In vitro androgenic responses in tropical genotypes of maize (*Zea mays* L.)

ABSTRACT

The production of doubled haploid (DH), a valuable technique for preserving hybrid vigor plant breeding, involves generating haploid individuals through *in vitro*. The dir embryogenesis or callogenesis from immature anthers using *in vitro* cultivation is used produce haploid individuals suitable for development of DH lines. *In vitro* anther cultu represents a viable approach albeit requiring the optimization of plant tissue cultu protocols for assessing genotypes. In this study, we systematically investigated tas collection with appropriate size of microspore developmental stages and the culture me with different growth regulator concentrations for effect on callus induction from anthers. genotype dependency of response is a very important factor we three YP basal media w different plant growth regulators for twelve different hybrid maize genotypes. The res revealed that three genotypes - Pusa HM8 Improved, Pusa HQPM1 Improved & APCH demonstrated significant outcomes having the induction frequency of calli 4% in Pusa HI Improved, 5% in APCH-2, 8% in Pusa HQPM1 Improved in YP with 2 mg/l IAA and 1 m Kinetin. Moreover, this study refines media compositions optimized for genotypes of tropi region, and it has potential for enhancing maize breeding programs.

Keywords: Androgenesis, Anther culture, Haploid, Callus, maize genotypes

1. INTRODUCTION

Doubled haploid (DH) development is a technique in plant breeding that accelerates the process of creating homozygosity. This approach significantly shortens the time required compared to traditional methods, which involve multiple generations of self-pollination [24]. DH lines are widely applied in commercial maize breeding programs, offering several key advantages i.e., greater genetic diversity from the outset, elimination of lethal alleles, shorter breeding cycles, adherence to criteria for protecting new varieties, cost savings, simpler logistics, and improved efficiency in selection and genetic modification.

Currently, two primary strategies exist for generating DH lines in maize. I*n vivo* strategies include using haploid inducer lines, wide hybridization and CENH3-based haploid inducer system. While *in vitro* strategies include utilization of plant tissue culture techniques like anther and microspore culture to produce haploid plants [2]. Commercial DH- line breeding programs of maize primarily use *in vivo* induction of maternal haploid inducer lines as the most successful method as compared to *in vitro* methods [6]. The availability of temperate haploid inducer lines, this method is straightforward and field-based, it is limited in tropical regions due to the unavailability of appropriate inducer lines capable of inducing haploids in tropical environments.

Maize haploids can be obtained through *in vitro* techniques known as androgenesis which involves the development of haploid plants from immature pollen using either anther culture

or microspore culture. In anther culture, microspores from the anther are induced to undergo androgenesis, forming direct embryo-like structures or calli. These structures can then either directly become haploid plants or indirectly regenerate through the formation of regenerable calli. However, achieving haploids and doubled haploids (DH) through androgenesis in maize has proven to be challenging. This approach offers a viable alternative for tropical regions, where inducer lines are not readily available. Success is highly genotype-dependent, with most maize genotypes showing little to no response to this approach. Even in responsive genotypes, success depends on various factors, including the anther's developmental stage, the donor plant's genetics, and anther pretreatment [3,13,20]. The future of maize breeding is expected to benefit significantly from DH lines, given their genetic and logistical advantages [12]. However, their success hinges on robust haploid induction technology and effective breeding strategies

Recognizing the significance of doubled haploids in plant breeding, ongoing research focuses on assessing the genotypes for androgenic responses under optimized conditions in maize genotypes suitable for tropical regions. The aim of the present study was to assess androgenic response of tropical maize genotypes with different treatments of growth regulators to improve DH production in tropical regions.

2. MATERIAL AND METHODS

2.1 Plant material and collection of immature tassels:

Tropical twelve maize hybrid genotypes released by Indian Agricultural Research Institute; New Delhi were used as material to collect immature anthers. The leaf collor method [1] was used to correlate plant morphology with microspore developmental stage. 2-3 top florets were selected before tassel emergence and anthers were extracted for microspore detection. After confirmation of the correct microspore stage, the plants were cut just below the tassel base, excess foliage was removed, and approximately 3-4 leaves were left around the tassel. The tassel was placed in a flask with water, disinfected immediately and stored in the refrigerator at 4°C for a minimum 3 days.

