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影响小麦成熟胚再生频率因素的研究(简报)*

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摘要 以小麦成熟胚为外植体，研究了基本培养基、预处理类型、接种方式、植物激素的浓度和不同组合以及分化培养基中是否添加抗生素对愈伤组织诱导和分化的影响，在此基础上建立了一套高效的小麦成熟胚植株再生系统。经过试验，我们选择在MS培养基上接种经无菌水预处理的纵切成熟胚作为起始的试验条件。在含2mg/L 2,4-D的MS培养基上，初级愈伤组织的诱导频率可达80%以上，在继代培养基中添加0.5mg/L 6-BA和0.2mg/L NAA可以显著提高胚性愈伤组织的产生。而在再生培养基中加入适当浓度的头孢霉素可以有效提高胚性愈伤组织再生出小植株的比例。利用该再生系统，我们从5个小麦优良主栽品种的成熟胚再生出了可育的植株，再生频率达15.3%-34.5%。

关键词：小麦 成熟胚 愈伤组织 组织培养 植株再生

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FACTORS AFFECTING EFFICIENT PLANT REGENERATION FROM WHEAT MATURE EMBRYOS

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Wheat is a widely grown cereal crop worldwide. Also, it is one of the most important fodder crops in industrialized countries and many developing countries. Under the pressures exerted by limited land, expanding population, and environmental stresses, the great demand for wheat of both quality and quantity requires more rapid genetic improvement of wheat. Genetically transformed wheat plants have been achieved by various approaches, such as biolistics[1] and *Agro-bacterium* infection[2]. However, an efficient plant regeneration system is a prerequisite for most of the approaches.

Compared with immature embryos, which were documented as the most frequently used explants, the use of mature embryos has remarkable advantages. Thus, the need for growing donor material in green houses requiring intensive labor and space can be avoided, and especially for winter genotypes, no additional time input due to vernalization is required. Moreover, dry seeds are available in large quantity and its physiological state shows minimal variability, an important trait for plant tissue culture. O'Hara and Street[3] first reported successful callus induction from wheat mature embryos, but failed to obtain regenerated plantlets. Zhou and Lee[4] investigated the effects of different auxins on wheat mature embryos culture and achieved successful plant regeneration for the first time, followed by a few reports demonstrating the a-

bility to undergo somatic embryogenesis and subsequent plant regeneration, but with low regeneration frequencies. The objective of this research was to develop an efficient plant regeneration system from mature embryos of wheat for the ultimate utility in genetic improvement of this crop.

Materials: mature dry seeds of five wheat genotypes (Yangmai 5, Yangmai 10, Yangmai 158, Jinan177 and Yumai 47) were used as sources of mature embryos. All seeds were surfaces sterilized with 70% ethanol for 1min, and 0.1% $HgCl_2$ for 15min. The sterilized seeds were rinsed six times with sterilized water then imbibed in 8mg/L 2,4-D or sterile water (5h, 33℃). The swollen mature embryos were removed from seeds with a scalpel and radicles were then separated from plumules on scutellar nodes. The plumule section was longitudinally sliced into halves and then plated cut-side down on induction media. In parallel, intact mature embryos were also plated to determine their callus induction response.

Primary callus induction: five basal media, MS, LS, N_6, B_5 and P[5] were tested for mature embryos culture. All these media were supplemented with 2mg/L 2,4-D (except for other optimization tests) and 30g/L sucrose. The pH was adjusted to 5.8 prior to the addition of agar (7g/L). Then the media were sterilized by autoclaving at 115℃ for 20min. Mature embryos were incubated for callus induction on the media at 25℃ in darkness. After 5-7 days, germinated shoots and roots from mature embryos were cut out completely and the

Table 1 Effects of combinations of three factors on callus culture and plant regeneration from wheat mature embryos(y158)

