Cotton Ovules Culture and Analysis

Jiafu Tan, Fenglin Deng, Wenxin Tang, Jie Han, Guo Kai, Lili Tu and Xianlong Zhang*

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China

*For correspondence: xlzhang@mail.hzau.edu.cn

[Abstract] The cotton ovules culture was innovated by Beasley and Ting (1973), and named after them. It is a convenient system to analyze the effect of chemical or environmental treatment on fiber development directly on ovules. This protocol was generated according to previous published papers and our practical experience.

Materials and Reagents

1. Flowers (-1 to 1 DPA of flowers are easy to manipulate)
2. 0.1% (w/v) $HgCl_2$ (caution: $HgCl_2$ is very dangerous. Please select an advantageous reagent to sterilize according to your lab condition. Otherwise 75% ethanol and DCCS (N,N'-Dicyclohexylcarbodiimide) could be the alternatives)
3. Toluidine blue O (Sigma-Aldrich, catalog number: T3260)
4. Glacial acetic acid-ethanol-water (10:95:5, v/v)
5. Vitamins:
   VB1(Vitamin B1, Thiamine) (Sigma-Aldrich, catalog number: T3902)
   VB6 (Vitamin B6, Pyridoxine) (Sigma-Aldrich, catalog number: P8666)
   VB3 (Vitamin B3, Nicotinic acid) (Sigma-Aldrich, catalog number: N0765)
6. Inositol (Sigma-Aldrich, catalog number: I3011)
7. IAA (Sigma-Aldrich, catalog number: I2886)
8. GA_3 (Sigma-Aldrich, catalog number: G7645)
9. KH_2PO_4 (Sigma-Aldrich)
10. $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich)
11. $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich)
12. KNO_3 (Sigma-Aldrich)
13. H_3BO_3 (Sigma-Aldrich)
14. $Na_2MoO_4 \cdot 2H_2O$ (Sigma-Aldrich)
15. KI (Sigma-Aldrich)
16. $CoCl_2 \cdot 6H_2O$ (Sigma-Aldrich)
17. $MnSO_4 \cdot H_2O$ (Sigma-Aldrich)

18. $ZnSO_4 \cdot 7H_2O$ (Sigma-Aldrich)
19. $CuSO_4 \cdot 5H_2O$ (Sigma-Aldrich)
20. $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich)
21. Na_2EDTA (Sigma-Aldrich)
22. BT medium preparation (see Recipes)
23. Hormone preparation (see Recipes)
24. Working BT medium preparation (see Recipes)

Equipment

1. Erlenmeyer flask (50 ml)
2. 1.5 ml microcentrifuge tubes
3. Spectrophotometer (Beckman Coulter, model: DU 800) or microplate reader (Tecan Trading AG, model: infinite® M200)
4. Clean bench laminar air-flow-hood with burner (Harbin East Electronic Technology , model: HD-1360)
5. Common plant tissue culture equipment (Measuring cylinder/volumetric flask, beaker, weighing machine, autoclave, ph-meter, scalpel, fine forceps)

Software

1. ImageJ

Procedure

A. Ovary sterilization

1. Flowers (Figure 1) are collected with 1 to 3 cm anthocaulus from plants (the length of anthocaulus is usually 1 to 3 cm in our used cotton species, if some others is longer, you can cut as you wish; but the shorter ones is not recommended to choose unless there is no alternative). Remove the petals, stamens and bracts with hand carefully and thoroughly (or you can do it with some special accessory appliances but make sure do not hurt the cotton boll).

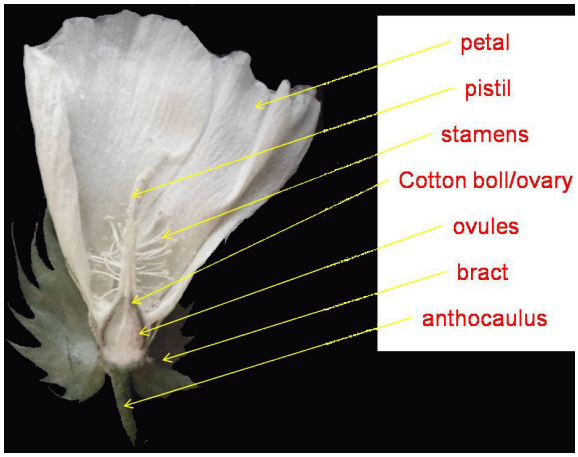

Figure 1. The organ of cotton flower

2. If the pistils are vigorous, such as those from in or before blooming flowers in which the pistils are linked with the cotton boll, while they will be physiological abscission after 2 day post anthesis (DPA), they should be kept to avoid liquid permeating into the cotton boll through the wound when sterilized.
3. Soak the whole remaining tissue (cotton boll linked with anthocaulus and/or pistils) into 0.1% (w/v) $HgCl_2$ for 15 min immediately (occasional shaking/stirring is needed to keep all the tissue fully soaked), discard $HgCl_2$ and rinse with sterilized ddH_2O three times. Each for 2 to 5 minutes.

