Screening of high somatic embryogenic soybean varieties and innovation of culture methods

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Abstract: Seventeen soybean (*Glycine max* L.) varieties representing 10 local, 5 Brazilian and 2 American ones were screened for their somatic embryogenic capacity using uniform *in vitro* culture methods. Auxin concentrations were optimized with five highest responsive varieties. The optimal concentration of 2, 4-D for the varieties of Lianjiang, Zhechun 2, Perry and BRS 07 were found to be 2 mg/L, whereas that for Chief was 5 mg/L. Moreover, synergistic enhancing effects of two additives, copper sulfate and silver nitrate on embryogenesis were detected in separated experiments with Perry and BRS 07 when the media were supplemented with both at 5 mg/L.

Key words: somatic embryogenesis; copper sulfate; silver nitrate; tissue culture; soybean
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高胚胎再生能力大豆品种的筛选和培养方法改进

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摘要: 应用离体培养的方法, 从本地 10 个, 巴西 5 个和美国 2 个合计 17 个大豆品种中筛选出廉江、浙春、Perry、BRS 07 和 Chief 等 5 个体细胞胚胎再生能力强的品种, 并在此基础上对这 5 个品种的培养基生长素浓度进行优化. 对于廉江、浙春、Perry 和 BRS, 最合适的 2, 4－D 质量浓度为 2 mg/L, 而对于 Chief 的最合适质量浓度为 5 mg/L. 此外, 在以 Perry 和 BRS 07 为材料进行的另一个试验中发现, 当同为 5 mg/L 的硫酸铜和硝酸银同时添加到培养基时对促进体细胞胚胎的形成具有协同作用.

关键词: 体细胞胚胎发生; 硫酸铜; 硝酸银; 组织培养; 大豆

Soybean has been identified as the most economical source of food protein and oil. The importance of the crop has led efforts to improve soybean characteristics via transformation and regeneration techniques to avoid some of the traditional limitations of breeding. However, it has now been recognized that soybean is one of the most recalcitrant crops to genetically engineer.

Many research works have been so far based on regeneration through somatic embryogenesis. Although many years have passed since the first report by Christianson et al.[1] on somatic embryogenesis of soybean, the frequencies at each step of regeneration are still far below relative to that of other crops[2]. In *in vitro* cultures, reformulation of medium constituents or physicochemical factors and optimization of hormone concentrations have a greater potential of improving the frequencies of somatic embryo-

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genesis[3,4]; and there has been evidence that different genotypes may have very different potentials of somatic embryogenesis[5]. The aims of this study were to screen some varieties with higher embryogenic potential and to improve the efficiency of cultures in soybean somatic embryogenesis.

1 Materials and methods

1.1 Plant material

Seeds of all the varieties were kindly provided by Prof. Nian Hai, a soybean breeding expert at the College of Agronomy, South China Agricultural University. Soybean plants were grown from seeds in this university farm, and 3—7 mm long immature cotyledons were excised from pods and used as explants for the cultures after surface-sterilization treatment of the immature seeds.

1.2 Preparation of media

Wide mouth glass bottles of about 300 mL inner space with transparent plastic screw caps were used for the cultures. Each bottle received 40 mL medium. All the media contained 30 g/L sucrose and 7 g/L agar, and the pH of all the media was adjusted at 5.8 before autoclaving. The media were autoclaved at 121 ℃ for 20 min.

1.3 *In vitro* culture methods

Ten excised cotyledons were placed abaxial side down on the medium in each culture bottle. In all the experiments, each treatment consisted of 5 bottles and was repeated once or twice. The cultures were maintained under illuminance of 2 000 lx and 12 h of photoperiod at 25 ℃ for 30 d and then the results were evaluated.

1.4 Evaluation and statistical analysis

In order to evaluate the results more precisely, terms not only of percent response but also of embryo quality and overall performance were recorded. Embryo quality is based on both morphology and colour. Tight and green embryos are the best for transformation and proliferation whereas elongated and yellow embryos cannot be transformed and proliferated[6]. Based on these facts, indices of embryo quality and overall performance were developed by Tomlin et al.[5] as follows: The highest score for the index of embryo quality is 200 when all the embryos are tight and green. When all the embryos are elongated and yellow, it receives the lowest score of zero.

