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Plants regeneration from protoplast and their genetic variation in foxtail millet

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Summary Compact calli derived from immature spikelet of a foxtail millet variety — Jigu 11 cann't be directly used for protoplast isolation because of its firm physical structure,and must be loosened with subculturing in M_1,M_2 and M_3 media successively and altering these media compositions. The loosened calli can be selected from the regulation and used for protoplast isolation successfully. Rate of protoplast division in KM_8P medium was 12. 3－33. 5%. Calli derived through protoplast division are loose and cann't be used directly for plan regeneration because of its soft physical structure. When they were subcultured in N_6-1,N_6-2,N_6-3 and N_6-4 media, in which the media compositions were changed,the compact calli were obtained and 129 plantlets were regenerated from them. 101 plants,which grew to maturity after transplanting the plantlets into field,exhibited sterility in some degree. Most of the subsequent lines derived from the regenerated plants were sterile and only two lines could get normal reproduction. The efficient protoplast culture and plantlet regeneration system were reported in this paper. The genetic variation of the progeny of the regenerated plants was also disscused.

Key words foxtail millet (*Setaria italica* Beau.),protoplast,plant regeneration,genetic variation

Introduction

Protoplast,as one kind of receptor for plant engineering operation,has particular and practical value for developing new germplasm and selecting new varieties. The great progresses were made in protoplast culture of cereal crops in recent years and plants were successfully regenerated from protoplast of rice,wheat,corn,foxtail millet and other crops. But there are several problems in the culture technique system still need to solve. An efficient protoplast culture system for foxtail millet including frequent cell division,callus formation and plant regeneration will be presented in this paper.

Materials and methods

Induction and culture of calli

Mature embryos of 109 genotypes and inflorescences of 4 genotypes of foxtail millet (*Setaria italica* Beau.)which came from several ecological zones and were representatives of different ecological strain groups were screened for callus induction. After sterilization the explants were inoculated in MS medium with 2. 0 mg/L 2,4－D and 0. 2mg/L KT. After 30-day culture,fresh calli were selected for subculture in the same medium. The subculture cycle was 30－40 days.

Regulation of status of callus growth

In the preliminary experiment,it was found that the compact calli derived from mature em-

bryo or inflorescence could not be directly used for protoplast isolation. Through consecutive subculture in M_1, M_2, M_3 media, in the order named, the compact calli were altered (Table 1), and the vigorous loosened calli could be obtained. Then they were subcultured in M_3 medium.

Isolation and culture of protoplast

Loosened calli subcultured about 10 days in M_3 medium were used for protoplast isolation. The enzyme liquid components used for enzymolysis included KH_2PO_4 (0.05%), $CaCl \cdot 2H_2O$ (1%), $MgSO_4 \cdot 7H_2O$ (0.1%), mannitol (10%), cellulase (2%), pectase (0.1%), and

Table 1 Compositions of media used for calli initiation, subculture and calli status regulation (mg/L)

Compositions		MS	M_1	M_2	M_3
Macro-elements	NH_4NO_3	1650	2500	2000	2000
	KNO_3	1900	2300	2000	2000
	$CaCl_2 \cdot 2H_2O$	440	600	400	400
	$MgSO_4 \cdot 7H_2O$	370	500	400	400
	KH_2PO_4	170	300	390	300
	KCl			1000	2000
	NaCl			1000	1000
Nutrilites		Same as MS	Same as MS	Same as MS	Same as MS
Organic matters	Glutamine		200		400
	Aspartic acid		150		300
	Casein hydrolysate		300		200
	Glycine	2	2	2	2
	VB_1	0.4	0.4	0.4	0.4
	VB_6	0.5	0.5	0.5	0.5
	Nicotinic acid	0.5	0.5	0.5	0.5
	Inositol	100	100	100	100
	Sucrose	30000	30000	30000	30000
	Sorbitol		30000	30000	30000
	Agar	10000	10000	10000	10000
Hormones	2,4—D	2	4	4	6
	KT	0.2	0.4	0.5	0.5
	pH	5.8	5.8	5.8	5.8