2.2 Morphometric attributes of maize genotypes:

The maize genotypes planted in pots with staggered sowing to get continuous anther for culturing & carefully managed using standard agronomic procedures followed at the National Phytotron Facility, New Delhi. Various morphometric attributes, including the number of days after sowing during sample collection, number of leaves per plant at the time of tassel collection, and the assessment of leaves that were appropriately aligned with tassels containing microspores for explants collection, have been recorded and are presented in Table 1.

2.3 Detection and confirmation of Microspore stage:

To observe the stage of microspore development, the anthers were isolated from the sampled spikelet using fine forceps. The anther was placed on a microscope slide and gently crushed with a razor blade to release the microspores. A few drops of DAPI staining solution was added to the crushed anthers, ensuring that it is covered, and a cover slip was placed over it. Fluorescence microscope (Bio-Rad ZOE™ Cell Imager) with a UV light source at a wavelength of 461 nm was used to observe and analyze the stained microspores. (Fig. 1). Immature pollen at the mid-unicellular stage was identified, and the

remaining anthers from the tassel were used as explants for anther culture.

2.4 *In vitro* anther culture:

Immature anthers that contained late uni-nucleate to early bi-nucleate microspores underwent cold pretreatment; the anthers were placed in pretreatment media in dark at 4°C refrigerator for 10 days [13]. Subsequently, the tassels were subjected to surface sterilization using a 2% (v/v) solution of sodium hypochlorite for a period of 20 minutes and washed thrice with distilled water. The isolated anthers were placed in 55×15 mm plastic Petri dishes, each containing 10 mL of modified YP medium, supplemented with different concentrations of plant growth regulators to determine the most effective combination for promoting callus/embryo formation. After inoculation the inoculated plates were covered with Aluminium foil and kept in dark in the primary growth room chamber at 28°C. Once in 15 days the anthers were subculture into new plates and observed for the morphological changes and embryo or calli induction under a light microscope (OLYMPUS CX33) at 1 micron resolution. The frequency of calli/ embryo induction were recorded and calculated as the percentage of cultured anthers.

The effect of plant growth regulator was determined by the following 3 treatments:viz., 1. YP without plant growth regulators, 2 . YP Media+ 2 mg/l IAA +2 mg/l Kinetin and 3.YP Media + 2 mg/l 2,4-D +1 mg/l BAP

2.5 Statistical analysis:

The study was carried out using a completely randomized design with 5 replications. Each replication cultured 25 anthers. One-way Analysis of variance (ANOVA) was carried out using SPSS statistical software package (SPSS, version 17.0). Numbers of calli/embryo induction were analysed through one-way ANOVA. Data were transformed using square root transformation of the form $(x + 0.5)$, {i.e.$\sqrt{(x + 0.5)}$} for No. of calli/embryo obersevd. Least Significant Difference (LSD) test was used to identify the pairwise significant differences among the main effects of treatment. A $p < 0.01$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Morphometric attributes of maize genotypes:

The development of immature anthers containing microspores in crop plants is significantly influenced by both plant growth conditions and the plant's genotype [13]. To determine the optimal stage for collecting anthers, various morphometric attributes were considered, including the number of days elapsed after sowing, number of leaves per plant at the time of tassel collection, and the assessment of leaves that were appropriately aligned with tassels containing microspores, as given in Table 1.

Table 1: Morphometric attributes of maize genotypes

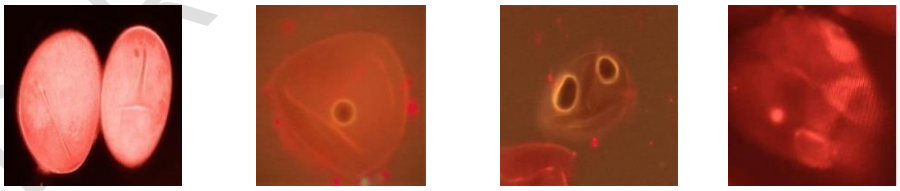
Sr. No	Genotypes	Sample taken DAS	No. of Leaf during sampling	Stage of Microspore
1	Pusa HM4 Improved	52	16	Mid-uninucleated to binucleated
2	Pusa HM8 Improved	54	14	Mid-uninucleated

