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Article

Optimization of Callus Induction Conditions from Immature Embryos in Maize and Plant Regeneration

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Abstract: This research uses the immature embryos of inbred maize lines (GSH9901, Hi01, Hi02, and Chang 7-2) as receptor materials to establish the callus induction system. These inbred lines provide the receptor materials for the genetic regeneration of maize and the verification of the genetic functions of maize. The factor experiment and orthogonal experiments were used to investigate the impacts of different genotypes, immature embryo size, shield orientation, 2, 4-D concentration, proline concentration, and folic acid concentration on the induction rate of embryogenic callus tissue. A sensitivity experiment testing glyphosate (Bar) and an antibiotic (Cefotaxime sodium) were also conducted. The results indicate that the immature embryos of inbred maize line GSH9901 were the most effective for callus tissue induction, and the immature embryos with a length of 1.6-2.0 mm produce the best result. The upward shield face is more successful for the formation of induced callus. Using orthogonal analysis, we found that the optimal combination for the induction system was A_3 (2,4-D concentration 0.25 mg mL^{-1}), B_1C_3 (proline concentration 0.8 mg mL^{-1}), and D_2 (folate Concentration 0.5 mg mL^{-1}) and the induction rate reached 84%. We found that cold storage at 4 °C for 1 d is more conducive for the formation of embryogenic callus than the other treatments tested. The sensitivity experiment for callus tissue screening revealed the critical concentration of glyphosate to be 10 mg ml^{-1}, and the critical concentration of antibiotic is 250 mg ml^{-1}. Using this combination of glyphosate and antibiotic resulted in regenerated plants. This study established the optimal conditions for immature embryo callus tissue induction in maize.

Keywords: Maize; optimization; genetic regeneration; immature embryos; function verification; regenerated plants

1 Introduction

Maize (Zea mays L.) belongs to the Gramineae plants (Gralnineae) group or Poaceae plant family and is an important human food and economic crop [1]. China plays a leading role in world food production due to its vast maize farms. After the United States, China is the world's largest corn producer with a total of 360 million acres [2]. Because of the importance of maize, there have been ongoing research efforts to improve the quality of the crops produced in China for many years.

Maize tissue culture is an integral part of maize genetic transformation. The rapid development of maize tissue culture technology allows regenerated plants to be successfully obtained with anthers [3-5], immature embryos [6-8], stem tips [9,10], and mature embryos [11,12].

The induction of callus from corn embryo as explant was first mentioned by Green et al. [13] and has the advantages of convenient inoculation, easy callus induction, and stable secondary culture. In this experiment, inbred maize lines GSH9901, Hi01, Hi02, and Chang 7-2 were used as experimental materials. Immature embryos were used as explants to explore the establishment of the immature embryo-induced callus system, and the influencing factors in the induction process were studied. Additionally, the sensitivity test of screening pressure on callus was performed. This study provides a good foundation for subsequent investigations into maize genetic transformation.

2 Materials and Methods

2.1 Corn Test Materials and Seed Disinfection

2.1.1 Test Material

The inbred maize lines GSH9901, Hi01, Hi02, and Chang 7-2 were sown in May 2017 in an experimental field site. After artificial pollination, the immature embryos from four inbred lines were selected as explants.

2.1.2 Medium

(1) Inducible medium: study of the induction of N6 medium was added to different adjunction proportion of callus.

(2) Subculture medium: the subculture medium added to the concentration of 2,4-D was 1 mg L^{-1}.

(3) Differentiation medium: IBA 0.8 mg L^{-1} and 6-BA 1.5 mg L^{-1} were added to the differentiated medium.

(4) Rooting medium: NAA 1.5 mg L^{-1} and activated carbon 2 g L^{-1} were added to the rooting medium.

