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Assessment of Gametophyte Stability and Seed Index Variability in Cotton Germpasm Accessions for Advanced Breeding Programs

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Abstract This study aimed to evaluate the cyto-embryological stability and agro-morphological characteristics of various *Gossypium hirsutum* genotypes, including semi-wild subspecies and modern cultivars, in order to determine their reproductive efficiency and potential value for breeding programs. Significant genotypic variation was detected ($P<0.05$). The cultivar Ravnak 1 demonstrated the highest reproductive performance, exhibiting an ovule penetration rate of 94.52 ± 0.42 and pollen fertility 96.78±0.94, indicating high stability of male gametophyte. In contrast, subsp. *purpurascens* showed the lowest fertility indicators. Meanwhile subsp. *gambia* demonstrated moderate meiotic stability with a meiotic index of 90.39 ± 1.19, despite the occurrence of micronucleated tetrads and polyads (3.14% and 6.47%, respectively). The analysis of the 1000-seed weight revealed substantial variability among genotypes, ranging from 81.78 ± 1.25 g in subsp. *gambia* to 133.89 ± 1.16 g in cultivar *Ravnak* 2. The genotype *Genofond* 2 exhibited remarkable population uniformity with a coefficient of variation of $V=1.07$, indicating high genetic stability. Overall, the obtained results demonstrate that the efficiency of pollen tube penetration and seed index stability can serve as reliable indicators for selecting highly productive cotton genotypes. These findings emphasize the importance of integrating reproductive biology parameters into cotton breeding programs aimed at developing high-yielding and genetically stable cultivars.

Keywords Cotton (*Gossypium hirsutum* L.), Pollen-ovule interaction, Male gametophyte fertility, Meiotic index, ANOVA, Micronucleated tetrads, Seed index, Cotton breeding

1. Introduction

Cotton is one of the most important industrial crops worldwide, serving as a major source of natural fiber and playing a crucial role in the global textile industry. According to recent estimates, global cotton production exceeds 25 million tons annually, highlighting its significant economic value and widespread cultivation in many regions of the world [5]. At present, cotton is grown on all continents except Antarctica, reflecting its high adaptability to diverse climatic conditions [1].

Among cultivated cotton species, *Gossypium hirsutum* L. is the most widely distributed and economically important. In its wild form, however, the natural distribution range of this species has considerably decreased. Currently, wild populations are mainly found in limited geographical areas such as the northern Yucatán Peninsula, Florida, southeastern Puerto Rico, and several islands of the Caribbean region [8]. The study of wild and semi-wild cotton forms is particularly important because they represent valuable sources of genetic diversity that can be utilized in breeding programs aimed at improving agronomic traits and environmental adaptability.

Embryological studies in angiosperms play a fundamental role in understanding the mechanisms of gametogenesis, fertilization, and early embryo development. One of the most critical stages of plant reproduction is the growth of pollen tubes through maternal tissues, which ensures the successful delivery of male gametes to the embryo sac. During fertilization, pollen tubes pass through three main anatomical structures: the stigma, the style, and the ovules located within the ovary. This precisely regulated process

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enables the transport of sperm cells to the egg cell and central cell, ultimately leading to double fertilization and seed formation [4].

The directional growth of pollen tubes toward the ovary is regulated by chemotropic signaling mechanisms. In cotton plants, the maturation of male gametes is completed within the pollen tubes during their elongation through the transmitting tissue of the style. Environmental factors, particularly temperature, also influence pollen tube growth. In most plant species, optimal pollen tube elongation occurs within the temperature range of 20–30 ℃, although species with delayed flowering periods may exhibit adaptation to lower temperatures. Furthermore, pollen tube growth rates vary considerably among plant taxa and are influenced not only by chemical signals but also by mechanical interactions with surrounding tissues. In this context, pollen tubes may respond to differences in tissue rigidity, a phenomenon referred to as durotropism, which facilitates efficient navigation toward the embryo sac [10].

