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Microspore selection and *in vitro* callus induction in maize anther culture

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Abstract
Productions of doubled haploid (DH) maize involve development of haploid through *in vitro* or *in vivo* technique, and convert to diploid by doubling the chromosomes using chemical agents. DH enables fixation of hybrid vigour of F1 plants. Several temperate seed production companies develop DH maize, and use them as a parent material to develop superior maize hybrids. DH helps to eliminate the presence of lethal alleles, and fixation of hybrid vigour for several generations. Presently two strategies are available for the development of DH lines in maize. The first strategy involves the crossing of maize with special donor parent called inducer lines which results increased occurrence of spontaneous haploids grown *in vivo* in the field. With the aid of marker capable to express only in haploids the haploids are directly identified in the field and used to develop doubled haploids. In the second strategy haploid microspores from immature anthers or ovules are collected and grown under *in vitro* condition by tissue culture and the haploids are used to develop DH lines. The *in vivo* strategy is easier way to produce haploids, but possess limitation of unavailable inducer lines suitable for tropical regions to induce haploids. The *in vitro* anther culture is a viable strategy to develop haploids in tropical regions. It is necessary to optimize a slightly modified plant tissue culture protocol for *in vitro* anther culture suitable to specific genotypes and different agro climatic regions. The present report showed the morphometric attributes and selection of immature anthers for haploid production in TNAU maize hybrid COH (M)6. In addition the modified media compositions are optimized for the induction of callus suitable for tropical region.

Keywords: Maize, Callus, Anther culture

Introduction
Development of doubled haploid is a rapid approach to homozygosity that shortens the time duration to development of new inbred lines compared to conventional methods which require six to eight generations of self-pollination (Zang *et al*., 2015) [10]. Haploid is also valuable to introduce desirable recessive trait through mutation, hybridization or genetic manipulation (Deimlings *et al*., 1997) [2]. Haploid techniques allow fixation of gene combinations which otherwise may not possible to isolate from a segregating populations through conventional means (Ham *et al*., 2010) [6]. An ideal situation would be the possible fixation of heterotic gene combination from an F1 generation to develop homozygous lines as good as heterotic F1 hybrids [Yiming *et al*., 2009] [9]. Doubled Haploid (DH) lines are routinely applied in many commercial hybrid maize (*Zea mays*) breeding programs. Major advantages of DH lines compared to selfed lines include (a) maximum genetic variance between lines for *per se* and testcross performance from the first generation, (b) reduced breeding cycle length, (c) perfect fulfillment of DUS (distinctness, uniformity, stability) criteria for variety protection, (d) reduced expenses for selfing and maintenance breeding, (e) simplified logistics, and (f) increased efficiency in marker-assisted selection, gene introgression, and stacking genes in lines (Geiger and Gordillo 2009) [3].

Present commercial DH-line breeding programs are based on *in vivo* induction of maternal haploids (Seitz, 2005; Barret *et al.*, 2008; Rotarenco *et al*., 2009) [8, 1, 7]. Other techniques have proven to be less effective or too genotype specific (Geiger and Gordillo 2009) [3]. An important aspect of the DH approach is to limit the loss of genetic variance due to random drift and selection by maintaining a minimum effective population size (Gordillo and Geiger 2008) [5]. To comply with this requirement, considerable numbers of DH parent lines need to be intercrossed for starting a new selection cycle (Gordillo and Geiger 2008) [5]. Because of the genetic, methodological, and logistic advantages, further progress in maize breeding is expected to increase considerably with the development of DH lines (Gordillo and Geiger, 2008) [5]. Yet, the success of employing DH lines depends on a robust and efficient haploid

induction technology as well as on breeding strategies that make optimum use of the breeder's genetic, technical, and monetary resources (Gordillo and Geiger 2008; Gayen *et al*., 1994) [5, 4].

Considering the importance and necessity of doubled haploids in maize for sustainable Agriculture, the present report optimized the morphometric traits to collect immature anthers, and induction of callus from immature anthers of maize suitable for tropical regions.

Materials and Methods

Plant material and Collection of immature tassels: Maize hybrid COH (M) 6 released by Tamil Nadu Agricultural university has been be used as material to collect immature anthers. Mother plants of maize COH (M) 6 was grown in the field with normal agronomical practices. Plant growth and development have been observed carefully, and immature tassel prior to emerging out from the plant was collected in an ice box. The tassels have been transferred to lab, and were used as a material to analyze the presence of microspores and source of explants.