2.2 Method

2.2.1 Stripping of Maize Immature Embryos

The immature maize embryos were stripped by selecting 10-13 day-old, pollinated, female ears of corn. The outer husk leaves were stripped off (leaving only a layer of young leaves) then soaked with 75% ethanol for 15 min. Next, the young leaves and the corn silk were removed. The remaining immature embryos were disinfected in a 0.5% NaClO solution for 10 minutes then rinsed with sterile water three to four times. Finally, we used a scalpel to remove the seed coat and endosperm at the top of the ear and picked out the immature embryos. The inbred lines with the strongest inducing ability were selected as the primary research objects.

2.2.2 Induction of Immature Embryo Callus in Maize

Effect of Different Inbred Maize Lines on Embryogenic Callus Induced by Immature Embryos

The immature embryos of GSH9901, Hi01, Hi02, and Chang7-2 that were pollinated on days 10-13 were then inoculated into the N6 induction medium. Each flask was inoculated with 25, five bottles in each group. Within six to eight days, young embryos began to swell and form callus, and the number of young embryos that developed callus was counted two weeks later.

Effects of Immature Embryo Length on the Formation of Callus

Immature embryos of the inbred line GSH9901 with the length of 0.6-1.0, 1.1-1.5, 1.6-2.0, and 2.1-2.5 mm were inoculated into N6 induction medium. Each flask was inoculated with 25, five bottles in each group. From the date of inoculation, the length of the immature embryo was measured every two days. After 10 measurements, the callus induction rate and the immature embryo expansion rate were counted. The results were analyzed by GraphPad prism 7.0 software.

Effects of Scutellum Orientation on Callus Formation

One immature embryo of the inbred line GSH9901 was the suitable length and thus was used to inoculate the N6 induction medium in both upward and downward directions. Each bottle was inoculated with 25, five bottles in each group. After two weeks, the callus induction rate was calculated.

Effects of 2,4-D Concentration, Proline Concentration, and Folate Concentration on the Formation of Callus

The immature embryos of the inbred line GSH9901 measuring 1.6-2.0 mm in length were inoculated into the N6 induction medium (Tab. 1) with varying add-on ratios. Each bottle was inoculated with 25 bottles; each group was repeated three bottles, using three factors and four levels of positive. The data from nine groups were processed by the L_9 (3^4), and the results were observed after 20 days of induction. The average browning rate and browning rate of callus were calculated.

Table 1: Maize immature embryos callus induction of different additive ratio

Test number	2，4-D（mg mL^{-1}）	proline （mg mL^{-1}）	folic acid （mg mL^{-1}）
1	1.00	0.60	1.00
2	1.00	0.70	0.50
3	1.00	0.80	0.25
4	0.50	0.70	0.25
5	0.50	0.80	1.00
6	0.50	0.60	0.50
7	0.25	0.80	0.50
8	0.25	0.60	0.25
9	0.25	0.70	1.00

Effects of Varying Cold Storage Time on Immature Embryo Induced Embryogenic Callus Formation

The inbred lines of the appropriate length for GSH9901 were selected for cold storage in 1°C, 2D, and 3D in a refrigerator at 4°C, 25 bottles per bottle, three bottles in each group, and then the treated immature embryos were inoculated into induction culture. Callus induction was observed after 20 days at the base. Type I callus was primarily used, and the callus healing rate was calculated. After two to three times, most of the callus was transformed into a stable type II callus. Once tissue was generated, the embryogenic callus induction rate was counted, and the results were analyzed by GraphPad prism 7.0 software.

2.2.3 Sensitivity of Corn Callus to Screening Material

Determination of Critical Concentration of Resistance Screening for Glyphosates

The callus induced by the immature embryos of inbred line GSH9901 was inoculated into the screening medium containing different concentrations of glyphosates. The browning of the callus was counted 20 days later to determine the optimal weeding of the callus during the screening process. The screening agent, which included the callus, was weighed with an electronic balance. After 20 days of screening, the weight was measured twice, and the weight gain index of the callus was calculated. The results were analyzed by GraphPad prism7.0 software.