3	Pusa HM9 Improved	56	15	Uninucleated
4	Pusa Vivek QPM9 Improved	54	16	Mid-uninucleated to binucleated
5	Pusa HQPM1 Improved	58	17	Mid-uninucleated
6	HQPM5 Improved	55	15	Uni-nucleate
7	Pusa HQPM7 Improved	52	16	Mid-uninucleated to binucleated
8	Pusa Biofortified Maize hybrid-	59	14	Mid-uninucleated
9	Pusa Super Sweet Corn-1	53	13	Uninucleated
10	Pusa Super Sweet Corn-2	53	14	Mid-uninucleated to binucleated
11	APCH-2	56	16	Mid-uninucleated
12	APCH-3	56	15	Mid-uninucleated to binucleated

3.2 Confirmation of microspore development Stage:

To precisely identify the microspore development stage, the leaf collar method was employed. The V13- V17 stage of the plant was found to contain the highest proportion of uninucleate stage microspores. Using fluorescence microscopy and staining with DAPI, different stages of microspore development, including tetrad, uninucleate, binucleate, and trinucleate stages (Fig.1), were observed. Notably, microspores in the mid-uninucleate stage were found to be the most responsive to anther culture.

Fig.1 Microspore development stages. (A) Tetrad stage (B) Uninucleated stage (C) Binucleate stage (D) Trinucleate stage.