Morphometric attributes: Maize COH (M)6 was grown in the crop cafeteria in ADAC&RI campus and maintained with proper agronomic practices. The morphometric attributes Days after sowing, Number of leaves in plant during the collection of tassel, Leaves covered tassel fit with tassels containing microspores suitable for explant collection have been identified for COH6 maize hybrids (Table 1).

Table 1: Morphometric attributes of COH6 for collection of immature tassel with microspore

S. No	Attributes	Optimum level
1.	Plant age to collect tassels	54 Days after sowing
2.	Number of leaves in plant during the collection of tassel	17
3	Leaves covered tassel	3
4	Microspore development stage observed	Late Unicellular stage

Detection and confirmation of Microspore stage in the explants: The leaf sheath was removed, tassels were briefly sterilized by immerse into 70% ethanol for 1 minute and washed with sterile distilled water thrice. Pollen grains of the immature tassels were collected by squeezing into a sterile slide, stained with methylene blue and the unicellular stage of microspores was confirmed by the phase contrast microscope at 400x magnification (Fig. 1). The immature pollen showing late unicellular stage was identified and the remaining anthers of the tassel were used as explant for anther culture. For cold pretreatment the tassels were placed in 4 °C refrigerator for 7 or 14 days.

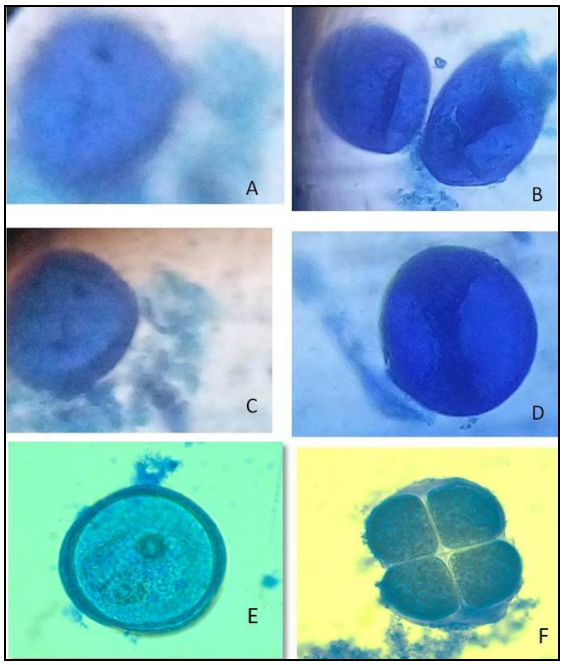

Fig 1: Microspores of immature maize anthers. A. Uninucleate microspore, B. Late Uninucleate, C. Microspore anaphase, D. Vacuolated microspore, E. Early Uninucleate, F. Tetrad stage

***In vitro* anther culture:** Immature tassels containing uninucleate to early binucleate microspores were pretreated in refrigerator with 7 °C for 10 days. The tassels were surface sterilize with 2% (v/v) sodium hypochlorite for 20 min, and the anthers were inoculated into the plates containing modified YP medium, N6 medium or MS medium with different concentrations of 2, 4-D, Kinetin, IAA or without additional supplementation (Fig. 2). After inoculation the inoculated plates were covered with black cloth to provide dark in the primary growth room at 28 °C. Once in 15 days the explants were sub cultured into new plates, and observed for the morphological changes and callus induction under a stereo zoom microscope.

Result and Discussion

Morphometric attributes of Maize COH (M) 6 and confirmation of microspore containing anthers stage

The plant growth conditions and genotype effects had a significant effect on the development and days to collect immature anthers containing micropores of the crop plants (Jäger *et al*., 2010) [11]. To identify the correct stage of the plant for the collection of anthers, the morphometric attributes namely Days after sowing, Number of leaves in plant during the collection of tassel, Leaves covered tassel fit with tassels containing microspores suitable for explant collection have been identified (Table 1).

