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Full Length Research Paper

Further improvement of N6 medium for callus induction and plant regeneration from maize immature embryos

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To explore the possibility to improve N6 medium for callus induction and plant regeneration from maize immature embryos, comparative experiments were conducted to screen the optimal calcium concentration for the callus induction medium and the subculture medium modified from N6 medium and to examine the effects of uniconazole and ABT root promoting powder supplement on plant regeneration and root generation. According to the results, the calcium concentration of N6 medium was increased from 1.13 to 5 mmol/l for callus induction and subculture. Uniconazole from 0.25 and 0.5 mg/l and ABT root-promoting powder of 0.5 mg/l were suggested to be supplemented to the regeneration medium and the root generation, separately. On these improved media, the frequency of callus induction, the relative proliferation rate of callus subculture, the regeneration rate and the average root biomass of the regenerated plantlets increased significantly. All these improvements facilitate the establishment of acceptor system from elite parental inbred lines of commercial hybrids and provide a solid basis for transgenic manipulation of maize.

Key words: ABT root-promoting powder, calcium, callus induction, immature embryo, maize, N6 medium, plant regeneration, uniconazole.

INTRODUCTION

The establishment of acceptor system, easy to be transformed and regenerated for plants, remains an obstacle to transgenic manipulation in maize. Up to date, the most efficient approach is to induce calli from immature embryos before or after transformation (Wang et al., 2009). Comparing to rice and some other cereal crops, maize is particularly challenging for callus induction and plant regeneration, which heavily depends on several model inbred lines, such as Hi-II, A188, H99 and their derivatives (Rafiq et al., 2005; Frame et al., 2006; Usman et al., 2007; Vega et al., 2008; Sidorov and Duncan, 2009), as well as some tropical germplasms (Valdez-Ortiz et al., 2007; Petrillo et al., 2008). Because their agronomic characters are poor for agricultural practices, any interested gene introduced into these maize lines has to be transferred into elite genetic background by backcross breeding with the help of marker assisted selection. It is necessary to improve the media used for callus induction and plant regeneration and directly introduce the interested genes into parental inbred lines of commercial hybrids.

For callus induction and plant regeneration in maize, the most frequently used is N6 medium (Ishida et al., 2003; Rafiq et al., 2005; Usman et al., 2007; Valdez-Ortiz et al., 2007; Vega et al., 2008; Sidorov and Duncan, 2009), which was first designed for anther culture of rice by Chu et al. in 1975. Subsequently, intense efforts have been made to improve the recipe of N6 medium by adjusting its growth regulator combinations (Zhao et al., 2001; Ishida et al., 2003; Valdez-Ortiz et al., 2007; Vega et al., 2008). Among these improvements, no attention has been paid to the adjustment of calcium concentration in the basal salt mixture or to the supplement of exogenous plant growth retardants.

For its ubiquitous functions as the second messenger of signal transduction and its influence to cell growth and proliferation (Shao et al., 2008; Mazars et al., 2009),

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calcium is used as a key element in the medium recipes for plant tissue culture (Murashige and Skoog, 1962; Chu et al., 1975), although, it is classified into major elements. Increasing calcium concentration of MS medium was reported to stimulate the increase of the content of protein and sugar, the activity of Ca^{2+}-dependent protein kinases and peroxidase in calli, facilitating callus induction in carrot (Overvoorde and Grimes, 1994), fig (*Fucus carica* L.) (Wang et al., 1999), *Eucalyptus urophylla* (Arruda et al., 2000) and sandalwood (Anil and Rao, 2000).