Embryo quality[5] = [(0)× Elongated embryo percentage + (1)× Loose embryo percentage + (2)× Tight embryo percentage] × (Green embryo percentage)/100.

Overall performance[5] = Response percentage× 0.01× Number of embryos per explant× Embryo quality.

Data were analyzed by the analysis of variance (ANOVA) as a Randomized Complete Block Design using the SAS for Microsoft Windows, release 8.01. Significant differences among means were determined by Duncan's multiple range tests at $P \leqslant 0.05$.

2 Results and discussion

2.1 Screening of high responsive varieties

In this experiment, immature cotyledons of 17 varieties were used to culture on MS media containing three different levels of 2,4-D for screening high responsive varieties of somatic embryogenesis. Results of the experiment are summarized in Tab. 1. In the experiment, different soybean varieties showed a great deal of variation in performance. These results confirmed the consequences of the previous works by Lazzeri et al.[7], Komasuda and Ohyama[8], Meurer et al.[9], and Tomlin et al.[5].

According to the results shown in Tab. 1, performances of different varieties vary from 1.667% of varieties No. 7 and 8 (local varieties) to 48.333% of No. 33 (Brazilian variety), from 0% of variety No. 19 (Brazilian variety) to 36.667% of No. 16 (American variety) and from 0% of varieties No. 1, 2, 3, 4, 5, 6, 7, 8 and 11 (local varieties) and No. 19, 21 and 29 (Brazilian varieties) to 25% of No. 17 (American variety) at the concentration of 2, 10 and 40 mg/L 2,4-D in the medium, respectively. Performance of No. 33, a Brazilian variety, was significantly different from other varieties at 2 mg/L 2,4-D. Of other concentrations of 2,4-D used, No. 16 (American variety) performed well at 10 mg/L whereas No. 17 (American variety) has equally responded in all the 3 concentrations. Most of the varieties showed better performances at 2 mg/L 2,4-D except the varieties of No. 7, 16 and 28 which performed comparatively well with 10 mg/L 2,4-D. It is clear that, with the increasing of auxin concentration, percent response in almost all the varieties declines, although some of the previous work have reported the high levels of auxin is more effective in producing somatic embryos[2,5,9].

Tab. 1 Culture response of embryogenesis in 17 varieties on media with three different levels of 2, 4-D

variety			ρ(2, 4-D) / (mg·L^{-1})		
region	code No.	name	2	10	40
south China	01	Lianjiang	35.000 b A[1)]	1.667 d B	0.000 c B
	02	Bendi 2	18.333 cdef A	15.000 bcd A	0.000 c B
	03	Bendi 62	21.667 bcde A	1.667 d B	0.000 c B
	04	Guangdali	13.333 cdefg A	3.333 cd AB	0.000 c B
	05	Guangzhongli	6.667 efg A	6.667 cd A	0.000 c B
	06	Yangchunxiaoli	6.667 efg A	1.667 d B	0.000 c B
	07	Yangchunchundou	1.667 g A	3.333 cd A	0.000 c A
	08	Puhuang	1.667 g A	1.667 d A	0.000 c A
	11	Jiahaishuangse	26.667 bc A	6.667 cd B	0.000 c B
	12	Zhechun 2	23.333 bcd A	18.333 bc AB	8.333 bc B
America	16	Chief	16.667 cdefg B	36.667 a A	13.333 b B
	17	Perry	26.667 bc A	13.333 cd A	25.000 a A
Brazil	19	Embrapa 58	13.333 cdefg A	0.000 d B	0.000 c B
	21	Embrapa 60	10.000 defg A	6.667 cd A	0.000 c B
	26	BRS 132	5.000 fg A	11.667 cd A	5.000 bc A
	29	BRS 157	18.333 cdef A	3.333 cd B	0.000 c B
	33	BRS 07	48.333 a A	28.333 ab AB	6.667 bc B