Table 2: Glyphosate sensitivity screening concentrations

Test number	Herbicide Bar (mg mL^{-1})
1	6
2	8
3	10
4	12
5	14

Determining the Critical Concentration of Antibiotic Resistance Screening

The callus induced by the immature embryo of inbred line GSH9901 was inoculated into the screening medium containing different concentrations of antibiotics. The browning of the callus was counted after 20 days to determine the optimal weeding of the callus in the screening process. The concentration of antibiotics was used to weigh the callus with an electronic balance. After 20 days of screening, the weight was indexed, and the callus weight gain index was calculated. The results were analyzed by GraphPad prism 7.0 software.

Table 3: Antibacterial antibiotic (Cefotaxime sodium) susceptibility screening concentrations

Test number	Cefotaxime sodium (mg mL^{-1})
1	0
2	100
3	150
4	200
5	250
6	300

2.2.4 Callus Redifferentiation and Plant Regeneration

The callus induced by the immature embryos in inbred line GSH9901 was placed in a differentiation medium for light treatment for 30 days, and the medium was periodically changed. If the differentiated seedlings did not grow, the roots were transferred to a rooting medium for a 30-day light treatment. When a differentiated seedling grew to about 10-15 cm, and the main root was thick enough, sterile water was added, and the treatment was stored in the greenhouse for three days. The seedling was then removed, and the root medium washed off with tap water. Next, the seedlings were planted in pots (sterilized soil with vermiculite) to grow for 8-15 days in a greenhouse setting. Lastly, they were planted in a field setting, and the final result is regenerated corn plants.

3 Results and Discussion

3.1 Induction of Immature Embryo Callus in Maize

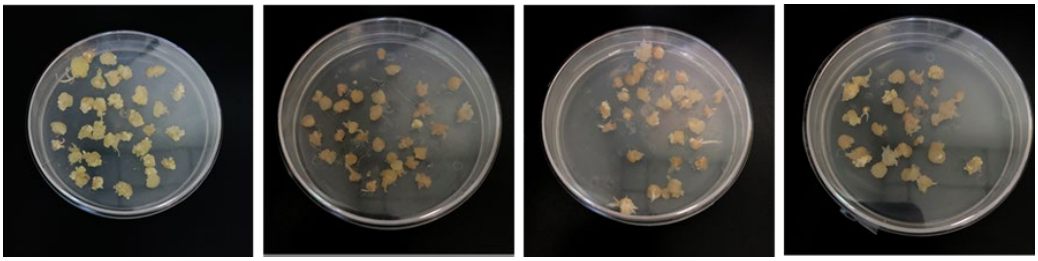
3.1.1 Effect of Different Inbred Maize Lines on Embryogenic Callus Induced by Immature Embryos

Tab. 4 shows that the rate of callus of four inbred lines, GSH9901, Hi01, Hi02, and Chang 7-2 was 92.3%, 80.7%, 81.5%, and 87.1% respectively. According to Fig. 1, GSH9901, the induced Type II callus were multiple, granular, fast-growing, loose, and easy to subculture. Genetic factors controlled the induction rate and regeneration ability of embryogenic callus, and different genotypes showed different results.

Table 4: 4 maize inbred lines out of the embryos

Inbred lines	Recovery rate (%)	Induction rate of type II callus (%)	Callus growth
GSH9901	92.3	83.1	Golden yellow, many sprouts,
Hi01	80.7	60.7	Milky white, less sprout, unloose
Hi02	81.5	62.3	Milky white, less sprout, unloose
Chang 7-2	87.1	80.5	Milky white, less sprout, unloose

* Note: The number of inoculations of different inbred lines is 500 per group.



