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影响决明无菌苗子叶原生质体分离和培养因素的研究

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摘 要：以决明(*Cassia obtusifolia*)无菌苗子叶为材料，对酶组合、无菌苗日龄，植物激素组合和培养方法对其原生质体的分离和培养的影响进行了研究。结果表明：用3%的纤维素酶和0.2% Pectinase Y-23的酶组合处理决明无菌苗子叶块8小时可以高效分离出有活力的原生质体；约14日龄的决明无菌苗子叶比较适合于原生质体的分离；适当浓度的2,4-D有利于原生质体的分离。促进原生质体分裂的理想的植物激素组合为0.4 mg/L 2,4-D，1.0 mg/L NAA and 0.1 mg/L KT；漂浮培养法最有利于原生质体的分裂和发育。找出了适合于决明无菌苗子叶原生质体的分离和培养的酶组合、植物激素组合、有效培养方法和决明无菌苗子叶日龄。这为有效地从决明无菌苗子叶原生质体再生植株奠定了基础。

关键词：决明；原生质体培养；酶组合；日龄；培养方法；植物激素

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Factors influencing protoplast culture of the seedlings of *Cassia obtusifolia* (Leguminosae)

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Abstract: In this work, the cotyledons of *Cassia obtusifolia* seedlings were used as materials. The effects of some factors such as different combinations of enzyme mixture, day-age of *C. obtusifolia* seedlings, culture methods and phytohormones in medium, on protoplast isolation and culture were investigated. The results demonstrated that protoplasts with high yield and quality were obtained by treating the cotyledon pieces with a mixture of 3% cellulase(Onozuka R-10) and 0.2% Pectinase Y-23 for 8h. The cotyledons from 14 day-old seedlings were appropriate for isolating protoplasts. The floating culture method was more suitable for protoplast division and the division frequency was 9.7%. The proper phytohormone combination was 0.4 mg/L 2, 4-D, 1.0 mg/L NAA and 0.1 mg/L KT 2,4-D, in which the division frequency 21.2% was gotten. The optimum combination of enzyme mixture, phytohormone combination, culture method and proper day-age of *C. obtusifolia* seedlings were determined. This laid the foundation of efficient plant regeneration from cotyledonary

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protoplasts of *C. obtusifolia* seedlings.

Key words: *Cassia obtusifolia*; protoplast culture; enzyme combination; day-age; culture method; phytohormone

1 Introduction

The higher frequency of plant regeneration from protoplasts is not only the foundation of plant genetic manipulation and plant improvement by means of cell engineering manipulation but also a good experimental system for the study of gene expression and *in vitro* cell differentiation. Therefore, scientists all over the word have been paying great attention to it. *C. obtusifolia* L. (Leguminosae) is a kind of common traditional Chinese herbs. Its seed is known as "false phaseolus radiatus" or "coffee bean", used for treating liver diseases, purging, reducing blood pressure, contracting the uterus, and dispelling rheumatism (Feng, 1993; Lian, 1986). So far, traditional breeding has been used in improving this species, but the limitations of traditional breeding method, such as the limited gene pool and the necessity for successive back crossing, could not be overcome. Accordingly, it requires improvement by biotechnology. In previous study, we had got the regenerated plants from its protoplasts (Zhou *et al*, 1998) and cotyledon cultures of *C. obtusifolia* (Zhou *et al*, 2001). In addition, because genetic manipulation and somaclonal variation are efficient ways to improve wild plant, they will be put to good use in *C. obtusifolia* production by increasing its plant regeneration frequency from protoplasts. In this work, some factors influencing its protoplast isolation and culture were investigated.

2 Materials and Methods

C. obtusifolia is a cross-pollinated species. Its seeds provided by Professor Yuan Baojun of Zhoukou Prefecture Institute of Agricultural Sciences, P. R. China were treated in concentrated sulfuric acid for 40 minutes and in 0.1% $HgCl_2$ for 5 minutes for sterilization, respectively, then washed 4～5 times with sterilized double distilled water and placed on agar-solidified MS medium (Murashige and Skoog, 1962) without any plant growth regulator in 100ml flasks (Zhou *et al*, 2001). Dry seeds were allowed to germinate for proper days under light conditions and then the cotyledons of its seedlings were harvested.