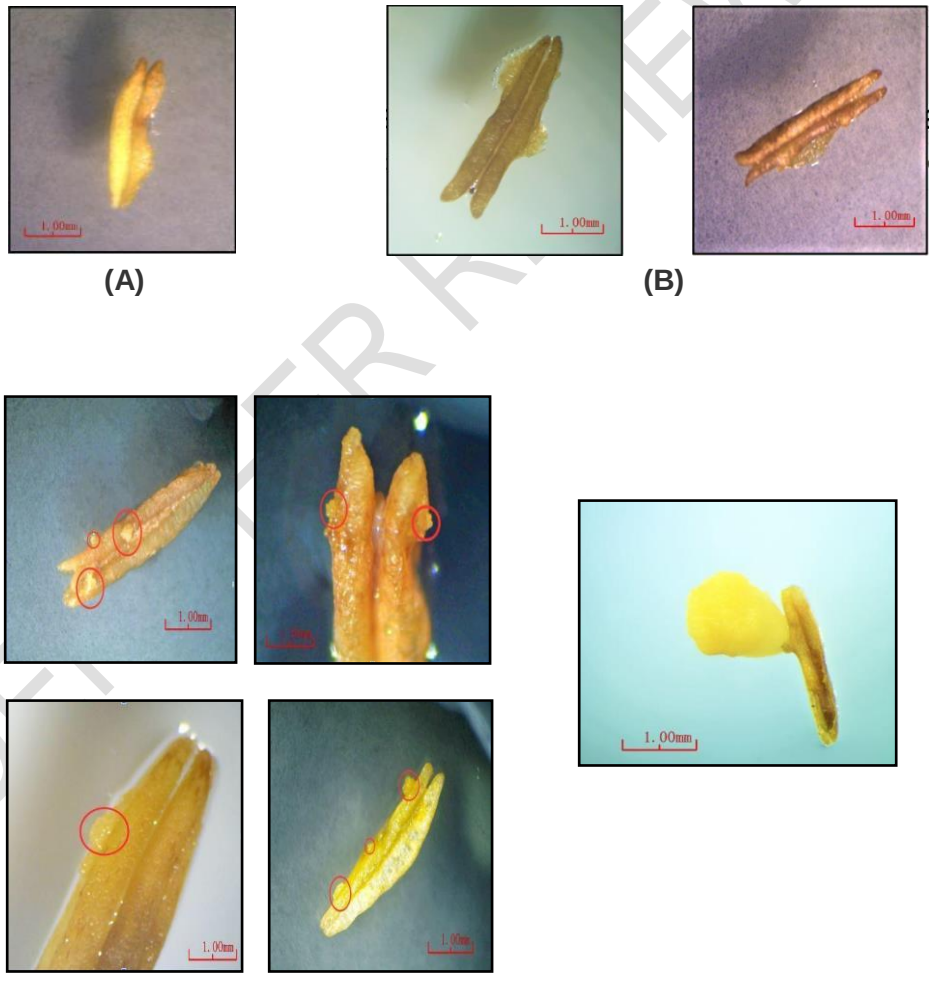
(A) **(B)** **(C)** **(D)**

3.3. Inoculation of anther on culture media:

The mid-uninucleate microspore containing anthers were kept in pretreatment media at 4°C for 10 days in the dark. The pretreated anthers were placed on three different YP media & incubated at 28°C in dark. After 7 days, the anthers were observed and the anthers were swollen and slightly brown & bent as seen through light microscope (Fig.2A), while after 14 days, anthers busted, and callusing occurred (Fig.2B). After 30 days of anther culture, callusing occurred (Fig.2C). Anthers were subcultured further to YP media for the next 15 days. After 45 days, the embryo-like structure was observed to be smooth and globular (Fig.

2D). The embryo-like structure showed an increase in size from 1mm to 2 mm, it was transferred into regeneration media and kept in light at a light intensity of 250 $Jmolm^{-2}s^{-1}$.

Fig.2 Observation of inoculated anther through light microscope. (A) After 7 days, swelling and browning of anthers. (B) After 14 days, bursting of anther wall and callusing occurs. (C) After 30 days, callusing in anther. (D) After 45 days, embryo induction occurred.



3.4 Effect of plant growth regulators on callus/embryo induction:

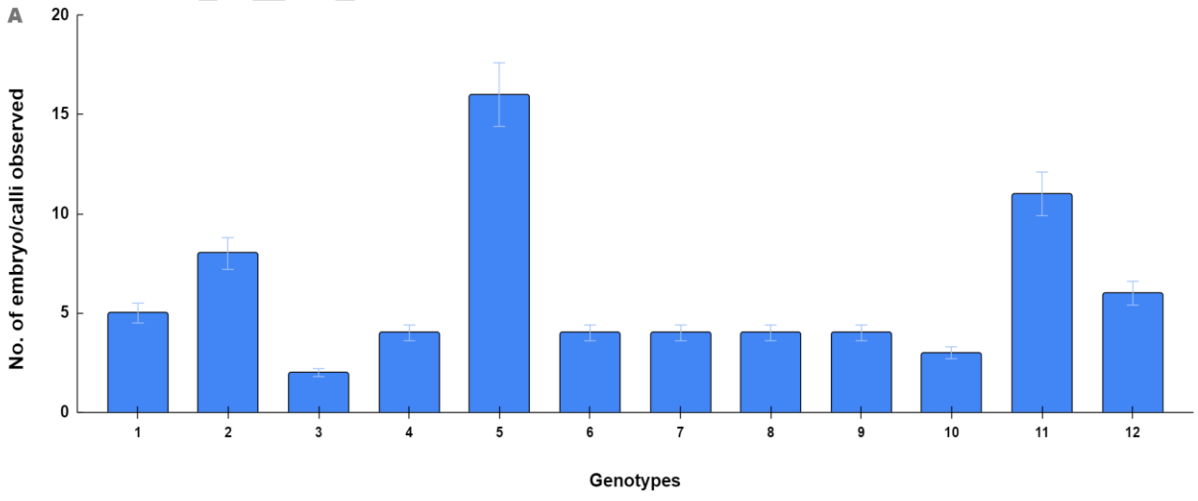
Plant growth regulators (PGRs), such as auxins and cytokinins, are essential for inducing callus and embryo formation in tissue culture. Auxins promote callus development, while cytokinins enhance cell division and the transition to embryo formation. Optimizing PGR concentrations and combinations is crucial for successful plant regeneration. Out of the

