

Jian GU, Kun LIU, Shaoxiang LI, Yuxian TIAN, Hexian YANG, Mujun YANG

Study on the *in vitro* culture of cut plants in wheat haploid embryo induction by a wheat × maize cross

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Abstract The wheat × maize system is one of the most effective ways to produce haploids in wheat. Whether and how it could be successfully applied in practical breeding mostly depends upon the efficiency of haploid embryo production. To perfect the protocols of haploid embryo induction, the efficiency of haploid embryo production between *in vitro* culture of cut plant and intact plant growth for hybrid spikes with two F₁ wheat hybrids and two maize varieties was compared. Effects of different cutting plant times and formulas of nutrient solutions for cut plant culture on haploid embryo formation were also studied. Results indicated that the embryo rate of *in vitro* culture was 3.29 times that of intact plant growth, with the figures of 31.6% vs 9.6%, respectively. The optimal time for cut plant culture was 24 h after pollination. Formulas of nutrient solutions significantly affected the efficiency of haploid embryo induction. With an embryo rate of 0–35.5%, adding calcium phosphate in the culture solution at 3 g·L⁻¹ could raise the caryopsis and embryo rates. According to this study, the best medium for cut plant culture was: 100 mg·L⁻¹ 2,4-D+40 g·L⁻¹ sucrose+10 mg·L⁻¹ silver nitrate+8 mL·L⁻¹ sulfurous acid+3 g·L⁻¹ calcium phosphate, with which a caryopsis rate of 95% and an embryo rate of about 30% could be obtained.

Keywords wide cross between wheat and maize, *in vitro* culture of cut plants, haploid embryo induction

1 Introduction

Wheat haploid induction through a wheat × maize cross is one of the most effective ways to produce wheat haploids (Laurie and Bennett, 1986; Aditya et al., 2006). The

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Jian GU (✉), Kun LIU, Shaoxiang LI, Yuxian TIAN, Hexian YANG, Mujun YANG (✉)
Food Crops Institute, Yunnan Academy of Agricultural Sciences, Kunming 650205, China
E-mail: gujian@yaas.org.cn; kmyangmj@163.com

key problems for its application in wheat breeding are the lower efficiency and poor stability of haploid embryo induction, normally at 20%–30%, ranging from 5% to 59% (Laurie and Reymondie, 1991; Li et al., 1996; Chen et al., 1998). The production of haploid embryos can be affected by many factors, including maize and wheat genotypes (Wang, 1998; Chen et al., 1998; Vinesh et al., 1999; Cai et al., 2005), quality of maize pollen (Laurie, 1989), ways and times of pollination (Laurie, 1989; Chen and Chen, 1998), wheat floret position and development status during pollination (Vinesh et al., 1999; Gu et al., 2005), the concentration, ways and times of 2,4-D treatment (Laurie, 1989; Sun et al., 1992; Almouslem et al., 1998; Wang, 1998), soil and air humidity, and light conditions after pollination (Chen and Chen, 1998; Lu et al., 2002; Gu et al., 2006). To minimize the influence of varying natural conditions on haploid induction, pollinated hybrid spikes were cut and cultured in controlled conditions instead of growing them in the field, which produced higher and more stable haploid embryo rates (Riera-Lizarazu et al., 1992; Suenaga et al., 1997; Inagaki, 1997; Cherkaoui et al., 2000; Cai et al., 2005). In our study, we conducted a group of extensive experiments to further perfect the technical details about the *in vitro* culture of cut plants.

2 Methods

2.1 Materials

Two wheat F₁s, C49S-87/01Y1-1069 (M₁) and K78S/01Y1-1069 (M₂), were used as females to cross with two commercial maize varieties, ‘Tiannuo 888’ in the Kunming site and Yunrui 8 in Yuanmou County, Yunnan Province, China.

2.2 Methods

Experiments were conducted in winter in Yuanmou in 2005, and in summer in Kunming in 2005 and 2006.