Uniconazole ((E)-l-(4-chlorophenyl)-4,4-dimethyl-2-(l,2,4-triazol-l-yl)-l-penten-3-ol), a kind of triazole compound, inhibits the three oxidation steps from kaurene to kaurenoic acid in the biosynthesis of gibberellins (Izumi et al., 1985). One of the obvious responses of plants to uniconazole is that, the treated plants become darker green with more chlorophyll than the control (Khalil and Rahman, 1995). The regulation and protection effects of uniconazole could also induce tolerance to water deficiency (Fletcher and Hofstra, 1990; Zhang et al., 2007). In agricultural practices, uniconazole is applied as exogenous plant growth retardant to reduce unwanted shoot elongation of plants in a desired way without changing developmental patterns or being phytotoxic (Rademacher, 2000). In plant tissue culture, the workable concentration of uniconazole supplemented to MS media was reported to significantly promote callus induction and plant regeneration in asparagus (*Asparagus officinalis* L.) (Li and Wolyn, 1995), wheat (An et al., 1997), rice (Feng and Xue, 2001), tall fescue (*Festuca arundinacea* Schreb) (Qian and Xue, 2004) and potato (Huang et al., 2009).

Aminobenzotriazole (ABT) root-promoting powder was developed for shoot cutting propagation by Tang et al. (2004). Its active components include indole-3-butyric acid and indole-3-acetic acid, which are plant growth regulators. ABT root-promoting powder has been evaluated to facilitate root generation and increase survival rate of transplanted plants under abiotic stress (Zhang et al., 1994; Tang et al., 2004). Few documents are available about its application to plant regeneration from embryonic callus.

The present study was conducted to explore the possibility to improve N6 medium by increasing calcium concentration and supplementing uniconazole and ABT root-promoting powder for callus induction and plant regeneration from maize immature embryos.

MATERIALS AND METHODS

Medium recipe

The basal medium contained N6 salt mixture (Chu et al., 1975), B5 vitamins (Gamborg et al., 1968) and 30 g/l sucrose. The pH was adjusted to 5.8 prior to solidification with 6 g/l agar. The callus induction medium was modified from the basal medium by supplement of 1.38 g/l L-proline, 500 mg/l casein hydrolysate and 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). The subculture medium was modified from the basal medium by supplement of 0.69 g/l L-proline, 100 mg/l casein hydrolysate, 20 g/l mannitol and 1 mg/l 2,4-D. The regeneration medium was modified from the basal medium by supplement of 100 mg/l casein hydrolysate and 1 mg/l kinetin. The root generation medium was modified from the basal medium with supplement of 100 mg/l casein hydrolysate.

Screening of calcium concentration for callus induction medium

The calcium concentration of the callus induction medium was increased from 1.13 mmol/l (the primary concentration of N6 medium, Chu et al., 1975) to 2, 3, 4, 5, 6, 7 and 8 mmol/l, respectively. About thirteen days after pollination, ears were harvested from the parental inbred lines (48-2, 18-599W, 18-599R, 478, Zheng22, 08-64, 87-1, Qi319 and A188) of commercial hybrids, surface-sterilized one by one husk leaf using 70% alcohol, and rinsed three times with sterilized distilled water. Immature embryos between 1.5 and 2 mm length were excised from the kernels and inoculated scutellum-side up onto the surface of the induction medium. For each calcium concentration, 210 immature embryos were inoculated for dark culture at 27℃. Adventitious buds and roots were removed in time. After six weeks, the number of calli induced on each embryo was investigated and used to calculate the frequency of callus induction (number of embryos with callus induced / number of inoculated embryos) and the callus number per embryo. These two indices were used to evaluate the effect of increasing calcium concentration on callus induction. The optimal calcium concentration was determined for the callus induction medium.

Screening of calcium concentration for subculture medium

Referring to the result of the stated experiment, the calcium concentration of the subculture medium was increased from 1.13 mmol/l to 3, 4, 5, 6 and 7 mmol/l, respectively. For each concentration, 100 pieces of the friable embryonic calli (type II), identified according to the standard proposed by Armstrong and Green (1985), were transinoculated into 10 tubes containing the subculture media. The net weight of the transinoculated calli was calculated by subtracting the weight of the tubes containing the medium from the total weight. After three weeks of dark culture at 27℃, the relative proliferation rate of the calli ((net weight after culture-net weight before culture)/net weight before culture) was investigated and used to evaluate the effect of increasing calcium concentration on subculture growth. The optimal calcium concentration was determined for subculture medium.