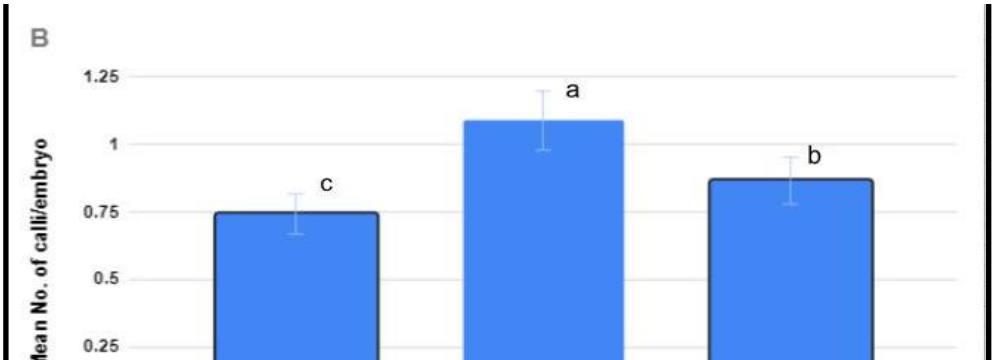
twelve genotypes assessed, only three exhibited a significant response, namely Pusa HM8 Improved, Pusa HQPM1 Improved, and APCH-2 as in Fig. 3A. The response was higher in YP media with 2 mg/l IAA + 1 mg/l kinetin as compared to other media (Fig.3B). The response rate of Pusa HM8 Improved, Pusa HQPM1 Improved, APCH-2 are 4%, 8% & 5% respectively in 2 mg/l IAA + 1 mg/l kinetin showing significant difference with other treatments. All the observations were subjected to statistical analysis and the results are presented in Table 2.

Table 2. Mean squares (MS) of the ANOVA for No. of embryo/calli induction in anther culture of *Zea mays* L. genotypes. (Significant difference at 1% probability level)**

Source of variati	Degree of Freedom	Mean sum of square	F value
Treatment	2	1.846	27.159**
Error	12	0.068	
Total	14		

Fig. 3. Effect of plant growth regulators on calli/embryo induction. (A) Analysis of androgenic response among twelve genotypes. [Error bars show mean +/- 1 SE]. (B) Effect of treatments on calli/embryo induction. 1- YP without plant growth regulator; 2-YP+2 mg/l IAA +1 mg/l Kinetin; 3- YP +2 mg/l 2,4D +1 mg/l BAP. [Error bars show mean +/- 1 SD]. Means in the bar graph were separated using Duncan Multiple Range Test at 1% significance. The same variable indicates that the values do not differ significantly.





4. DISCUSSION

Doubled haploids (DH) represent a powerful tool in maize breeding, enabling faster and more efficient development of homozygous lines with desired traits. This technique not only streamlines the breeding process but also enhances the precision and effectiveness of maize improvement programs. As agricultural challenges evolve, the use of DH in maize breeding will continue to play a critical role in enhancing food security and agricultural sustainability. Androgenesis involves the development of a plant from a haploid microspore (pollen grain) or anther tissue, resulting in a haploid plant. Androgenesis involves two stages. The former stage is induction, where somatic cells acquire embryogenic potential through changes in cellular physiology, metabolism, gene expression etc. and the latter stage is regeneration, where cells differentiate into somatic embryos. Critical factors, such as the composition of plant growth regulators and endogenous hormone levels, influence the embryogenic competence of explants [10, 11].It has been hypothesized that endogenous growth regulator levels in maize are sufficient for embryo development, making exogenous supply unnecessary [22]. Data suggest that regulators requirements may vary based on genotype and donor plant growth conditions [17]. Differences in embryogenic competence among maize genotypes may arise from varying cellular responses to exogenous plant growth regulators [9]. A key distinction between more and less competent lines is the distribution of IAA within embryos cultured on 2,4-D-containing medium [4]. The results of this investigation affirm the possibility of generating haploid plants directly through the process of anther culture, despite a relatively minimum success rate in calli/embryo induction. The findings within the realm of maize anther culture lend support to the occurrence of the direct generation of somatic embryoids from anthers which closely resemble zygotic embryos. This phenomenon, as observed in our study and previously reported by Kuo *et al.*(1986). Generating plantlets from these embryoids is a more straightforward task compared to deriving them from callus, as demonstrated in the study by Cheng *et al.* (1978). Elevated levels of auxin have been documented to enhance the response of anther culture in maize, although they are also linked to a reduction in the number of embryo structures [5]. Furthermore, a higher concentration of exogenous IAA has been correlated with an increased ability to induce calli/embryo in maize. An elevated natural level of auxin has been demonstrated to be linked with an augmented ability