A1 B1 C1 D1

Figure 1: Growth status of immature embryos from 4 maize inbred lines. A1: inbred line GSH9901; B1: inbred line Hi01; C1: inbred line Hi02; D1: inbred line Chang 7-2

3.1.2 Effects of the Length of Immature Embryo on the Formation of Callus

The initial growth length of GSH9901 embryos was 0.6-1.0, 1.1-1.5, 1.6-2.0, and 2.1-2.5 mm. The results obtained from the analysis software are shown in Fig. 2. The young embryo length was in the range of 0.6-2.5 mm. The average daily enlargement rate of immature embryos tended to decrease gradually following inoculation and was typically stable 14 days after inoculation. At the same number of days after inoculation, the immature embryos with initial lengths of 1.0-1.5 mm had a higher daily average enlargement rate; the immature embryos with initial lengths of 1.6-2.0 mm had the highest daily average enlargement rate, with the slow growth of embryo, strong enlargement ability of callus, and good callus status. In summary, the immature embryo length range was found to be most effective between 1.6-2.0 mm, and that finding is consistent with previous studies.

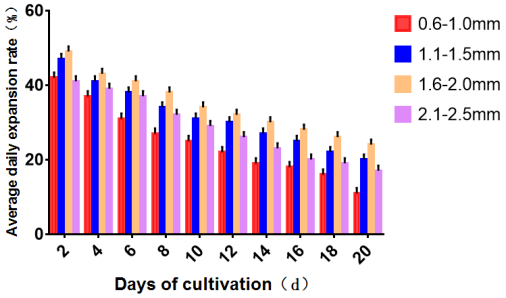


Figure 2: Daily average expansion rate of different lengths of GSH9901 immature embryos

3.1.3 Effects of Shield Face Orientation on Callus Formation

In a 25°C culture, the inoculation of GSH9901 immature embryo scutellum in the upward orientation can effectively inhibit the growth of germ and radicle, and easily induce the formation of embryogenic callus. The callus induction rate was 66.4%, which was higher than that of the shield face (41.6%). Callus formation is more successfully induced when the shield of the young embryo is facing upward (Tab. 5).

Table 5: Effect of shield face orientation of GSH9901 immature embryos on callus formation

Shield face	Callus number (a)	Callus induction rate (%)
Upward	83	66.4
Downward	52	41.6

3.1.4 Effects of 2,4-D Concentration, Proline Concentration, and Folate Concentration on the Formation of Callus

The results shown in Tab. 6 were obtained by the orthogonal test. The induction rate of callus varied significantly with the presence of three different additives-he greater the value of R, the greater the impact of this factor. According to the R-value in the table, the factors influencing the induction rate of callus were as follows: 2,4-D concentration (A) > proline concentration (C) > folic acid concentration (D); According to the average value of the callus induction rate at each level of each factor, the optimal level of each factor was determined as A_3 (2,4-D concentration 0.25 mg mL^{-1}), B_1C_3 (proline concentration 0.8 mg mL^{-1}), and D_2 (Folic acid concentration 0.5 mg mL^{-1}). The results of variance analysis (Tab. 7) showed that there was no significant difference between the third group, while the seventh group, the eighth group, and the other remaining groups did. The seventh group was the optimal combination.

Table 6: Range analysis of callus induction rate of GSH9901

Test number	2,4-D (mg mL^{-1})	Blank column	Proline (mg mL^{-1})	Folic acid (mg mL^{-1})	Callus induction rate
1	1 (1)	1	1 (0.6)	1 (1)	67
2	1	2	2 (0.7)	2 (0.5)	63
3	1	3	3 (0.8)	3 (0.25)	74
4	2 (0.5)	1	2	3	50
5	2	2	3	1	57
6	2	3	1	2	56
7	3 (0.25)	1	3	2	84
8	3	2	1	3	74
9	3	3	2	1	65
K_1	2.03	1.99	1.95	1.91	
K_2	1.62	1.93	1.80	2.00	
K_3	2.22	1.95	2.12	1.96	
K_1	0.68	0.66	0.65	0.64	
K_2	0.54	0.64	0.60	0.67	
K_3	0.74	0.65	0.71	0.65	
R	0.20	0.01	0.11	0.03	
Main order of factors	A > C > D > B				
Optimal level	A_3	B_1	C_3	D_2	
Optimal combination	$A_3B_1C_3D_2$				

* Note: The number in parentheses represents the concentration of the corresponding hormone.

