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A sterilization protocol for field-harvested mature maize seed used for in vitro culture and genetic transformation

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In vitro tissue culture techniques require not only an aseptic work environment but also contaminant free starting materials (Dixon, Plant Cell Culture, a Practical Approach, IRL Press, 1985). Two common contaminants affecting in vitro-cultured tissues are bacteria and fungi. Enrichment of the media used for in vitro culture makes explants susceptible to these microorganisms (Leifert and Waites, Int. Soc. Plant Tissue Cult. Newsletter 60:2-13, 1990). Despite careful attention while applying sterilization techniques, contamination still may cause 100% loss from field-grown material due to large numbers of microorganisms present in the explants (Skirvin et al., In Vitro Cell Dev. Biol. Plant 34: 278-280, 1999). One of the first steps in the process of plant genetic transformation is to acquire sterile plant materials for in vitro tissue culture.

For maize, a well-established protocol exists for the sterilization of initial material when immature embryos are used (Frame et al., In Vitro Cell Dev. Biol. Plant 36:21-29, 2000). However, no effective sterilization protocols are reported for mature maize seeds, especially for field-harvested seeds that typically carry large amounts of air- and soil- borne pathogens. One strategy to overcome the contamination problem in mature seed sterilization is simply to start with large quantities of seeds, which increases the chances of obtaining adequate numbers of sterilized seed for in vitro culture. Alternatively, a method that prevents introduction of these contaminants into the laboratory in the first place would be ideal.

Here we report an efficient and reproducible seed sterilization method, which was developed specifically for mature maize seeds harvested from field-grown plants. The method includes three major disinfection stages. First, the maize seed surface is disinfected and soaked in sterile water, then the softened seeds are disinfected and the mature embryos isolated. Finally, the dissected mature embryos are disinfected before being placed on plant media for culturing. Using this step-wise sterilization method, an average of 98% sterile mature embryos are obtained, and plant vigor (measured by Seedling Growth Rate; ISTA, Seed Sci. Technol. 27(Suppl.):27-32, 1999) is not compromised compared to the non-sterilized control (Martinez, 2008).

Materials. 1) Mature maize seeds harvested from field-grown plants. 2) 80% Ethanol (~300 ml for ~200 seeds). 3) 50% bleach solution: mix 450 ml of commercial bleach (5.25% hypochlorite) with 450 ml of Millipore water containing 2 drops of the surfactant Tween-20. Use ~900 ml for ~200 seeds. 4) 15% bleach solution: mix 15 ml of commercial bleach (5.25% hypochlorite) with 85 ml of Millipore water containing 1 drop of the surfactant Tween-20. Use ~100 ml for ~200 dissected embryos. 5) Millipore water (autoclaved). 6) Petri dishes: 60 x 20 mm, 100 x 15 mm, 150 x 15 mm

Methods. First seed surface disinfection and seed softening: 1) Place 50 seeds in a 250 ml beaker along with a stir bar. 2) Add ~ 75 ml of 80% ethanol, cover with the aluminum foil and place the beaker on a stir plate. Stir at medium speed for 3 minutes. 3) Take the beaker to the flow bench and decant the ethanol into a liquid-waste container. 4) Add ~ 75 ml of 50% bleach solution, cover with the aluminum foil and stir for 15 minutes on medium speed. 5) In the flow bench, decant the bleach into the liquid-waste container. 6) Sterilize the seeds a second time by repeating Steps 4 and 5. 7) Rinse the seeds 5 times with sterile Millipore water (~ 75 ml each time). 8) After the last rinse, keep seeds in ~50 ml sterile water (just enough to cover the seeds--do not overfill the container), cover with aluminum foil and leave the beaker inside the flow bench for 24 hours. This treatment serves to soften the seed coat and endosperm for ease of embryo dissection.

Second seed surface disinfection and embryo dissection: 9) After 24 hours, sterilize the softened seeds once with 50% bleach solution for 2 minutes while stirring (covered). 10) Decant the bleach and rinse the seeds 5 times with sterile Millipore water. 11) Leave seeds covered with last wash so they do not dry out. 12) Move sterilized seeds to a 150 x 15 mm petri dish in groups of 3 or 4 for embryo dissection. 13) Dissect the mature embryo using bent nose forceps to hold the middle of the seed with the embryo facing up. First, cut longitudinally along both sides of the embryo to remove the endosperm flanks. Next cut the base of the seed away, including two thirds of the root (tip) portion of the embryo axis. Finally, cut away the remaining endosperm from the top end of the embryo being careful not to injure the apical end of the embryo. The remaining endosperm can then be peeled away from beneath the embryo. Gently lift the embryo away from the seed carcass using the scalpel and place it in a 60 x 20 mm petri dish containing 10 ml sterile distilled water. 14) Repeat Step 13 until you dissect all the seeds.