to induce embryonic development in maize.[17]. A better induction of embryogenesis and direct regeneration of plantlets in maize was obtained by using 2mgl-1 kinetin and 2 mgl-1 IAA [15]. While in our study, a more effective induction of embryonic development was achieved by employing a combination of 2 mg/L of IAA and 1 mg/L of Kinetin.

CONCLUSION

The present report showed the androgenic response, via examination of 12 different hybrid varieties of maize. Among these varieties, three genotypes, specifically Pusa HM8 Improved, Pusa HQPM1 Improved, and APCH-2, exhibited positive response under the given conditions of pretreatment & plant growth regulators. The induction frequency of calli/embryogenesis ranged from 4% to 8% per 125 anthers plated on the YP media. Notably, hybrid Pusa HQPM1 Improved exhibited the highest induction frequency of 8%, making it the most responsive genotype among the tested genetypes. This may help to develop doubled haploids to boost maize breeding in tropical countries.

DEFINITIONS, ACRONYMS, ABBREVIATIONS
BAP: Benzyl aminopurine
IAA: Indole-3-acetic acid
2,4-D:2,4-Dichlorophenoxyacetic acid CENH3: Centromeric Histone H3
DAPI: 4',6-Diamidino-2-Phenylindole
DH: Doubled Haploid
YP media: Yu-Pei media
.
Term: Definition for the term

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APPENDIX

1.1 Sampling of tassels for anther culture

1. For every genotype, a correlation between plant morphology and developmental stage of micro spores can be established by leaf collar method and microscope method.
2. Select 2-3 top florets from a tassel before it emerges from the boot.
3. Extract anthers from the florets and crush them with a glass rod in a DAPI solution. Score the developmental stage of microspores on microscope slides using fluorescence microscopy.
4. Evaluate most microspores for their developmental stage, focusing on the late uninucleate to early binucleate stages.
5. Cut the plants at 1-2 nodes below the tassel base and remove excess foliage, leaving only 3-4 leaves around the tassel. Trim the remaining leaves slightly longer than the tassel and place the tassel in a flask with distilled water.
6. Bring the flask back to the laboratory for further processing. Disinfect the harvested tassels immediately if possible, or store them in a refrigerator at 4°C for short-term storage.
7. Tassels can be stored for 1-3 days without significant detrimental effects, but prolonged storage without processing is discouraged.

1.2 Detection of microspore development stages

1. Collect anthers containing microspores from the maize tassel.
2. Crush with needle to expose microspore, remove debris & fix using a suitable fixative Conroy's solution for 1-2 hours.
3. Wash the fixed anthers multiple times with a PBS buffer.
4. Prepare a DAPI staining solution by diluting 1mg/ml DAPI dye in a buffer.

5. Add the DAPI staining solution to the washed microspore and incubate in the dark for 15-30 minutes.
6. Transfer the stained anthers to a microscope slide. Apply a mounting medium and cover with a coverslip. Examine the stained microspores under a fluorescence microscope with 461nm filters.

1.3. Pretreatment of anthers.

1. In a laminar flow hood, remove the remaining foliage around the tassel and take it out of the boot for disinfection.
2. The tassel can be disinfected as a whole or separated into florets before disinfection. Submerge the whole tassel or florets in a 20% commercial bleach (or 1.2% sodium hypochlorite) solution for 20 minutes, periodically shaking it.
3. Pour out the bleach solution and rinse the tassel or florets three times with autoclaved distilled water.
4. Place the disinfected florets into a Petri dish for androgenesis preculture media. If the florets were separated before disinfection, transfer them directly to the Petri dish for pretreatment. Float 150-200 florets over 10- 15 ml of preculture medium in each Petri dish.
5. Seal the Petri dishes with Parafilm and incubate them in the dark at temperatures of 4°C for 10 days.