These cotyledons were cut into about 1 mm-long pieces. Firstly, the pieces were put in CPW-13M solution containing 13% mannitol (Wei and Xu, 1990) for preplasmolysis for 1.5 h. Secondly, about 1 g pieces were placed in eight milliliter enzyme solution (Table 1) and incubated at 26 ℃ in the dark while vibrated at forty r/pm. Third, the isolated protoplasts during the enzyme digestion were observed by microscope every other one hour. The adopted isolation time was determined when mean protoplast density did not increase any longer in the last hour. In the best isolation time, the isolation condition was shown by percentage of protoplasts in enzyme-protoplast suspension.

Floating, collection, purification and harvest of protoplasts were carried out using th the method described by Zhang *et al* (1994). The cotyledons from different day-age seedlings of *C. obtusifolia* were used to study the effect of day-age on the isolation of protoplasts. The effect of several phytohormones such as NAA, 2, 4-D and KT on protoplast culture was investigated. The protoplasts released from the cotyledons were cultured in CK8P thin layer liquid medium (Reinert and Yeoman, 1989) at a density of 5.1×10^4 numbers/ml (1 mL protoplasts suspension in a 3.3 cm × 1 cm dish). Percentage of survival protoplasts was calculated after a culture of two days. The effect of phytohormones on the division frequency of protoplasts was studied as stated by Zhang *et al* (1999).

3 Results and Discussion

3.1 Effect of different combinations of enzyme mixture on the protoplast yield from the cotyledons of *C. obtusifolia* seedlings

After the cotyledon pieces were digested for 8 ~24 h in the enzyme solution(Table 1), 1.8×10^2 ~1.6×10^5 protoplasts/g. fresh weight were successfully produced (Fig. 1). The results showed that the proper duration of protoplast isolation and protoplast yield and quality in different enzyme compositions were different. It was very clear that protoplasts with high yield and quality were obtained by treating the cotyledon pieces for 8h in a mixture of 3% cellulase(Onozuka R-10)and 0.2% Pectinase Y-23. This result was consistent with Johnson *et al*(1982). However, the enzyme combinations of cellulase(3% Onozuka R-10)with 0.2% Macerozyme R-10 and 1% Hemi cellulase(Sigma) or 0.5% Pectinase(Serva)and 0.5% Hemi cellulase (Sigma)gave poor protoplast yield in longer duration.

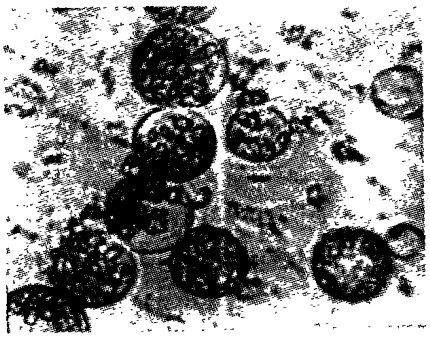

Fig. 1 Protoplasts isolated from cotyledon segments

Table 1 Effect of different combinations of enzyme mixture on the protoplast yield from the cotyledons of *C. obtusifolia* seedlings

Enzyme composition	Isolation time (h)	Enzyme-protoplast combination	
		Yield of protoplast(No./g · FW)	Clusters of mesopyll
3% Onozuka R-10,0.5% Pectinase,0.5% Hemi cellulase	24	1.8×10^2	+++
3% Onozuka R-10,0.2% Macerozyme R-10,1% Hemi cellulase	18	5.4×10^3	++
3% Onozuka R-10,0.2% Pectinase Y-23	8	1.6×10^5	+

Note: Enzymes was dissolved in CPW-9: 9% Mannitol CPW(Reinert and Yeoman, 1989); +: 2~5 Clusters, high isolation frequency; ++: 6-10 Clusters, low isolation frequency; +: 11~15 Clusters, very low isolation frequency

3.2 Effect of the age of seedlings on protoplast isolation of *C. obtusifolia*

The age of *C. obtusifolia* seedlings also affected the protoplast isolation. It was more difficult to isolate protoplasts from older cotyledons than that from younger ones. However, very young cotyledons were not suitable for the protoplast isolation, because their protoplasm membranes were easily damaged. The experimental results showed that the cotyledons from 14 day-age seedlings were appropriate for the isolation of protoplasts(Table 2)

Table 2 Effect of the age of seedlings on protoplast isolation of *C. obtusifolia*

Age of seedlings (d)	Cotyledon color	Protoplast yield (No./mL)	Initial isolation time of protoplasts (d)	Percentage of survival protoplasts (%)
5~6	Light yellow	1.5×10^6	5	30
14~15	Light green	8.1×10^5	7	70
20~21	Green	7.0×10^5	8	60

3.3 Effect of different culture methods on protoplast division and development

The effect of different culture methods on protoplast division and development was seen in table 3. It was obvious that the culture methods indeed influenced protoplast division and further development; floating culture was the best suitable for protoplast division (Fig. 1 ~ 2). The frequency was 9.7%. As a result, small calli formed and browning decreased; agarose-embedded culture was more suitable for protoplast division than thin layer liquid culture.