the pH was regulated to 5.8. The enzymolysis was carried out in flasks with the help of shaking at 30 rpm and at 28℃ constant temperature for 7 to 10 hours. After filtration through a nickel screen with 400 meshes/cm^2 and centrifugation at 1000 rpm for 5 minutes, the protoplasts collected were cleansed twice by centrifugation at 1000 rpm for 5 minutes in the same liquid from which the cellulase and pectase were excluded. The cleansed protoplasts were transfered at a density of 4×10^5 protoplasts/ml into double layer medium KM_8P (Table 2) which was made by firstly pouring 1 ml solid medium of KM_8P in a culture dish (diameter 3.5 cm) and then adding 0.5 ml liquid KM_8P to it. The protoplast culture was carried out in the dark at 25℃. About 0.25 ml liquid KM_8P in which glucose consentration reduced one half was added into the original medium in the 10th day and the 20th day, respectively. After 30-day culture, small cell aggregates derived from protoplast

were inoculated into solid KM_8P. Calli obtained through 60-day culture in solid KM_8P were transfered in MS medium supplemented with 0. 2 mg/L 2,4—D and 0. 2 mg/L KT to subculture.

Table 2. Compositions of medium used for protoplast culture (mg/L)

Components	Consentration
Basic element *	Same as KM_8P
Casein hydrolysate	250
Glutamine	200
Aspartic acid	150
Sucrose	10000
Glucose	0. 55M
Coconut milk	2%
2,4—D	1
KT	0. 2
NAA	0. 2
pH	5. 6

* :Basic elements include macro-elements, nutrilites, molysite and organic components.

Regulation of the regenerated callus growth status

The loosened calli derived from protoplast culture were inoculated into MS medium supplemented with 0. 2mg/L 2, 4 — D and 0. 2mg/L KT and N_6-1 medium (Table 3) for subculturing. In the whole regulation process, more compact calli were selected for subculture.

Regeneration of protoplast plantlet

The compact callus obtained through the above process was inoculated successively into N_6-2, N_6-3 and N_6-4 media (Table 3) for embryo body initiation and plantlet regeneration.

Observation on genetic variation of the regenerated plants

Notes were made on the genetic variation of the regenerated plants and their first and second generations in aspects of fertility and other main characters.

Results and discussion

Induction and utilization of callus

The callus is the basic material for protoplast isolation, so the callus status plays a key role in the protoplast culture. There are more than 15 000 strains of foxtail millet in China, and calli derived from different genotypes exhibit difference in the physiological status and the physical structure which have close relation with the protoplast culture. 113 representative strains were choosed from different ecological zones and different types to observe their calli initiation and growth status. The results showed that all varieties could induce calli and the rate of calli induction was 80—95%. The calli can be classified into 3 types: (1) compact calli which grow slowly and have a firmer physical structure; (2)brittle calli which grow much faster than compact one and have a soft and brittle physical structure; (3)loosened calli which grow vigorously and has a loose and tender physical structure. The compact calli can differentiate and grow into plantlets, but can not be directly used for protoplast isolation. The loosened calli can be directly used for protoplast isolation but can not grow into plantlets. Only brittle callus can be used both for protoplast isolation and direct induction of plantlets. Unfortunately, the brittle calli (about 0. 89%) can be induced by only one genotype, and the loosened (20. 35%) or compact calli (78. 76%) were induced by other 112 strains. Something must be done before using the latter two kinds of calli for protoplast culture.