Supplement of uniconazole to regeneration medium

To the regeneration medium containing 5 mmol/l of calcium, 0 (negative control), 0.25, 0.50 1 and 2 mg/l uniconazole was supplemented, respectively. For each concentration of uniconazole, 72 pieces of the embryogenic calli with the same duration of subculture were transinoculated into 24 tubes and cultured at 27℃ under illumination of 1000 μmol/m^2·s for a photoperiod of 12 h light/12 h dark. After seven weeks, the regenerated plantlets more than 1 cm high were counted and used to calculate the regeneration rate (100 × number of regenerated plantlets/number of transinoculated calli).

Table 1. Frequency of callus induction and callus number per embryo of eight inbred lines on induction media with varying calcium concentrations.

Inbred line		Calcium concentration (mmol/l)							
		1.13 (Control)	2	3	4	5	6	7	8
48-2	Frequency of callus induction (%)	60.5	58.6	68.1*	71.0**	71.4**	65.2	52.4	53.3
	Callus number per embryo	1.2	1.8**	2.3**	2.7**	3.2**	2.2**	1.9*	0.8**
18-599W	Frequency of callus induction (%)	56.7	69.5**	68.6**	75.7**	89.5**	73.3**	65.2*	50.5*
	Callus number per embryo	2.8	3.5**	3.3**	4.1**	4.5**	3.6**	2.4*	2.1**
18-599R	Frequency of callus induction (%)	54.8	60.5*	78.6**	86.7**	91.4**	88.1**	67.6**	61.4*
	Callus number per embryo	3.7	3.9	4.6**	5.0**	5.3**	4.6**	3.2*	1.6**
478	Frequency of callus induction (%)	42.4	50.5**	54.8**	64.3**	69.5**	70.5**	63.3**	44.8
	Callus number per embryo	0.4	0.8**	1.3**	1.7**	2.5**	2.7**	1.6**	1.1**
Zheng22	Frequency of callus induction (%)	37.1	39.5	48.1**	53.3**	56.2**	54.3**	40.5	38.6
	Callus number per embryo	0.2	0.7**	1.2**	1.5**	1.8**	1.4**	1.2**	0.4**
08-64	Frequency of callus induction (%)	50.5	53.3	61.4**	58.6**	62.4**	63.3**	52.4	48.1
	Callus number per embryo	0.1	0.7**	1.7**	2.2**	2.1**	1.6**	0.4**	0.5**
87-1	Frequency of callus induction (%)	56.3	55.5	59.8	69.2**	75.6**	63.3*	59.6	45.6
	Callus number per embryo	2.1	1.9	2.7**	3.5**	4.3**	2.5**	2.3	0.9**
Qi319	Frequency of callus induction (%)	50.5	55.7*	66.7**	68.6**	73.8**	61.0**	57.6*	50.0
	Callus number per embryo	1.1	1.7**	2.5**	3.7**	3.9**	2.1**	2.3**	0.9**
A188	Frequency of callus induction (%)	63.3	61.0	76.7**	82.4**	87.6**	75.7**	69.5	62.4
	Callus number per embryo	2.6	3.1**	4.2**	4.5**	4.7**	3.7**	2.3*	2.8

Note: * and ** stand for significant difference comparing to the control calcium concentration at $P < 0.05$ and $P < 0.01$ levels, respectively.

Supplement of ABT root-promoting powder to root generation medium

The root generation medium containing 5 mmol/l calcium, 0 (negative control) and 0.5 mg/l ABT root-promoting powder were supplemented, respectively. For each concentration of ABT root-promoting powder, 40 regenerated plantlets were transferred into 40 bottles and cultured at 27°C under illumination of 1000 µmol/m^2·s for a photoperiod of 12 h light/12 h dark. After three weeks, the root of each plantlet was cut off, washed and dried at 95°C to a constant weight. The root biomass per plantlet was investigated and used to evaluate the effect of ABT root-promoting powder supplement on root generation of the regenerated plantlets.

