli differentiated into green buds one month after transfer. However these buds developed rather slowly. To improve shoot growth, different concentrations of gibberelic acid (GA_3) were added in the medium. As given in Tab 6, 0.2-0.5 mg /L GA_3 played a promotive role in shoot elongation. When differentiated buds together with linked calli were transferred on the medium containing 0.2 - 0.5 mg/L GA_3, numerous shoots elongated in 3-4 weeks. When shoots grew up to over 4 cm, they were transplanted to root induction medium , i.e. MS medium with 1 mg/L IAA or without any auxin. Three weeks later, plantlets with roots were produced (Fig 7).

Tab 6. The effects of GA_3 concentration on shoot elongation*

GA_3(mg/L)	Shoot elongation**
0	-
0.1	+
0.2	+++
0.5	++++
1.0	++
1.5	-
2.0	-
3.0	-

* Basal medium used in this experiment was MS medium with 3% sucrose, 500 mg/L CH, 2 mg/L 6-BA and 0.2 mg/L NAA.

** -, noneffective; +, a little positive result; + + , apparent positive result; +++, remarkable promotive effect; + + + + , strong promotive result.

Chromosome number

10 regenerated plants were examined fot chromosome number. It was revealed that all checked plants had normal chromosome number, i.e., 2n =28 (Fig 8).

Resistance of protocalli and regenerated plants to HYP or NaCl

Protoplast-derived calli could grow well on the inedium containing 10 mmol /L HYP, and expressed a certain tolerance to 0.8% NaCl (Tab 7) . The growth rate in the presence of 10 mmol /L HYP in the mediuln corresponded to 78% of the control.

Tab 7. Resistance of protoplast-derived calli to HYP and NaCl*

Stress factor	Concentration	Rate of relative growth (%)	
		HYP-resistance	Wild type
CK	0	100	100
HYP	10 mmol/L	78	0
NaCl	0.8%	56	0

* Basal medium used was the same as MS shown in Tab 2.

All 20 regenerated plants tested could grow normally on the medium containing 10 mmol /L HYP or 1.0% NaCl. However, the regenerated plants from control calli, i.e. its wild type, could not grow on MS medium containing 5 mmol/L HYP or 0.8% NaCl. This indicated that the tolerance of the resistant cell line to HYP was still retained after long term protoplast culture. Resistance test of seedling couhl not be made since the seeds have not yet been obtained from protoplast-regenerated plants.

The protoplast culture system of this investigation would be useful to study somatic hybridization. The protoplast fusion between this cell line and alfalfa has been undertaking in our laboratory.

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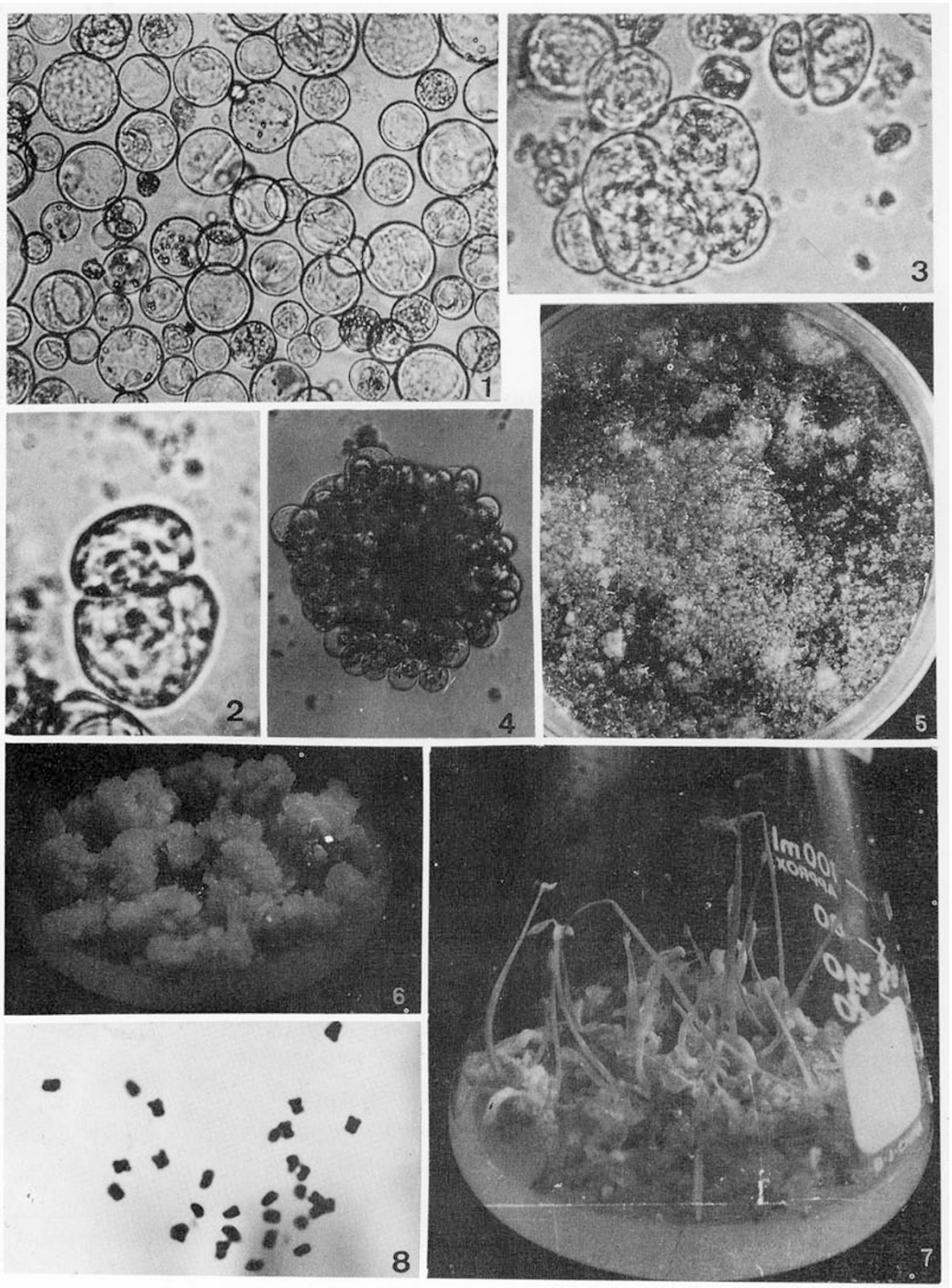
▷ **Fig 1.** Protoplasts isolated from calli of sainfoin, × 400.
Fig 2. First division after 5 d in culture used SH mediuin, × 600
Fig 3. A small cluster after 8 d in protoplast culture used SH medium, × 300
Fig 4. A cell colony formed from a protoplast, × 150.
Fig 5. Protoplast-derived calli forined in liquid medium after 6 weeks in culture.
Fig 6. Proliferating calli formed on ag/ar-solidified MS medium with 1 mg/L 2, 4-D and 0.5 mg/L 6-BA.
Fig 7. Plantlets regenerated from protocalli.
Fig 8. Chromosome set from root tip of protoplast-derived plants (2n=28).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