The immature anthers sap is squeezed over a clean slide and

stained with methylene blue and presence of microspores have been confirmed under a phase contrast microspore (Fig. 1). The COH (M)6 maize at 54 days after sowing and the tassels were completely covered by top three leaves are suitable explants contained microspores with early and late uninucleate stage. Further the immature anthers looks milky white and anthers were half the length of the florets, but in later days the matured anthers looked yellowish and the anther length was increased to the length of the florets.

Modified Yu-Pei or N6 or MS medium with various dose of Plant Growth regulators:
1 Standard Medium
2 Medium +2 mgL^{-1}kinetin
3 Medium +3 mgL^{-1}kinetin
4 Medium +2 mgL^{-1}kinetin+2 mgL^{-1} IAA
5 Medium +2 mgL^{-1}kinetin+2 mgL^{-1} NAA
6 Medium +2 mgL^{-1}kinetin+2 mgL^{-1} 2-4-D

Fig 2: Nutrient medium with different levels of growth hormones

Effect of nutrient medium in callus induction

To assess the efficiency of different nutrient medium, modified MS, Yu-Pei and N6 medium containing various concentrations of growth regulators were used (Fig. 2). The MS and Yu-Pei medium containing kinetin at 2mg/L and 3mg/L showed induction of callus in anthers. N6 medium with 2mg/L myoinositol also showed induction of callus. At twenty days post inoculation the anthers become brown, and Watery callus like structures visualized after 30 days of inoculation. At forty days after inoculation the callus emerged by piercing through the anther walls (Fig. 3).

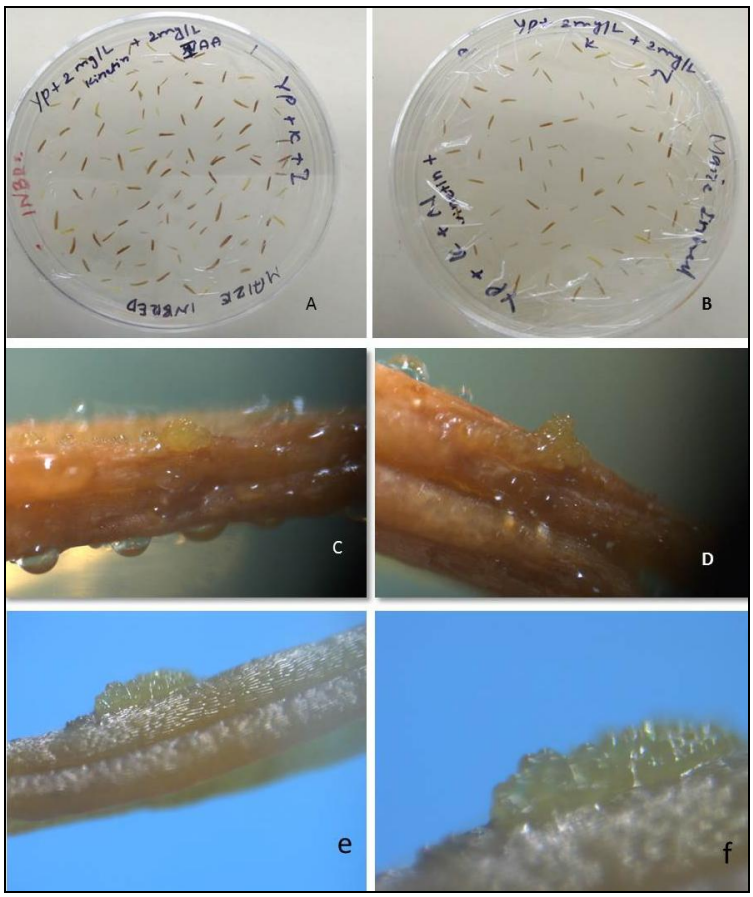

Fig 3: Yupei MS medium supplemented with kinetin started bulging after four weeks. (A-B) Anthers after twenty days after inoculation (C-D) Thirty days after inoculation. Anthers started bulging. Watery callus induction after 30 days of inoculation. (E-F) Callus emerged piercing through the anther wall after 40 days of inoculation

Conclusion

The present report showed the induction of callus in tropical maize hybrid COH (M)6. The morphometric attributes determined in this report may fasten the collection of explants for another culture. Even though the haploid nature of microspores confirmed further confirmation of ploidy level using flowcytometry. This may help to develop doubled haploids to boost maize breeding in tropical countries.

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