1) All the data are percentages of explants which showed response of producing somatic embryos; Means followed by the same small letters in the columns and by the same capital letters in the rows are not significantly different at 5% level in Duncan's Multiple Range Test

Out of the local varieties, No. 1, 11 and 12 showed higher responses at 2 mg/L 2, 4-D. Of No. 11 and 12, only No. 12 responded for a wide range of the auxin concentration. Both No. 16 and 17 (USA varieties) performed comparatively well in all 3auxin concentrations used, even at higher concentration of 40 mg/L. Only No. 33 of Brazilian varieties showed a better performance for a wide range of auxin concentration. Hence, No. 1 and 12 of local, No. 16 and 17 of USA and No. 33 of Brazilian varieties were screened as high responsive varieties for further experiments in this study.

2.2 Optimization of 2, 4-D concentration

Five high responsive varieties screened from the above experiment were used for this experiment. Immature cotyledons were placed on MS medium containing different concentrations of 2, 4-D of 2, 5, 12.5 and 31.25 mg/L. Furthermore, instead of 2, 4-D, 10 mg/L of NAA was used in another treatment for the comparison of the responses for different auxins as it has been pointed out that NAA induces more normal embryo formation[7, 8].

Results of the experiment summarized in Tab. 2 clearly showed that all four varieties except No. 16 have achieved the highest response at the lowest concentration of 2, 4-D. The optimal concentration for No. 16 was 5 mg/L 2, 4-D which is significantly different from other concentrations at 5% probability level. However, No. 17 still showed higher responses both at 2 and 5 mg/L with no significant different between these two concentrations. All the varieties performed similarly showing lower response at 10 mg/L NAA than that at optimal concentrations of 2, 4-D. It was observed, that the auxin type has an effect on embryo morphology, which confirmed the previous results obtained by Lazzeri et al.[7] and Komatsuda and Obyama[8]. Normal embryos with two-or poly-cotyledons were obtained in NAA whereas embryos produced with 2, 4-D were usually horn-shaped. Moreover, explants in the medium containing NAA produced calli and roots.

From Tab. 2 it is clear that the higher indices of embryo quality in all the varieties except No. 33 were resulted at the optimized concentrations of 2, 4-D. No. 33 yielded the highest embryo quality with 12.5 mg/L 2, 4-D. Fairly higher values of embryo quality could be seen at 10 mg/L NAA with no significant differences among varieties. Meanwhile, the highest values for the mean number of embryo clusters per total number of explants cultured from all the varieties have been obtained at the optimal concentrations of 2, 4-D in each variety whereas the values are not significantly different among varieties with NAA in the medium (data not shown).

Tab. 2　Embryogenenic responses of five highly responsive varieties under different levels of auxins

variety code	items investigated	ρ(2, 4-D)/(mg·L^{-1})				10.0 mg/L
		2.0	5.0	12.5	31.25	NAA
1	response[1]/%	29.00 a AB[2]	14.00 b C	4.00 c B	0.00 c C	19.00 b A
	embryo quality	118.05 a AB	67.59 a B	100.00 a A	0.00 b B	105.22 a AB
	overall response	46.71 a C	10.46 bc E	4.00 bc B	0.00 c B	24.63 b A
12	response/%	39.00 a A	21.00 b BC	19.00 b A	12.00 c A	18.00 bc A
	embryo quality	131.46 a AB	112.02 ab AB	107.72 ab A	90.00 ab A	71.60 b B
	overall response	94.98 a AB	37.45 b C	26.82 bc A	12.01 c A	14.27 c A
16	response/%	22.00 b B	40.00 a A	17.00 bc A	8.00 d AB	15.00 c A
	embryo quality	79.40 ab B	119.36 a AB	60.05 ab A	20.00 b AB	122.67 a AB
	overall response	23.43 b C	89.37 a A	15.28 b AB	2.00 b B	19.33 b A
17	response/%	36.00 a A	24.00 ab B	2.00 c B	2.00 c C	18.00 b A
	embryo quality	166.48 a A	133.89 ab A	50.00 bc A	25.00 c AB	148.87 a A
	overall response	118.92 a A	63.12 b B	1.00 b B	1.00 b B	43.81 b A
33	response/%	34.00 a AB	15.00 bc C	11.00 bc AB	5.00 c BC	18.00 ab A
	embryo quality	96.26 a AB	112.60 a AB	138.89 a A	56.06 a AB	69.86 a B
	overall response	62.36 a BC	29.60 b D	17.70 b AB	3.34 b B	20.48 b A