K value: Corresponding level under each factor

R value: R = K max-K min

Table 7: Variance analysis table of callus induction rate of GSH9901

Test number	Callus induction rate (%)			Standard deviation	Significant degree
	I	II	III		1%
1	65	66	70	0.0265	C
2	65	59	65	0.0346	D
3	72	75	75	0.0173	B
4	46	55	49	0.0458	G
5	60	57	54	0.0300	E
6	55	57	56	0.0100	F
7	89	82	81	0.0436	A
8	78	71	73	0.0360	B
9	67	63	65	0.0200	CD

3.1.5 Effects of Different Cold Storage Time on Immature Embryo Induced Embryogenic Callus Formation

The cold storage pretreatment was completed at 4°C before the inoculation of the immature embryos. As shown in Fig. 3, cold storage exposure had little effect on the induction rate of primary callus, and the minimum callus induction rate was 81%. Cold storage did greatly influence the induction rate of embryogenic callus, and the minimum callus induction rate was only 7.0%. Cold storage at 4°C for 1 d is more conducive to the formation of embryogenic callus of inbred maize line GSH9901.

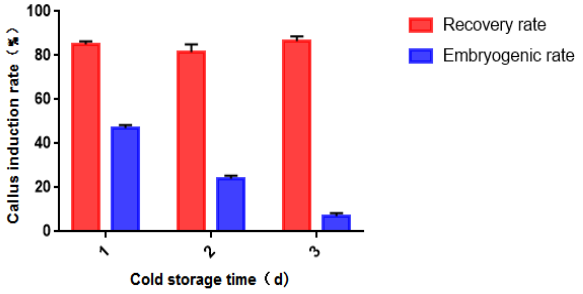


Figure 3: Effect of cold storage on callus induction rate at different times

3.2 Study on the Sensitivity of Corn Callus to Screening Material

3.2.1 Determination of Critical Concentration of Resistance Screening for Glyphosates

Fig. 4 shows that the callus weight gain index becomes significantly smaller, with the increase of glyphosate concentration. When the glyphosate reaches a specific concentration, the callus is completely sieved and will not be killed. The weight gain index was regenerated. With the increase of glyphosate concentration, the browning rate of callus increased correspondingly, and most of the callus browned until death. The survival of callus grew slowly and almost lost embryogenicity. Therefore, the optimum glyphosate (Bar) screening concentration of callus is 10 mg ml^{-1}.

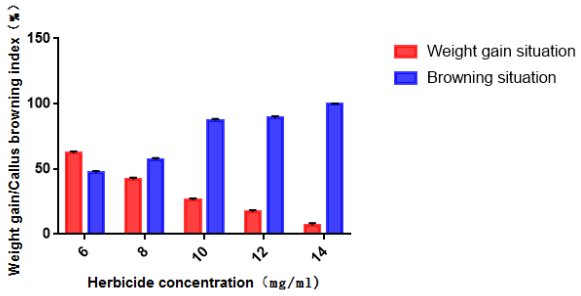


Figure 4: Effect of glyphosate concentration on GSH9901 callus

3.2.2 Determination of Critical Concentration of Antimicrobial Resistance Screening

Fig. 5 shows that with the increase of the concentration of antibiotics, callus weight index decreased significantly. After reaching a certain concentration of callus of all screen death will no longer generate weight index; Callus Browning rate also increased, and the most direct Callus Browning death, survival of the callus growth is relatively slow, the partial loss of embryogenic callus, therefore The Cefotaxime sodium screening concentration of 250 mg ml^{-1}.