2.2.1 Wheat × maize crossing

In summer seasons in Kunming, wheat and maize materials were planted in the field according to sowing dates previously determined by Gu et al. (2006). In Yuanmou, with maize growing all year round, wheat was planted in late September. Wheat spikes were emasculated 2 to 3 days before anthesis, with 34–40 basal florets of middle parts of a spike left. When most florets were flowering, ‘rolling pollination’ (putting 1 to 3 spikes into fresh maize pollens and rolling spikes aided by slightly shaking the pollen container up and down) was conducted (Gu et al., 2005).

For the same experiment, spikes with similar development status and size were selected in emasculation to minimize the error.

2.2.2 *In vitro* culture of cut plants and intact growth in the field for pollinated spikes

After pollination, two treatments were conducted for hybrid spikes from M₂, including B₁: Half tillers with pollinated spikes were immediately injected with 2,4-D at 100 mg·L⁻¹ into upper internodes of wheat stems. After 24 h, these spikes were sprayed again with 2,4-D at 100 mg·L⁻¹. Spikes were kept wrapped with parchment bags and growing in the field, 17 d after pollination, embryos were excised under a microscope; B₂: Another half were cut near the root along the ground 24 h after pollination and inserted in nutrient solution A₃ (see Section 2.2.4). Simultaneously, spikes were sprayed with 2,4-D at 100 mg·L⁻¹ and then cultured under indoor natural conditions with natural dispersing light regime, 70% humidity and temperatures of 22°C–23°C during the day and 16°C–17°C at night. Two weeks after pollination, embryos were excised.

2.2.3 Optimal time of cutting plants

The tillers with hybrid spikes from M₁ were cut 4, 24, 48, 72 and 96 h after pollination, respectively. Later procedures were the same as described in B₂ above.

2.2.4 Comparison of different formulae of nutrient solutions for cut plant culture

The cut tillers with hybrid spikes were separately cultured in the following three reported formulae and in an improved one based on A₂:

A₁: 100 mg·L⁻¹ 2,4-D+40 g·L⁻¹ sucrose+8 mL·L⁻¹ sulfurous (Cai et al., 2005),

A₂: 100 mg·L⁻¹ 2,4-D+40 g·L⁻¹ sucrose+10 mg·L⁻¹ silver nitrate+8 mL·L⁻¹ sulfurous (Inagaki, 1997),

A₃: 100 mg·L⁻¹ 2,4-D+40 g·L⁻¹ sucrose+10 mg·L⁻¹ silver nitrate+8 mL·L⁻¹ sulfurous+3 g·L⁻¹ calcium phosphate,

A₄: 100 mg·L⁻¹ 2,4-D+40 g·L⁻¹ sucrose+10 mL·L⁻¹ ethyl alcohol (Cherkaoui et al., 2000)

2.3 Data collection and calculation

The number of pollinated spikes, florets, caryopsis, and embryos from all treatments was recorded. The caryopsis and embryo rates were calculated as follows:

Caryopsis rate

(%) = No. of caryopsis/pollinated florets × 100%,

Embryo rate

(%) = No. of embryo/pollinated florets × 100%.

3 Results

3.1 *In vitro* culture and intact plant growth

In vitro culture of cut plants (B₂) was obviously better than intact plant growth (B₁) in haploid embryo induction (Table 1). The embryo rate of B₂ was 3.29 times that of B₁, with figures of 31.6% vs 9.6%. B₂ also had a caryopsis rate 20% higher than B₁. In addition, although the duration of caryopsis and embryo growth in B₂ was 14 d, 3 d shorter than that in B₁, the plumpness of caryopsis was better and the size of the embryo was bigger in treatment B₂ compared with that in B₁. This suggests that the conditions of *in vitro* culture were more advantageous for haploid embryo induction and development.