1) Percent response is based on the number of explants cultured; 2) Means followed by the same small letters in the same rows and by the same capital letters in the same columns are not significantly different at 5% level in Duncan's Multiple Range Test

2.3　Effects of additives silver nitrate and copper sulfate

Immature cotyledons from two high responsive varieties, No. 17 and 33 were used in this experiment to investigate the effects of silver nitrate and copper sulfate on embryogenesis of the explants. All the media contained 5 mg/L of 2, 4-D as the only hormone, and were supplemented with silver nitrate or/and copper sulfate both at 5 mg/L. Results of the experiments are summarized in Tab. 3.

Copper enhances the regeneration ability of *in vitro* cultures[10] as this is an essential element regulating many important physiological pathways in plant growth and development. Silver nitrate, an ethylene inhibitor, can be used in the medium to promote the initiation and growth of buds by inhibiting the ethylene production in tissues. Surprisingly, in this study, although copper sulfate in all the media tested improved the percent response and overall response, silver nitrate decreased the performances of embryogenesis drastically and produced more calli. However, both copper sulfate and silver nitrate together in the media significantly improved the performance. Improvement of the performances by both of these additives is much greater than that by copper sulfate alone in the media. This can be explained by their synergistic effects when both additives combined together in the medium.

Tab. 3　Effects of media additives silver nitrate and copper sulfate on embryogenesis

basal medium	additive	response[1]/%		no. of embryos/explant		embryo quality		overall response	
		Var. 17	Var. 33	Var. 17	Var. 33	Var. 17	Var. 33	Var. 17	Var. 33
MS	—	24.00 d[2]	15.00 bc	1.96 a	1.73 a	133.89 ab	112.60 ab	63.12 b	29.60 b
	$CuSO_4$	32.00 c	23.00 b	1.77 abc	2.18 a	109.06 ab	54.82 c	61.83 b	25.30 b
	$AgNO_3$	14.00 e	15.00 bc	1.78 abc	1.30 a	96.00 ab	47.30 c	21.65 c	10.03 b
	$CuSO_4$+$AgNO_3$	50.00 ab	37.00 a	1.59 abc	1.57 a	115.11 ab	117.16 ab	90.61 a	67.87 a
B5	$CuSO_4$	34.00 c	24.00 b	1.84 ab	1.98 a	112.14 ab	51.04 c	69.54 b	24.01 b
	$AgNO_3$	9.00 e	8.00 c	1.43 bc	1.70 a	148.75 a	83.68 bc	19.00 c	10.72 b
	$CuSO_4$+$AgNO_3$	43.00 b	36.00 a	1.93 a	1.43 a	119.09 ab	137.48 a	98.67 a	72.26 a

1) All the percentages are based on number of explants cultured; 2) Means with the same letter in the same columns are not significantly different at 5% level in Duncan's Multiple Range Test

3 Conclusion

Various genotypes of soybean show different responses in somatic embryogenesis in terms of both quantitative and qualitative responses at different auxin levels. Some varieties poorly respond whereas some others highly respond at the same auxin level. All the local varieties used in this study responded well in the lowest concentration of auxin used. However, their responses could be improved by using additives such as copper sulfate and silver nitrate. According to our study, silver nitrate could be used with copper sulfate to obtain their synergistic effects on somatic embryogenesis. The present results have high application value for improvement of soybean when bio-technical means are to be used.

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