Recent studies have demonstrated that the kinetics of pollen tube elongation plays a decisive role in successful fertilization. For example, Wang et al. (2023) reported that in certain Malvaceae species, failure of pollen tubes to reach the embryo sac within 24 hours often results in seed abortion [12]. In addition, previous embryological investigations conducted by V.A. Rumi provided detailed insights into the morphometric characteristics and elongation dynamics of pollen tubes during the fertilization process in flowering plants. These studies highlighted the importance of pollen tube growth dynamics in determining reproductive success. Furthermore, stigmatic secretions have been shown to influence pollen germination and pollen tube development, emphasizing the complex biochemical interaction between the male gametophyte and maternal tissues [3].

In cotton breeding research, yield-related traits such as the weight of seed cotton per boll and the 1000-seed weight (seed index) are considered key agronomic indicators. These parameters are widely used for evaluating the productivity of cultivars and hybrids and for identifying promising genotypes in breeding programs. Previous studies have demonstrated that the variability and heritability of these traits play an essential role in the development of high-yielding cotton varieties through targeted selection strategies. For instance, Meyer (1972) reported significant differences in the number of stamens per flower between New World and Old World cotton species, which are associated with their ploidy levels. According to this study, the tetraploid species *G. hirsutum* L. possesses an average of 121.6 stamens per flower, whereas the diploid species G. arboreum L. has approximately 78.6 stamens, indicating clear differences in reproductive morphology between these taxa [7].

Considering the importance of reproductive stability for cotton productivity, the evaluation of embryological characteristics such as pollen fertility, pollen tube growth, and meiotic stability can provide valuable information for breeding programs. Therefore, the present study aims to assess the cyto-embryological stability and seed index variability in different *Gossypium hirsutum* genotypes, including both cultivated varieties and semi-wild forms, in order to identify promising genetic resources for future cotton breeding efforts.

2. Materials and Methods

Plant Materials and Germplasm Collection

The study was conducted during a three-year period (2022-2024) in the experimental fields of the Institute of Genetics and Plant Experimental Biology of the Academy of Sciences of Uzbekistan (Tashkent region). Several *Gossypium hirsutum* L. genotypes, including modern cultivars and semi-wild forms, were selected for the investigation. The plant material was obtained from the Uzbekistan Cotton Germplasm Collection maintained at the Institute of Genetics and Plant Experimental Biology. The analyzed genotypes included four cultivated varieties (*Namangan* 77, *Ravnak* 1, *Ravnak* 2, and *Genofond* 2) and three semi-wild forms (subsp. *purpurascens*, subsp. *glabrum* var. *marie-galante*, and subsp. *punctatum* var. *gambia*). These genotypes represent diverse genetic backgrounds and were selected in order to evaluate their reproductive stability and agronomic potential (Table 1).

Table 1. List of *Gossypium* L. genotypes used in experiment

№	Genotypes	Country of Origin
1	Namangan 77 cultivar	Uzbekistan
2	Ravnak 1 cultivar	Uzbekistan
3	Ravnak 2 cultivar	Uzbekistan
4	Genofond 2 cultivar	Uzbekistan
5	subsp. *purpurascens*	West India and Colombia
6	subsp. *glabrum* var. *marie-galante*	West India and Mexico
7	subsp. *gambia*	West Africa

Embryological Analysis

Successful fertilization in flowering plants depends on proper pollen germination and the growth of pollen tubes through the style toward the ovules within the ovary. In angiosperms, microsporogenesis consists of two successive meiotic divisions that produce a tetrad of haploid microspores. These microspores subsequently undergo microgametogenesis, during which each microspore divides mitotically to form a vegetative cell and a generative cell. The generative cell later divides to produce two sperm cells responsible for double fertilization. In the present study, embryological observations were focused on the efficiency of pollen tube penetration into ovules. The number of ovules per ovary and the proportion of ovules penetrated by pollen tubes were determined in order to assess fertilization efficiency. All embryological procedures and anatomical analyses were carried out according to the microtechnical methods described by *Barykina* et al. [2].