3.2 Effect of plant-cutting time

The caryopsis rates of cutting plants 4 h, 24 h and 48 h after pollination were all above 95% (Table 2), without obvious differences among them. After that, the figures went down sharply; at 96 h, a caryopsis rate of only 69.4% was obtained. However, the highest embryo rates came from cutting plants 24 h and 48 h after pollination, with the figures at 34.2% and 30.7%, respectively. Cutting at 96 h produced an embryo rate of only 5%. Therefore, the optimum time to cut plants for *in vitro* culture was 24 h after pollination. In addition, the average embryo rate within 48 h after pollination was 29.7%, 1.74 times higher

Table 1 Comparison of haploid embryo induction between *in vitro* culture and intact plant growth (Kunming, 2005)

treatment	pollinated spikes	pollinated florets	rate of caryopsis/%	no. of embryo	rate of embryo/%
B ₁ : intact plant growth	34	1099	75.0	106	9.6
B ₂ : <i>in vitro</i> culture	48	1758	95.0	555	31.6

Table 2 Effect of different cut-plant times on efficiency of haploid embryo induction (Kunming, Yunnan, 2005)

time of cut-plant	pollinated spikes	pollinated florets	rate of caryopsis/%	no. of embryo	rate of embryo/%
4 h after pollination	18	658	97.0	159	24.2
24 h after pollination	15	587	96.4	201	34.2
48 h after pollination	15	521	95.2	160	30.7
72 h after pollination	17	624	88.1	104	16.7
96 h after pollination	17	595	69.4	30	5.0

Table 3 Effect of different formulae of cut-plant culture solutions on haploid embryo induction

place and time	wheat material	formula of cut plant culture	pollinated spikes	pollinated florets	caryopsis rate/%	no. of embryo	embryo rate/%
Yuanmou, 2005	M ₁	A ₁	13	537	87.0	10	1.9
		A ₂	13	479	87.7	13	2.7
		A ₃	13	482	99.4	17	3.5
		A ₄	13	480	0.0	0	0.0
Kunming, 2006	M ₁	A ₁	15	557	57.8	54	9.7
		A ₂	15	614	92.7	176	28.7
		A ₃	20	767	98.7	225	29.3
		A ₄	15	610	0.0	0	0.0
	M ₂	A ₁	10	395	41.8	41	10.4
		A ₂	10	386	92.2	126	32.6
		A ₃	10	380	95.5	135	35.5
		A ₄	10	390	0.0	0	0.0

Note: M₁ indicates C49S-87/01Y1-1069; M₂ indicates K78S/01Y1-1069. A₁: 100 mg·L⁻¹ 2,4-D+40 g·L⁻¹ sucrose+8 mL·L⁻¹ sulfurous; A₂: 100 mg·L⁻¹ 2,4-D+40 g·L⁻¹ sucrose+10 mg·L⁻¹ silver nitrate+8 mL·L⁻¹ sulfurous; A₃: 100 mg·L⁻¹ 2,4-D+40 g·L⁻¹ sucrose+10 mg·L⁻¹ silver nitrate+8 mL·L⁻¹ sulfurous+3 g·L⁻¹ calcium phosphate; A₄: 100 mg·L⁻¹ 2,4-D+40 g·L⁻¹ sucrose+10 mL·L⁻¹ ethyl alcohol.

than that at 72 h and 96 h (10.9%), suggesting that the hybrid spikes should be cut within 48 h for *in vitro* culture to obtain better efficiency in embryo induction.

3.3 Effects of different formulae on embryo induction

In December of 2005, a comparative experiment was done with four formulae of cut plant culture in Yuanmou, Yunnan (Table 3). Results indicated that the efficiency of embryo induction by all these formulae was very low, with an embryo rate ranging from 0 to 3.5%. Among the four formulae, A₃ showed the best caryopsis and the highest embryo rates; A₄ did not even yield caryopsis and embryo, but produced early fading of spikes, leaves and stems during culture.