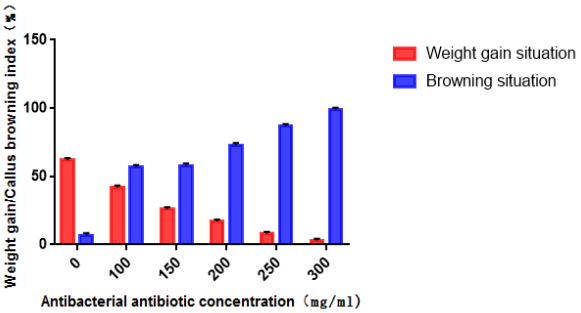


Figure 5: Effect of antimicrobial concentration on GSH9901 callus

3.3 Redifferentiation and Plant Regeneration of Callus

The calluses were placed in the differentiation medium. The callus tissue differentiated into green shoots after about 20 days Fig. 6(A2). 15% of the seedlings differentiated into rooted seedlings and 85% of them were differentiated into rootless seedlings, and were transferred to rooting cultures. Basalt As Fig. 6-B2 indicates, 85% of seedlings were rooted, and a small percentage were albino. Once the differentiated seedlings reach 10-15 cm and grow two to three roots, they are transplanted with hardening seedlings Fig. 6(C2). After growing into vigorous seedlings, they are transplanted into pots (Fig. 6(D2)) and finally transplanted into the field Fig. 6(E2) as regenerated maize plants.

A2 B2 C2 D2 E2

Figure 6: Callus Regeneration from maize inbred line GSH9901. A2: differentiation and culture； B2: rooting culture; C2: acclimatization; D2: transplant; E2: field

3.4 Discussion

Lu et al. [14] believe that maize genotypes are often important factors in determining the in vitro response. There are great differences in the regeneration ability of different genotypes, and adding different additives to the medium is one of the key factors to success. This study demonstrated that the young embryo-induced callus of inbred maize line GSH9901 is the most successful of the lines tested. The optimal combination of additives is A_3 (2,4-D concentration 0.25 mg mL^{-1}), B_1C_3 (proline concentration 0.8 mg mL^{-1}), and D_2 (folate Concentration 0.5 mg mL^{-1}). This combination of additives laid the foundation for the induction of high-quality embryogenic callus. When the immature embryos were inoculated into the N6 medium at 1.6-2.0 mm, the callus formation and differentiation of seedlings should be accounted for. The scutellum positioned in the upward orientation was more conducive to callus formation. Cold storage at 4°C for 1 d is more conducive to the formation of embryogenic callus. The sensitivity of the same inbred line to the glyphosate is also very different. The growth state of the good callus is more tolerant of the screening agent. According to reports in the literature, a high concentration of glyphosate is very important for the formation of callus. For large damage, this experiment establishes the basis for future experiments by determining the critical concentration of glyphosate 10 mg ml^{-1}. The antibiotic Cefotaxime sodium can effectively inhibit the contamination of the bacteria in the culture medium. In this experiment, the critical concentration of antibiotics was determined to be 250 mg ml^{-1} to more effectively reduce the bacterial infection rate in the culture process. During the process of callus re-differentiation, the inbred maize line GSH9901 has exhibited the phenomenon of low regeneration rate, browning, and emergence of albino seedlings.

4 Conclusion

At the stage of embryogenic callus induction, induction rate and regeneration ability is controlled by genetic factors of embryogenic callus. Different genotypes showed varying results, while embryo induction of inbred line GSH9901 was the most successful at callus production. The most effective immature embryo length proved to be in the range of 1.6-2.0 mm with the scutellum in the upward orientation. According to the average value of the callus browning rates at each level of each factor, the optimal level of each factor was A_3 (2,4-D concentration 0.25 mg mL^{-1}), B_1C_3 (proline concentration 0.8 mg mL^{-1}), and D_2 (folate Concentration 0.5 mg mL^{-1}). The induction rate was 84%, and cold storage at 4°C for 1 d was the most conducive to the formation of embryogenic callus. In the susceptibility test stage of callus screening, the optimal concentration of callus for glyphosate (Bar) was 10 mg ml^{-1}, and the optimum concentration of antibiotic (Cefotaxime sodium) was 250 mg ml^{-1}. This study provided a sound basis for the early induction of maize embryo callus induction technology.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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