B. Ovules preparation

1. Hold the tissue with the anthocaulus, and remove the shuck of the ovary (or the young cotton boll) with a sterilized forceps carefully, then float and disperse the intact ovules on the liquid BT medium gently. Make sure the ovules are not injured, broken or attached the brown gossypol from the broken shuck.
2. For same batch culture, equal ovules should be put in each flask and usually less than 20 ovules for each batch.
3. If the ovules are going to culture for more than 10 days, the ovules sum in each flask should be less than 10 at the beginning.
4. For young ovules that have not grown fiber, they could be separated easily, while for ovules with longer fiber that are sticking together, such as the ovules in 2 day post anthesis (DPA), they need to be carefully separated one by one with forceps before floating on the medium.
5. Label the flask with the date, the age of the ovules and the hormones contained.

C. Ovule culture

1. The floating ovules are incubated in 30 °C without light and shake.
2. The fiber should be easily visible after 4-5 days of culture for the 0 DPA ovules (Figure 2).

Figure 2. 0 DPA ovules after 5 days culture

3. The ovules or fiber can be used for further analysis, such as fiber production measurement, RNA extraction and biochemical analysis *etc*.

D. Fiber measurement

There are two methods to measure fiber production.

1. Total fiber units (TFU) measurement, which was also innovated by Beasley *et al*. (1974).
   a. For 12 days cultured ovules, 10 ovules for each assay. Ovules are dried out of medium with filter paper. Sunk in boiling water for 2 min, then dried again with filter paper for 2 to 3 min.
   b. Stain the ovules in a small beaker with 20 ml 0.02% Toluidine blue O for 30 sec, then discard the liquid with a fine sieve and wash in running water for 1 min immediately to remove the non-absorbed dye.
   c. Dry the ovules, then destain in flask with 20 ml glacial acetic acid-ethanol-water (10:95:5) for 2 h. The flask should be sealed to avoid liquid evaporation which can cause differences in concentration of destaining liquid.
   d. Measure the absorbance of the destaining liquid in 624 nm and count as the relative yield of fiber.
2. Fiber length measurement (Figure 3)
   a. Ovules are sunk in boiling water for 2 min or in 75% ethanol for 15 min, then put the ovules in slowly running water to make the fiber flow to one side and measured with a ruler.
   b. Or put the ovules on a glass slide, comb the fiber gently with a dissecting needle, then take photos and measure the length with a ruler or you can use software for

automation of the measurement (*e.g.* Image J).

Figure 3. Cultured ovule (1) is soaked in 75% ethanol (2) to separete fiber and measured on a glass slide (3)

Notes

1. This protocol can be used for analyzing the effects of dozen of chemicals, including hormones, plant growth regulators, plant growth inhibitors and flavonoids (Tan *et al*., 2013) *etc.*, on fiber development in our lab. We found it functioned similarly in *Gossypium hirsutum* (more than 4 cultivars were applied), *G. arboreum* and *G.herbaceum*. But for *G. barbadense*, there will be several differences in the fiber growth rate and hormones response. The fiber will grow later and fewer, and GA_3 can't promote fiber grow for the 0 DPA ovules independently.
2. While it is a very sensitive system, many factors are involved in a success assay, especially for that of ovule developmental stage. According to our unpublished data, there are dramatic differences for 0 DPA ovules from 7 am to 12 am in response to IAA and GA_3. And GA_3 could promote fiber growth independently on the 0 DPA ovules post 12 am except for that from *G. barbadense*. The ovules before 0 DPA may be not easy to float on the liquid medium, should be taken more gently. And ovules of different developmental stages are also response differentially to chemical treatment (Tan *et al*., 2012). So for the same assay, the ovules should be from the same stage and all the manipulations should be strictly consistent and control should be set up for each. The samples from different batches could not analyze together.
3. $CaCl_2 \cdot 2H_2O$ should be separately prepared and stored with KH_2PO_4 and $MgSO_4 \cdot 7H_2O$, be carefully mixed and diluted before use.
4. Other chemical should be sterilized first and added into the medium with IAA and/or GA_3. Each new chemical should be undergone a dose test to determine the optimal dose.
5. Infection rate of ovules from the glasshouse is less than that from the field.
6. Take a simple plan before start. It will take a lot of time to release the ovules from the ovary (approximately 10 ovules per hour for freshman).
7. For TFU assay, it is 10 ovules for each assay usually, while it should be changed depending on the days of culture and the bulk of ovules.