Serial	Factor			Primary callus*	Embryogenic callus**	Culture***
Number	A	B	C	induction	induction	efficiency
1	MS	D+	I	90.4±5.1^{ab}	35.0±3.2^{c}	20.4±4.0^{bc}
2	MS	D+	L	95.3±3.4^{a}	43.8±1.3^{a}	22.8±1.1^{ab}
3	MS	D-	I	88.3±6.7^{bc}	37.9±2.1^{b}	21.3±1.8^{bd}
4	MS	D-	L	92.8±1.8^{a}	40.3±2.9^{ab}	24.9±0.8^{a}
5	LS	D+	I	82.9±8.0^{cd}	32.8±3.6^{cd}	19.8±3.5^{bc}
6	LS	D+	L	87.8±5.8^{bd}	33.4±7.0^{bd}	21.0±2.8^{bc}
7	LS	D-	I	84.3±7.3^{c}	28.9±0.9^{d}	17.8±0.9^{bcde}
8	LS	D-	L	86.6±1.9^{bc}	30.0±5.9^{bcd}	20.3±2.3^{bde}
9	N6	D+	I	78.5±8.1^{d}	27.3±3.8^{ce}	14.9±1.9^{cdf}
10	N6	D+	L	83.7±4.8^{bc}	30.1±3.9^{de}	16.3±5.9^{bd}
11	N6	D-	I	69.0±2.9^{e}	28.9±3.5^{d}	15.3±3.8^{bd}
12	N6	D-	L	79.3±2.1^{bcd}	29.9±4.0^{cd}	12.8±4.1^{ef}
13	B5	D+	I	53.9±2.8^{f}	19.3±7.0^{f}	8.3±5.1^{g}
14	B5	D+	L	57.8±0.7^{ef}	20.0±1.8^{ef}	9.8±1.0^{fg}
15	B5	D-	I	50.7±6.9^{g}	16.5±2.9^{g}	7.8±3.7^{g}
16	B5	D-	L	51.7±3.9^{fg}	18.6±5.0^{fg}	10.0±2.8^{ef}
17	P	D+	I	78.3±4.1^{cd}	28.8±4.0^{bd}	11.8±0.8^{cde}
18	P	D+	L	73.9±7.8^{cde}	29.0±2.3^{cd}	12.0±1.8^{ef}
19	P	D-	I	80.9±1.2^{cd}	27.3±0.3^{bce}	12.3±2.0^{ef}
20	P	D-	L	83.0±0.9^{bc}	24.0±1.7^{ce}	14.3±3.3^{bcdf}

Means denoted by different letters (a-g) in a column are significantly different at $P<0.05$.

Factor A: the basal medium; Factor B: D+, preinoculation with 8mg/L 2,4-D, D-, preinoculation with distilled water; Factor C: I, intact embryo, L, longitudinally bisected embryo.

*Primary callus induction was counted as the number of mature embryos that formed callus/the number of mature embryos cultured × 100.

**Embryogenic callus induction was assessed as the number of mature embryos forming embryogenic callus/the number of mature embryos explanted × 100.

***Culture efficiency was estimated as the total number of shoots developed/ the number of mature embryos explanted × 100.

callus responding explants plated again in the same Petri dish for further callus proliferation. Four weeks later, primary callus induction rate was evaluated.

Embryogenic callus formation and maintenance: to test the effects of plant growth regulators on embryogenic callus formation, different combinations of 2,4-D, NAA and 6-BA were added to the subculture medium. Cultures for embryogenesis were incubated under low light conditions (10 μmol m^{-2} s^{-1}) at 25℃ and a 16/8h (light/dark) photoperiod. The cultures were transferred onto fresh subculture medium every 2 weeks. After 6 weeks of culture on subculture medium, the embryogenic calli were evaluated.

Plant regeneration, root strengthening and trans plant of regenerated plants: embryogenic calli were then designated for regeneration. We investigated the effects of plant growth regulators and antibiotics (cefotaxime and carbenicillin) on plant regeneration. Filter-sterilized antibiotics were added to the media after autoclaving and cooling. Cultures were maintained at 25℃ under a 16/8h photoperiod with dime light (30μmol m^{-2} s^{-1}). After 4 weeks, the percentage of plant regeneration and culture efficiency were calculated. Well-developed plantlets (5-6cm in length) were then transferred to rooting medium consisting of 1/2 MS medium + 20g/L sucrose + 7g/L agar devoid of plant growth regulators. Cultures for rooting were exposed to a high light intensity (80-90 μmol m^{-2} s^{-1}, 25℃, 16/8h photoperiod). After 2-3 weeks, the plantlets with healthy roots were removed from culture, rinsed in water to remove media, and transplanted into sterilized soil, and grown under humid conditions in a growth room for 2 weeks. Plants were vernalized for an additional 3 weeks at 4℃ then grown to maturity.

Determination of initial culture conditions

By careful selection of uniform and healthy seeds, the first genotype, Y158, was cultured to investigate basal media, inoculation methods and pretreatment methods on callus culture and plant regeneration. Our statistical results were presented in Table 1. On MS media, the longitudinally bisected embryos gave the highest embryogenic callus induction rate and culture efficiency. Given that higher concentrations of 2,4-D might result in a greater possibility of mutation, we determined the optimal combination as follow: MS basal medium + longitudinally bisected mature embryos + pretreatment of seeds with distilled water.