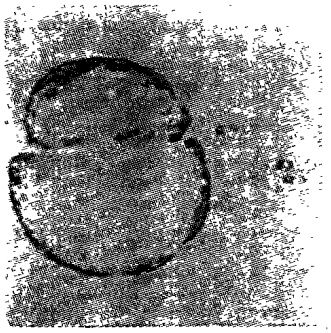

Fig. 2　The first protoplast division

3.4 Effect of different phytohormones on the division frequency of protoplasts

In this experiment, the effect of several plant phytohormones on cell division frequency of protoplasts was summarized in Table 4.

Table 3　Effect of different culture methods on protoplast division and development

Culture method	Protoplast division frequency (%)	Last development stage
Thin layer liquid culture	2.9	Cell mass
Agarose-embedded culture	7	Second division
Floating culture	9.7	Small callus

As shown in Table 4, (1) When only 2,4-D was used, high concentration of 2,4-D was better than the low one for the protoplasts division. Higher division frequency (18.8%) of protoplasts was obtained in composition Ⅱ at the higher concentration of 1.0 mg/L 2,4-D than that (12.6%) in composition Ⅰ at the low one of 0.4 mg/L 2,4-D. (2) The results from composition Ⅲ to composition Ⅳ revealed that the division frequency of protoplasts was improved from 11.7% to 21.2% with the increase of the concentration of 2,4-D from 0.2 to 0.4 mg/L in the compositions plus the same concentration of NAA and KT. To put (1) and (2) together, it was concluded that the higher the concentration of 2,4-D, the higher the division frequency of protoplasts. After that, by comparing composition Ⅰ with composition Ⅳ and composition Ⅱ with composition Ⅴ, it was found that NAA and KT could also improve the frequency. The positive effect of 2,4-D, NAA and KT was stronger than that of 2,4-D alone on the protoplast division. However, NAA and KT improved less protoplast division frequency than 2,4-D did. This was more obviously shown by comparing composition Ⅰ with composition Ⅲ, because high concentration of 2,4-D (0.4 mg/L) could produce higher protoplast division frequency than low one of 2,4-D (0.2 mg/L), together with 1.0 mg/L NAA and 0.1 mg/L KT, could. In addition, it could be known from composition Ⅳ and composition Ⅴ that the higher division frequency (21.2%) of protoplasts was gotten in the composition of 0.4 mg/L 2,4-D and 1.0 mg/L NAA and 0.1 mg/L KT than that (20.5%) in the one of 1.0 mg/L 2,4-D and 1.0 mg/L NAA and 0.1 mg/L KT at the same concentration of NAA and KT. The possible reason was that the ratio of auxin to cytokinin affected the frequency. This ratio in composition Ⅳ was more suitable for the frequency than that in composition Ⅴ. Meanwhile, the frequency (21.2%) in composition Ⅳ was the highest among al' the 5 compositions. Hence, it was believed from the above analyses that composition Ⅳ was the best suitable for the improvement of the frequency in this work. In a word, different phytohormones had a positive impact on the division frequency of protoplasts; phytohormone composition was very important for the cell wall regeneration of protoplasts and cell division at the initial stage of protoplast culture; 2,4-D was indispensable to cell division under its proper concentration.

Table 4　Effect of several phytohormones on the division frequency of protoplasts

Phytohormone composition (mg/L)	Division frequency (%)
Ⅰ. 2,4-D 0.4	12.6
Ⅱ. 2,4-D 1.0	18.8
Ⅲ. 2,4-D 0.2, NAA 1.0, KT 0.1	11.7
Ⅳ. 2,4-D 0.4, NAA 1.0, KT 0.1	21.2
Ⅴ. 2,4-D 1.0, NAA 1.0, KT 0.1	20.5

In conclusion, the optimum combination of enzyme mixture (3% cellulase (Onozuka R-10) and 0.2% Pectinase Y-23), phytohormone combination (0.4 mg/L 2,4-D, 1.0 mg/L NAA and 0.1 mg/L KT), culture method (floating culture) and better

day-age (14 to 15 d) of *C. obtusifolia* seedlings were determined. This laid the foundation of efficient plant regeneration from cotyledonary protoplasts of *C. obtusifolia* seedlings and will greatly contribute to its breeding via somaclonal variation, germ line improvement and genetic transformation.

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