Statistical analysis

Multiple variance analysis of two factors was done using SPSS 18.0 software (http://www.spss.com/) to test the significance of the frequencies of callus induction, the number of induced calli per embryo, the relative proliferation rates of callus subculture, the regeneration rates and the average root biomass of the regenerated plantlets among the inbred lines and different concentrations of calcium, uniconazole and ABT root-promoting powder. The levels of significance were set at $P < 0.05$ and $P < 0.01$.

RESULTS

Optimal calcium concentration for induction medium

The result of variance analysis showed that the frequencies of callus induction and the callus numbers per embryo were significantly different among the eight the calcium concentrations and among the eight inbred lines. The interaction effect between the calcium concentrations and the inbred lines was not significant. Except 478 and 08 to 64, all the other seven inbred lines had the highest values of the frequency of callus induction and the callus number per embryo at 5 mmol/l of calcium concentration (Table 1). Therefore, the optimal calcium concentration for the induction medium was determined to be 5 mmol/l.

Optimal calcium concentration for subculture medium

Although, the eight inbred lines showed different responses to the increase of the calcium concentration of the subculture medium, the highest values of their relative proliferation rates distributed around 5 mmol/l of calcium concentration. Except Qi319, all the other eight inbred lines had the highest values of relative proliferation rates at 5 mmol/l of calcium concentration (Table 2). On the subculture medium of this calcium concentration, the calli looked friable and fresh yellow and the average of their relative proliferation rates was 80% higher than that of the control concentration. Therefore, the optimal calcium concentration for the subculture medium was determined

Table 2. Relative proliferation rate of embryonic calli on subculture media with varying calcium concentrations.

Inbred line	Calcium concentration (mmol/l)					
	1.13 (Control)	3	4	5	6	7
48-2	2.52	2.89*	3.67**	5.03**	4.85**	4.52**
18-599W	3.73	5.27**	6.73**	8.16**	7.64**	7.35**
18-599R	4.03	5.31**	5.89**	7.01**	6.48**	6.29**
478	3.22	3.36	4.79**	4.80**	4.67**	3.57*
Zheng22	2.80	5.32**	5.57**	5.43**	4.68**	4.53**
08-64	2.95	3.29*	4.42**	4.89**	4.56**	3.45**
87-1	3.76	3.68	4.57**	5.41**	5.38**	3.21*
Qi319	3.80	4.67**	5.79**	6.34**	6.76**	5.93**
A188	4.00	4.73**	6.48**	7.42**	7.35**	5.69**

Note: * and ** stand for significant difference comparing to the control calcium concentration at $P < 0.05$ and $P < 0.01$ levels, respectively.

Figure 1. Effect of uniconazole supplement on plant regeneration. (A) The plantlets of inbred line 18-599W regenerated on the regeneration medium supplemented with 0.5 mg/l uniconazole. (B) The plantlets of inbred line 18-599W regenerated on the regeneration medium without uniconazole supplement.

to be 5 mmol/l.

Effect of uniconazole to plant regeneration

The supplement of uniconazole showed significant promotion effect to plant regeneration. The regenerated plantlets became stronger than the negative control (Figure 1). For inbred lines 18-599R, 478 and Qi319, the highest values of regeneration rate were obtained at 0.25 mg/l of uniconazole concentration, while the other six lines had the highest values at 0.5 mg/l of uniconazole concentration (Table 3). Therefore, the optimal uniconazole concentration for the regeneration medium should be between 0.25 and 0.5 mg/l.

Effect of ABT root-promoting powder to root generation

The supplement of 0.5 mg/l ABT root-promoting powder showed significant promotion to root generation of the regenerated plantlets (Figure 2). The average root biomass per regenerated plantlet regenerated on the root

Table 3. Regeneration rate of embryonic calli induced from eight inbred lines on regeneration media with varying concentrations of uniconazole.