Table 3. Compositions of madia used for regulation of regenerated callus status and regeneration of plantlet(mg/L)

Compositions	N_6-1	N_6-2	N_6-3	N_6-4
Basic elements *	N_6	N_6	N_6	N_6
KNO_3	3800			
L-proline		1500	1000	
Casein hydrolysate		200		
Coconut milk		2%		5%
L-aspartic acid		800		
VB_1	10			
VB_6	1			
Nicotinic acid	1			
Inositol	100			
Mannitol	3%			
Sucrose		4%	3%	2%
2,4-D		0.5	0.3	
KT	2	1	1	1
6-BA	0.5	0.5	0.5	0.5
Agar	1%	1%	1%	1%
pH	5.8	5.8	5.8	5.8

* :Basic elemants include macro-elements,nutrilites,molysite and organic components.

Regulation of calli growth status

Calli derived from Jigu 11 belong to compact ones. When they were inoculated into M_1 medium,they grew much fast within about 45 days. When calli were moved from M_1 to M_2 medium and subcultured for 120 days,the light yellow and loose particles could be seen on the surface of calli,but they grew slowly. After this kind of calli was subcultured in M_3 medium and grew for about 80 days,they became loose in structure and light yellow in color and grew vigorously. Based on the above work,the vigorous and loose cell lines were established.

Protoplast isolation and culture

After 10-day subculturing,loose calli which were at early cell division stage were excellent materials for protoplast isolation. When they were placed into the enzyme liquid for about 9 hours,they would be fully enzymolysed,and more than 95% of protoplasts could be obtained. The protoplasts,which were uniform in size and dense of cytoplasm,were inoculated into double layer medium KM_8P. The first division of protoplast occured in 3—4 days, and from then on much divisions took place. Small cell aggregates could be seen in 14 days culture and small pieces of calli were established in 50 days. If the protoplasts density was controlled in the range of 4×10^5 per ml medium,and when the protoplasts were at division stage and the small cell aggregates were in the formation stage,some liquid medium should be added. The experiment results indicated that most of protoplasts would not form cell aggregates if extra medium was not added in the 10th day of culture,and that most of the small cell aggregates would not continuously grow and would brown if extra medium was not added in the 20th day of culture. The rate of cell division was 12.3% in the 7th day of culture and 33.5% in the 14th day. The protoplast culture experiments were repeated 21

times and nearly the same resultes were obtained in all the trials.

Regulation of regenerated calli growth status

Calli derived from protoplasts of Jigu 11 belong to loosened calli and cann't be directly used for plantlet regeneration. They were inoculated into MS medium supplemented with 0.2 mg/L 2,4—D and 0.2 mg/L KT for subculture and regulation of calli status. After 80 days the calli were moved into N_6-1 medium for another 25 days subculture, and finally the compact calli were developed from the loose ones and used for plant regeneration.

Plantlet regeneration

After 25-day subculture in N_6-2 meduim, the compact calli might derive white and firm embryo bodies. In order to get more plantlets, the embryo bodies were dibbled into N_6-3 meduim, and 15 days later shoots could be obtained. Then the shoots were transfered to N_6-4 medium which excludes hormone. After 20 days 129 plantlets fully developed. The plantlets were then transplanted to experimental field and 101 plants grew well until maturity.

Genetic variation of the regenerated plast and their progency

(1)Regenerated plants: All of the 101 plants directly derived from protoplast culture grew to maturity and exhibited sterility in some different degrees.
(2)The first generation: Seeds coming from 80 regenerated plants were sowed in the summer of 1991. Among these 80 different lines, only 2 lines showed normal reproduction and other 78 lines exhibited sterility in some degrees as the regenerated plants.
(3)The second generation: Fifty lines coming from the first generation were cultivated in the summer of 1992. Plants derived from the two normally reproducing lines could get normal seed, while the other lines still exhibited sterility in some degrees as before.

The results above have indicated that protoplast is characterized by the variability of heredity especially in sterility. This character is of certain practical value in somatic variation breeding, but in other biotechnology processes such as somatic crossing and gene transfering this variability would make the experiment complicated.

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