Similar experiments done the next summer at Kunming with M₁ and M₂ materials also showed the same trend as that in Yuanmou, although the caryopsis and embryo rates here were raised significantly. A₃ produced the highest caryopsis and embryo rates in both M₁ and M₂, especially with caryopsis rates all above 95%. A₂ had the same efficiency in haploid induction as A₃. No caryopsis and embryo was obtained in A₄ again. In addition, there were obvious differences in caryopsis quality and embryo size among the four formulae: A₃ had the biggest embryo and caryopsis full of water; A₂ ranked in the middle in terms of embryo size, with less water in caryopsis; the embryo from A₁ was very small, and the caryopsis was tender and brittle, making it difficult to peel the embryo.

Results from two years of research covering two sites suggested that the improved A₃ formulae was the best and most stable in terms of efficiency, quality of caryopsis and embryo production.

4 Discussion

In vitro culture of cut plants is one of the most effective improvements in wheat × maize systems during the past 20 years. It is proven to be obviously better than intact plant growth in the field in this study, which confirms previous reports (Inagaki, 1997; Ballesteros et al., 2003; Cai et al., 2005). In summer at Kunming, although wheat and maize could be synchronously planted in the field in quantities for moderate climates, rains cause frequent changes in temperature and humidity outdoors. This explains the considerable difference in embryo rates between *in vitro* culture and intact plant growth in this study, which was different from the results reported by Lai et al. (2007).

The time of cutting plants after pollination had a significant influence on embryo induction. According to this study, the optimum time was 24 h after pollination, as cutting too early or too late was disadvantageous to raising the embryo rate, which was possibly related with 2,4-D treatment. 2,4-D is indispensable for the induction and development of caryopsis and embryo within 24 h after pollination (Inagaki and Tahir, 1992). To simplify the

operation, we changed the approach of 2,4-D treatment from injecting or/and spraying ear by ear (Laurie, 1989) to spraying the collected cut plants (24 h after pollination). Therefore, cutting too late may leave the hybrid spikes short of enough 2,4-D supply because the plant itself can only make limited 2,4-D. On the other hand, the decrease in embryo rate when cutting plants 4 h after pollination was possibly related to the interference of elongation of pollen tubes or fertilization in parts of florets (Zhang et al., 1996; Campbell et al., 2001).

In addition, the formula of culture solution for cut plants is another important factor influencing the efficiency of haploid embryo induction. In this study, although formulae A₁, A₂ and A₃ produced haploid embryo, there were obvious differences in the efficiency of induction and quality of the embryo. Among them, A₃ showed the best overall efficiency; its difference with A₂ was the addition of calcium phosphate, which could help promote the transportation of nutrients, synthesized by leaf and stem to caryopsis, and thus improve the nutrient supply for embryo development. A₄ did not produce caryopsis and embryo, which was possibly due to the application of ethyl alcohol. In 2003, we injected or sprayed the hybrid spikes with 2,4-D separately dissolved with ethyl alcohol and NaOH. As a result, clear leaf fading and presenility occurred in spikes treated with 2,4-D dissolved in ethyl alcohol. The reason concerned is to be further investigated.

Environment temperature after pollination strongly affected the efficiency of haploid embryo induction, and the optimum temperature was 20°C–25°C according to previous results (Riera-Lizarazu et al., 1992; Suenaga et al., 1997; Inagaki, 1997). Therefore, it is estimated that the very low embryo rate in the winter experiment at Yuanmou was possibly due to the lower indoor temperature (15°C–17°C) during cut plant culture.

Based on the favorable natural conditions in Kunming and the improved and highly effective pollination method, a simplified protocol of haploid embryo induction was established as follows: mass emasculated wheat spikes were crossed with maize pollen by rolling pollination in the field. Tillers with hybrid spike were then cut and sprayed with 2,4-D 24 h after pollination, and later cultured in A₃ solution under indoor natural temperature and light condition until 14 d after pollination. It could produce a caryopsis rate of 95% and haploid embryo rate of about 30%. It has been successfully applied in the construction of a DH population, purification of wheat sterile and restoring lines and as part of a breeding program. However, further experiments should be done with different wheat materials or combinations to evaluate the efficiency. In the meantime, factors and the mechanism affecting an increase of the embryo rate need to be further investigated.

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