Inbred line	Uniconazole concentration (mg/l)				
	0 (Control)	0.25	0.5	1	2
48-2	32.5	42.8**	43.6**	45.0**	34.5
18-599W	63.7	75.2**	87.7**	78.1**	57.4
18-599R	74.0	85.9**	75.3	70.0	36.4**
478	43.2	54.7**	53.3**	49.8**	41.6
Zheng22	42.8	54.3**	65.7**	55.3**	34.8**
08-64	32.9	30.9	44.4**	40.8**	34.5
87-1	41.1	51.6**	58.7**	4.37	30.6**
Qi319	53.8	75.7**	64.7**	60.3*	36.6**
A188	64.0	74.3**	86.4**	71.4*	47.3**

Note: * and ** stand for significant difference comparing to the negative control at $P < 0.05$ and $P < 0.01$ levels, respectively.

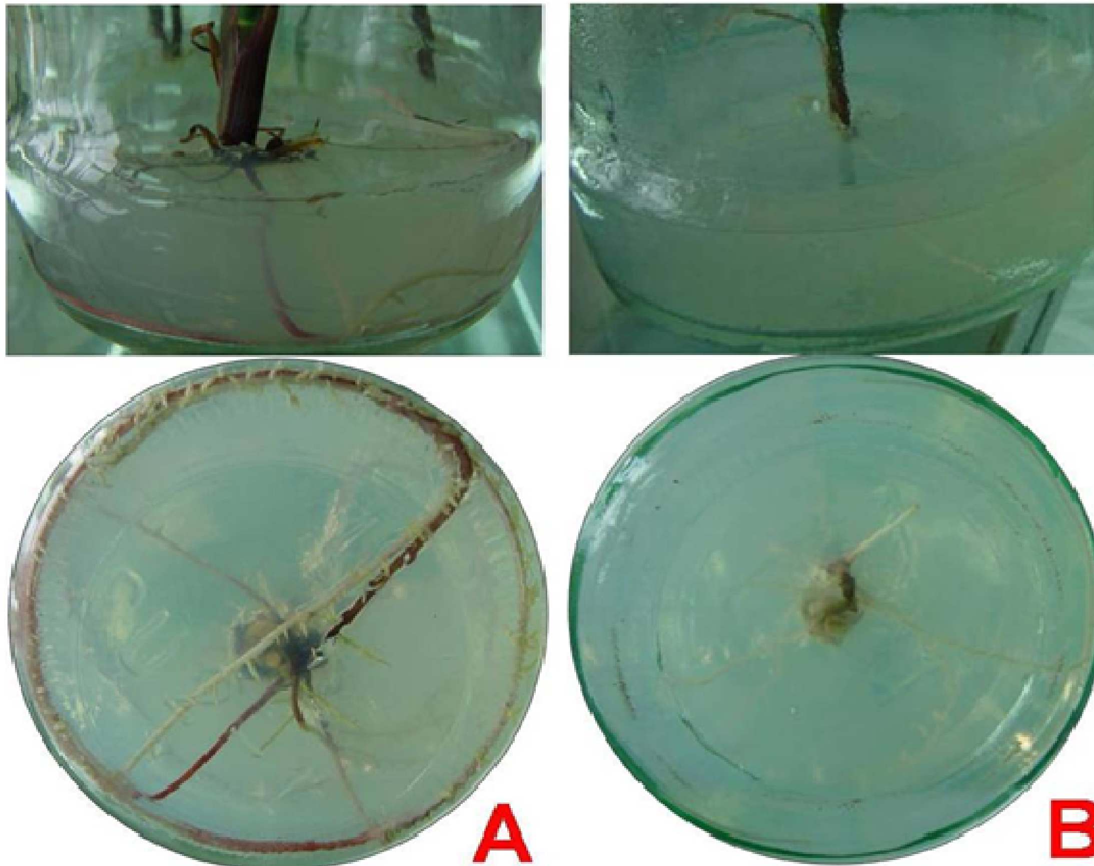

Figure 2. Effect of ABT root-promoting powder supplement to root generation of regenerated plantlets. (A) The plantlet and root of inbred line 18-599W regenerated on the root generation medium supplemented with 0.5 mg/l ABT root-promoting powder. (B) The plantlet and root of inbred line 18-599W regenerated on the root generation medium without ABT root-promoting powder supplement.