Embryo sterilization: 15) Using a sterile pipet, remove water from the petri plate containing the dissected embryos. 16) Add ~15 ml of 15% bleach solution to the petri plate, close the lid and place on a table top shaker (low speed) for 5 minutes. 17) Remove the bleach solution using a sterile pipet. 18) Rinse the embryos 4 times (15 ml each time) with sterile Millipore water. 19) Embryos are ready to be placed on a plant medium. Place about 10 embryos per 100 x 15 mm petri plate of medium. 20) Check the plates after 2, 3 and 4 days and if contamination is found, move uncontaminated explants to fresh medium.

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Studies on Zhuo-Zi No.1, a purple hybrid in maize (*Zea mays* L.)

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In this paper, we describe the research status quo on Zhuo-Zi No.1, a purple hybrid in maize (*Zea mays* L.). We have a breeding program for purple maize, using information about genetic loci summarized by Coe et al. (1977).

Parents for Zhuo-Zi No.1 are ZP 99-01 (Female) and ZP 99-02 (male). ZP 99-01 was developed by continued inbreeding and selection from a local variety with purple plants and grain, selecting for ears of superior plants from large populations. ZP 99-02 was developed by continued inbreeding and selection from the improvement population No. 02. Both parents have good or high general combining ability; high specific combining ability; high vigor; normal to high production; resistance to biological stress; late maturity; medium tall plant and ear height; fair stalks; vigorous roots; semi-erect and mid-sized leaves; 1~2 ears per plant and purple-black grain on a deep-purple cob. The main agronomic traits of Zhuo-Zi No.1 are summarized in Tables 1 and 2, and Figure 1. It produces a purple color in the seedling, leaf tip, leaf periphery, leaf ear, leaf sheath, stalk, tassel and its branches, anther surface, silk, husk leaf, ear handle, cob, pericarp, aleurone, and leaf blade of the main plant and in tillers at maturity. It has normal color (pale-yellow or nearly white) endosperm, embryonic bud, shield blade, shoot sheath and root system. The biomass yield and grain yield can reach 75000~7950 kg/ha and 7500~8025 kg/ha respectively. The anthocyanidin-3-monoglucoside (maize morado color) content of purple maize Zhou-Zi No.1 is estimated to be 225~300 kg/ha. Anthocyanin content varies in different tissues and organs. It is 0.023% in the stalk and leaf blade, 1.007% in the tassel, 2.228% in the husk leaf, 0.268% in the silk, 0.728% in the cob, 0.106% in the grain, and 0.869% in the stalk coat (Table 3.). Thus, the husk leaf and tassel have a higher anthocyanin content.

Figure 1. Ear of Zhuo-Zi No. 1.

Table 1. The agronomic traits of Zhuo-Zi No. 1.

No.	Trait name	Average value, character and resistance
1	Plant height cm	313.5
2	Ear height cm	124.7
3	Tassel length cm	42.6
4	Tassel branch number	14.0
5	Leaf number	23.5
6	Ear length cm	18.3
7	Ear diameter cm	4.5
8	Row number per ear	15.7
9	Grain numer per row	37.8
10	Weight of 100 grain g	398.6
11	Ear number each plant	1.6
12	Husk number each ear	14.8
13	Grain type	Semi-dent
14	Ear form	Cylinder
15	Resistance to	
	E. turcicum	HR
	B. maydis	HR
	C. lunada	HR
	C. zeae-maydis	MR
	U. zeae	MR
	F. moniliforme and *P. inflatum*	MR
	R. solani and *R. cerealis, zeae*	R
	S. holci-sorghi	HR
	MRDV	MR
	SCMV-MDR	MR
16	Tolerance lodging	
	drought	

Table 2. The biochemical composition of grain of Zhuo-Zi No. 1.

Component	% (g/100g grain), g/L
Protein	10.86
Lipid	5.02
Starch	74.23
Lysine	0.36
Water	10.7
Unit Weight (g/L)	792

Table 3. The anthocyanin content of different tissues and organs of Zhuo-Zi No. 1.

Tissue name	Anthocyanin % (g/100g)
Stalk and leaf blade*	0.023
Tassel	1.007
Husk leaf	2.228
Silk	0.268
Ear cob	0.728
Grain	0.106
Stalk sheath	0.869

*Sampled at maturity

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The *opaque2* and *opaque7* mutants reveal extensive changes in endosperm metabolism as revealed by transcriptome-wide analyses

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The changes in storage reserve accumulation during maize (*Zea mays*) grain maturation are well established; however, the key molecular determinants controlling carbon flux to the grain and the partitioning of carbon to starch and protein are more elusive (Motto et al., Cellular and Molecular Biology of Plant Seed Development, Larkins and Vasil, eds., 1997). The *Opaque-2* (*O2*) gene, one of the best-characterized plant transcription factors, is a good example of the integration of carbohydrate amino acids and storage protein metabolism in maize endosperm development. Evidence also indicates that the *Opaque-7* (*O7*) gene plays a role in affecting endosperm metabolism.

To advance our understanding of the nature of the mutations associated with an opaque phenotype, we used nearly isogenic inbreds for *o2* and *o7* mutants, and for the double mutant combination *o2o7*, to provide genome-scale information about gene expression patterns by cDNA microarray. Classifying genes based on similarities or differences in transcript profile with phenotype can confirm existing knowledge, lead to the dissection and revela-