Effect of 2,4-D on the induction of primary callus and embryogenic callus

The appearances of small calli from mature embryos were observed 3-4 days after the mature embryos were plated. These were the primary calli observed as translucent, rough and somewhat watery structure (PLATE Ⅰ, Fig.A, B). The induction of primary calli ranged from 71.6%-98.7% depending on the 2,4-D concentrations (Fig. 1). Calli could not be induced in induction medium devoid of 2,4-D and mature embryos readily germinated within 2-3 days of culture. Embryogenic callus induction frequency reached the highest level with 2 mg/L 2,4-D in the induction medium. Higher concentration of 2,4-D did not significantly change induction of the primary callus. Because higher concentrations of 2,4-D might result in a greater possibility of somatic mutation, 2.0mg/L 2,4-D was used for further optimization studies.

Further optimization of culture protocol with different combinations of 2,4-D, 6-BA and NAA

The primary calli were then transferred onto fresh subculture medium containing 2.0mg/L 2,4-D in combinations with 6-BA and NAA. Following 3 subcultures, the embryogenic calli became visible as compact, friable, irregularly shaped, light-yellow or creamy structure (PLATE Ⅰ, Fig. C). Organized somatic embryos in the surface of callus were visible under the anatomical len (PLATE Ⅰ, Fig.D). Other calli gradually turned brown and died. Fig.2 showed the effects of different combinations of 2,4-D, 6-BA and NAA on embryogenic callus formation. The frequency of embryogenic callus formation varied depending on the combinations of applied plant growth regulators. Low concentrations of 6-BA (0.2mg/L, 0.5mg/L) promoted embryogenic callus formation, although brown embryogenic callus and restraint of subsequent plant regeneration was noted with high levels (1mg/L). Interestingly, low concentrations of

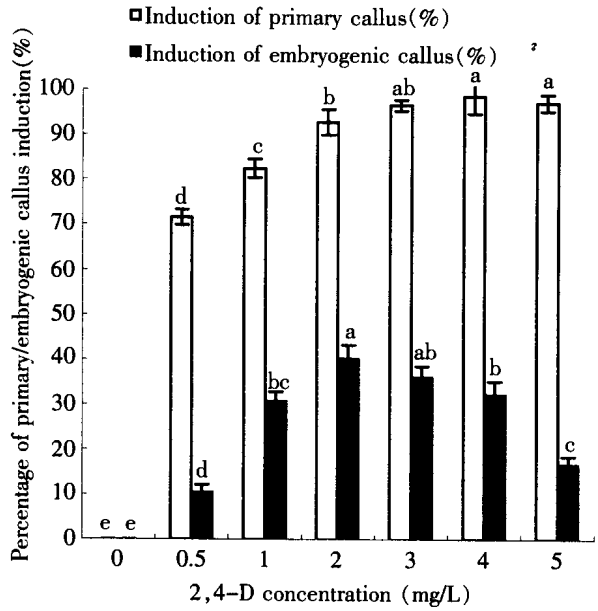

Fig.1 Effects of 2,4-D on primary callus induction and embryogenic callus from wheat mature embryos(y158)

Columns denoted by different letters (a-e) are significantly different at $P<0.05$.

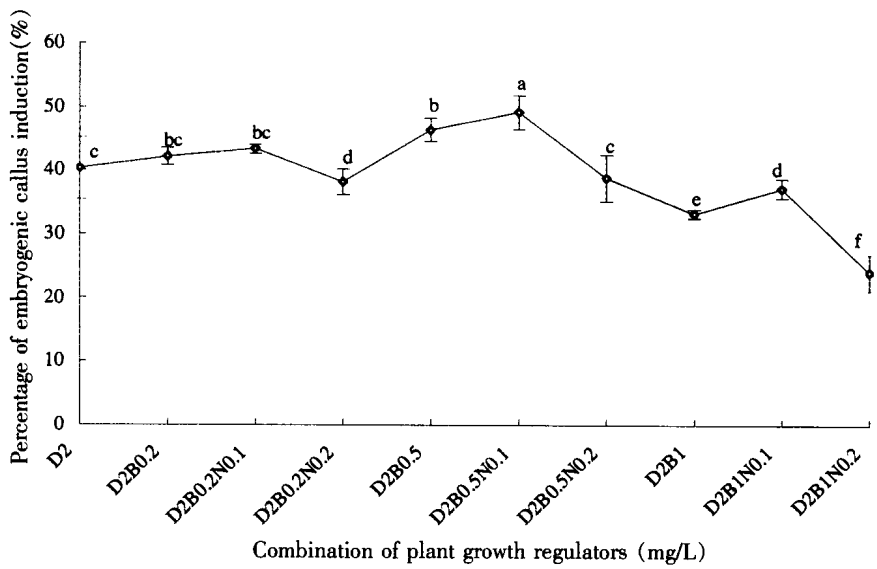

Fig.2 Effects of plant growth regulators on embryogenic callus formation

D2B0.2N0.1 under the spot represents 2,4-D 2.0 mg/l, BA 0.2 mg/l and NAA 0.1 mg/l, etc. Spots followed by different letters (a-f) are significantly different at $P<0.05$.