Recipes

1. BT medium preparation

Preparation of 20x Macro element stock solution (stored in 4 °C)						
Chemicals (company)	Mol.wt	Final conc. (mg/L)	Final conc. (mM)	Conc. stock (mM)	mg to take	Final volume of stock
KH_2PO_4	136.09	272.18	2	40	5,443.6	1,000 ml
$CaCl_2{\cdot}2H_2O$	147.01	441.06	3	60	8,821.2	
$MgSO_4{\cdot}7H_2O$	246.47	493.00	2	40	9,860	
KNO_3	101.10	5,055.50	50	1,000	101,110	
Preparation of 100x Micro element stock solution (stored in 4 °C)						
H_3BO_3	61.83	6.183	0.1	10	618.3	1,000 ml
$Na_2MoO_4{\cdot}2H_2O$	241.95	0.242	0.001	0.1	24.2	
KI	166	0.830	0.005	0.5	83	
$CoCl_2{\cdot}6H_2O$	237.93	0.024	0.0001	0.01	2.4	
$MnSO_4{\cdot}H_2O$	169.02	16.902	0.1	10	1,690.2	
$ZnSO_4{\cdot}7H_2O$	287.56	8.627	0.03	3	862.7	
$CuSO_4{\cdot}5H_2O$	249.69	0.025	0.0001	0.01	2.5	
Preparation of 100x Fe salt stock solution (stored in brown bottle and 4 °C)						
$FeSO_4{\cdot}7H_2O$	278.01	8.341	0.03	3	834.1	1,000 ml
Na_2EDTA	372.24	11.167	0.03	3	1,116.7	
Preparation of 1,000x Vitamins mixture stock solution (stored in 4 °C)						
VB1	337.27	1.349	0.004	4	1,349	1,000 ml
VB6	205.64	0.822	0.004	4	822	
VB3	123.11	0.492	0.004	4	492	
Preparation of 100x Inositol stock solution (stored in 4 °C)						
Inositol	180.16	180.160	1	100	18,016	1,000 ml

2. Hormone preparing

The general hormones for cotton ovules culture are IAA and GA_3.The stock solutions of IAA and GA_3 are 5 mM and 0.5 mM, respectively. Both are 1,000x solutions, pre-dissolved in a small volume (500-1,000 µl) of 95% ethyl alcohol, then brought to volume with sterilized double-distilled H_2O water, aliquoted into 1.5 ml microcentrifuge tubes, sealed and stored in -20 °C. Usually 10 to 50 ml stock solution is prepared for each time.

3. Working BT medium preparation

Stock	Conc. of stock	Amount of stock soln to take	Final volume of media
Macro-element mixture	20x	50 ml	1,000 ml
Micro-element mixtures	100x	10 ml	
Fe salt solution	100x	10 ml	
Vitamin mix	1,000x	1 ml	
Inositol	100x	10 ml	
Glucose		24 g	
pH 5.0			

Medium is prepared in 100, 200 ml or other certain volumes before used, after sterilized, added suitable hormones, mixed and aliquoted into sterilized 50 ml flasks with about 10 ml each. Label the flask with what kinds of hormones contained, preparation and expiration dates (the medium should be used within less than 15 days).

Acknowledgments

The protocol was based on Beasley and Ting's original work (Beasley and Ting, 1973), and adapted from two of our previous published work (Tan *et al.*, 2012; Tan *et al.*, 2013). This work was supported by the University Scientific and Technological Self-innovation Foundation, the National Natural Science Foundation of China (grant no. 30871560 and 31230056) and the National High-Tech Program of China (grant no. 2012AA101108).

References

1. Beasley, C. and Ting, I. P. (1973). The effects of plant growth substances on *in vitro* fiber development from fertilized cotton ovules. *Am J Bot* 130-139.
2. Beasley, C. A., Birnbaum, E. H., Dugger, W. M. and Ting, I. P. (1974). A quantitative procedure for estimating cotton fiber growth. *Stain Technol* 49(2): 85-92.

3. Tan, J., Tu, L., Deng, F., Hu, H., Nie, Y. and Zhang, X. (2013). A genetic and metabolic analysis revealed that cotton fiber cell development was retarded by flavonoid naringenin. *Plant Physiol* 162(1): 86-95.
4. Tan, J., Tu, L., Deng, F., Wu, R. and Zhang, X. (2012). Exogenous jasmonic acid inhibits cotton fiber elongation. *J Plant Growth Regul* 31(4): 599-605.