generation medium supplemented with 0.5 mg/l ABT root-promoting powder was significantly heavier than that regenerated on the negative control of the root generation medium (Table 4).

Table 4. Average root biomass (g) per plantlet of eight inbred lines regenerated on root generation medium supplemented with ABT root-promoting powder.

Concentration of ABT root-promoting powder	48-2	18-599W	18-599R	478	Zheng22	08-64	87-1	Qi319	A188
0 (control)	0.47	0.43	0.48	0.23	0.38	0.36	0.29	0.40	0.35
0.5 mg/l	0.59**	0.62**	0.64**	0.31**	0.51**	0.42**	0.42**	0.47**	0.47**

Note: ** stands for significant difference comparing to the negative control P < 0.01 level.

DISCUSSION

In the present study, the effect of increasing calcium concentration of N6 medium was evaluated to improve embryonic callus induction and plant regeneration from maize immature embryos. The optimal calcium concentration (5 mmol/l) for callus induction and subculture is in accordance with the optimal calcium concentration of MS medium for callus induction in carrot (3 mmol/l, Overvoorde and Grimes, 1994) and *E. urophylla* (6.62 mmol/l, Arruda et al., 2000). However, the optimal calcium concentration of MS medium for callus subculture in fig (*F. carica* L.) is as high as 40 mmol/l (Wang et al., 1999). This may be due to species specificity of acceptable calcium concentration. Otherwise, because the primary calcium concentration of N6 medium (1.13 mmol/l) is much lower than that of MS medium (3 mmol/l) (Murashige and Skoog, 1962; Chu et al., 1975), the effect of increasing calcium concentration of N6 medium is intelligibly more significant.

In the previous reports about uniconazole supplement to improve callus induction and plant regeneration on MS medium, the workable concentrations of uniconazole supplement spanned an extensive range from 0.01 to 5 mg/l (Li and Wolyn, 1995; An et al., 1997; Feng and Xue 2001; Qian and Xue, 2004; Huang et al., 2009). In the present study, the optimal uniconazole concentration for the regeneration medium was screened to be between 0.25 and 0.5 mg/l, which was moderate in the earlier mentioned range. Higher concentrations of uniconazole supplement inhibited the growth of the regenerated plants to a great extent, resulting in abnormal development of ear and tassel.

The promotion of aminobenzotriazole (ABT) root-promotion powder supplement to root generation of the regenerated plants should be ascribed to effective combination of its active components, indole-3-butyric acid and indole-3-acetic acid (Tang et al., 2004), which are the key auxins for the modulation of cell division and elongation, regulating vascular development, apical dominance and organ patterning (Woodward and Bartel, 2005; Tognetti et al., 2010). Combined with the effect of uniconazole, the supplement of ABT root-promoting powder adjusts endogenous hormones, facilitates chlorophyll formation and root generation of the regenerated plants and improves tolerance to water deficiency when transplanted from the regeneration medium to nutrient soil. The detail mechanism remains to be researched.

In summary, we have further improved N6 medium by increasing its calcium concentration and supplementing with uniconazole and aminobenzotriazole root-promoting powder to its recipes for plant regeneration and root generation. The frequency of callus induction, the relative proliferation rate of callus subculture, the regeneration rate and the root generation of the regenerated plantlets were increased significantly. All these improvements facilitate the establishment of acceptor system from elite parental inbred lines of commercial hybrids and provide a solid basis for transgenic manipulation of maize.

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