NAA may cooperate with 6-BA in promoting embryogenic callus formation. However, increased level of NAA (0.2mg/L) resulted in root hair-like structures on the calli surface, which inhibited the subsequent plant regeneration. Of the combinations tested, 2.0mg/L 2,4-D, 0.5mg/L 6-BA and 0.1mg/L NAA was the most effective, with the highest frequency of forming embryogenic callus (49.3% for Y158).

Effects of plant growth regulators and antibiotics (cefotaxime, carbenicillin) on plant regeneration

Spontaneous formation of green spots was observed after embryogenic calli were transferred onto regeneration medium free of plant growth regulators (PLATE Ⅰ, Fig.E) and plantlet regeneration was readily noticeable within two weeks (PLATE Ⅰ, Fig. F). We found that adding plant growth regulators to the regeneration medium not only did not enhanced re-

Table 2 Effects of plant growth regulators and antibiotics (cefotaxime, carbenicillin) on plant regeneration(y158)

Regeneration medium (mg/L))		Number of explants*	Regeneration capacity (%)**	Number of shoots per explant
MS_0		40	39.2±1.6^{c}	2.21^{a}
0.1mg/L 2,4-D+10mg/L zeatin		40	30.7±1.0^{e}	1.02^{d}
Cefotaxime (mg/L)	50	38	46.7±2.3^{bc}	1.76^{ab}
	75	35	51.9±3.0^{b}	2.02^{a}
	100	40	55.4±2.1^{a}	2.37^{a}
	125	41	49.8±0.9^{b}	2.16^{a}
Carbenicillin (mg/L)	40	38	38.4±2.9^{cd}	1.17^{b}
	60	40	36.4±3.8^{cd}	1.29^{b}
	80	40	33.9±1.6^{de}	1.08^{d}
	100	40	31.4±2.5^{e}	1.19^{c}

* Embryogenic calli transferred onto regeneration medium.

** Regeneration capacity was calculated as the number of green plants regenerated/number of explants plated × 100. Means denoted by different letters (a–e) in one column are significantly different at $P<0.05$.

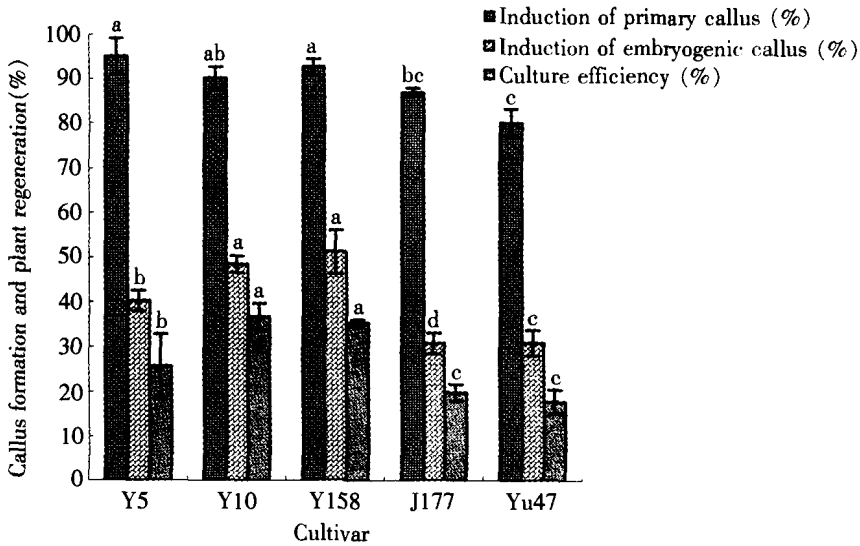

Fig.3 Callus induction and plant regeneration from wheat mature embryos in five elite genotypes

Columns followed by different letters (a–d) are significantly different at $P<0.05$.

generation capacity, but significantly decreased the number of shoots per embryogenic callus (Table 2).

In this study, we also tested the effects of cefotaxime and carbenicillin on plant regeneration. Interestingly, the stimulative effect of cefotaxime on plant regeneration resulted from higher regeneration capacity, not from higher average number of shoots per explant (Table 2). Cefotaxime at 100mg/L appears to be the optimal level, giving the highest regeneration capacity (55.4% for Yang158). As to carbenicillin, no significant differences were detected among 40, 60 and 80mg/L for regeneration capacity although regeneration capacity decreased gradually. But it was noted that higher level (100mg/L) strongly inhibited plant regeneration, with regeneration capacity decreasing to 31.4%. Many of the regenerated plantlets became browny gradually and exhibited teratogenesis to some extent. As to average number of shoots per explant, it was significantly affected by carbenicillin at all tested levels.

Establishment of the protocol for plant regeneration in other genotypes

For the induction of a strong root system, well-developed plantlets were transferred to rooting medium. Plantlets with well – developed roots were then transferred to soil (PLATE Ⅰ, Fig. G). After vernal-

ized for 3 weeks, they were grown to maturity in a greenhouse. Nearly all of the established plants were morphologically normal and producing normal seeds (PLATE Ⅰ, Fig. H).

Using this optimized protocol, plantlets were regenerated from mature embryos of all 5 genotypes. Concerning the induction of primary callus, all genotypes showed positive responses, with the frequency higher than 80% in all genotypes (Fig.3). With respect to embryogenic callus formation, significant genotype specific differences were observed. The cv. Y158 gave the highest percentage followed by cv. Y10, whereas a significantly lower percentage of callus formation was recorded for cvs Y5, J177 and Yu47. Besides that, genotype-specific difference also appeared for culture efficiency where cv.Y10 gave the best result. In short, culture efficiency presented in the current research ranged from 17.8% to 36.8%.

According to Delporte[5], a segmentation of mature embryos was shown to improve somatic embryogenesis and plant regeneration in wheat mature embryos culture. Culture medium was another key factor in callus culture. In addition, pretreatment of cereal crop seeds in 2,4-D solution before culture was well documented in recent researches[6]. However, these studies only investigated the effect of one factor. It is widely known that callus growth and development are influenced by a complex relationship between the explants status and the components of the medium. Consequently, we utilized a randomized complete block design with a factorial arrangement of 3 experimental factors. This calculation considered the interaction between factors, improving experimental efficiency and reducing experimental error. It also paved the way for further optimization tests.

Our data suggested the addition of 2,4-D in culture medium was essential for wheat callus induction and embryogenic callus formation from mature embryos and the optimal level was identified as 2mg/L. Former studies indicated that addition of cytokinin into culture medium was important for embryogenic callus formation. In the current study, addition of 6-BA during subculture greatly promoted embryogenic callus formation in wheat mature embryo culture. Our data also suggested that lower levels of NAA displayed a cooperative effect with 6-BA in promoting embryogenic callus formation.

Antibiotics, two in particular, cefotaxime and carbenicillin, have been used extensively to eliminate *Agro-bacterium* from the culture medium after transforming the explants. However the influences of these antibiotics on callus morphogenesis and plant regeneration were also noted for some plant species[7,8]. So the influences of these antibiotics on callus morphogenesis and plant regeneration should be further investigated. Our results showed that 100mg/L cefotaxime was beneficial to shoot regeneration. A possible explanation for the activity of cefotaxime in culture is that it is converted by cell metabolism to an unknown compound with phytohormone activity. But there have not been convincing results up to now. As to carbenicillin, it may break down to give physiologically active level of the auxin phenylacetic acid[9]. As a result, the normal endogenous auxin/cytokinin balance in the explants would be changed. This could be an explanation for the reduced plant regeneration observed in our research. However, to our knowledge, there are no references in the literature to the degradation products of carbenicillin. So related investigations should be carried out based on HPLC and other chemical analysis.

In conclusion, we have investigated the factors affecting wheat regeneration from mature embryos and established an efficient plant regeneration system. These results suggest that it might be possible to improve regeneration from mature embryos by optimizing the compositions of both subculture and regeneration media for specific genotypes. This system could pave the way for genetically transforming elite genotypes of wheat independent of the gene transfer method chosen.

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EXPLANATION OF FIGURES

PLATE I

Callus induction and plant regeneration from isolated mature embryos of wheat (bar=1mm).

Fig.A Longitudinally sliced mature embryos on induction medium;

Fig.B The primary calli on induction medium;

Fig.C The embryogenic calli on subculture medium;

Fig.D Morphology of an embryogenic callus (Arrow for developing somatic embryo);

Fig.E Green spots formed on embryogenic calli;

Fig.F Green shoots of embryogenic calli formed on regeneration medium;

Fig.G Well-developed plantlets;

Fig.H Normal fertile plantlets regenerated from mature embryos growing in greenhouse

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