Sample Preparation and Microscopy

For embryological observations, flowers were artificially pollinated and immediately covered with isolation bags in order to prevent uncontrolled cross-pollination. After 24 hours, the samples were collected and fixed in Carnoy's solution (ethanol: acetic acid=3:1).

To improve the visualization of internal structures, the fixed samples were subjected to a clearing process using a potassium hydroxide (KOH) solution for seven days until complete tissue decolorization was achieved. After thorough washing with distilled water, the ovules were carefully isolated and examined under a microscope to determine the presence and penetration of pollen tubes. Cytological and morphological observations were carried out using a Leica CME light microscope. Digital imaging was performed using Leica CM E and XSP-500SM microscopes equipped with a Leica EC3 camera.

Cytogenetic Analysis

The cytogenetic stability of the studied genotypes was evaluated through the analysis of meiotic processes and pollen fertility. Floral buds at appropriate developmental stages were fixed in an ethanol-acetic acid solution (3:1) and stained with acetocarmine according to the cytological methodology described by *Pausheva* (1988) [14]. The meiotic index was determined by analyzing tetrads formed during microsporogenesis in microspore mother cells. The proportion of normal tetrads relative to abnormal sporads (monads, triads, pentads, hexads, and heptads) was calculated to assess meiotic stability. Pollen fertility was evaluated using fresh pollen samples incubated at 4 ℃ for 24 hours to enhance staining contrast. Microscopic observations were performed in ten randomly selected fields of view, and the percentage of fertile pollen grains was calculated.

Measurement of Yield-Related Traits

Yield-related parameters were evaluated during the stage of physiological maturity when approximately 75% of the cotton bolls had opened. To obtain representative samples, bolls were collected from the 5th to the 8th sympodial nodes of the main stem of each plant. The collected samples were used to determine the 1000-seed weight (seed index), which is considered an important indicator of productivity and seed development in cotton breeding programs.

Statistical Analysis

Statistical analyses were performed using OriginPro 2022 software [6,9,13]. To evaluate the significance of differences among genotypes, one-way and two-way analyses of variance (ANOVA) were applied. For traits influenced by multiple factors, the two-way ANOVA model was used to assess the main effects of genotype and environmental factors, as well as their interactions. Differences between mean values were evaluated using Tukey's Honestly Significant Difference (HSD) test at a significance level of $P < 0.05$. All quantitative results are presented as Mean ± Standard Error (SE).

3. Results

Embryological Characteristics of the Studied Genotypes

Embryological traits were analyzed in both cultivated varieties and semi-wild forms of *Gossypium hirsutum*. The investigation focused on several reproductive parameters, including the total number of ovules per ovary, the number of ovules penetrated by pollen tubes, and the overall efficiency of fertilization (Fig. 1).

Among the studied genotypes, the cultivar *Ravnak* 1 demonstrated the highest proportion of ovules successfully penetrated by pollen tubes (94.52 ± 0.42%), with 311 out of 329 ovules showing successful fertilization. Similarly, *Namangan* 77 (92.42 ± 0.73%) and *Ravnak* 2 (90.58 ± 2.59%) also exhibited high embryological performance. The cultivar *Genofond* 2 (90.12 ± 1.54%) showed slightly lower but still relatively stable fertilization efficiency. In contrast, the semi-wild genotype subsp. *purpurascens* (83.72 ± 1.22%) exhibited the lowest ovule penetration rate. Other semi-wild forms, such as subsp. glabrum var. marie-galante (88.45 ± 0.78%) and subsp. *punctatum* var. *gambia* (85.63 ± 1.04%), demonstrated intermediate fertilization efficiency. The variability of ovule fertilization also differed among genotypes. For example, *Ravnak* 2 showed the highest variability (SD= 8.20), indicating greater heterogeneity in reproductive performance. In contrast, *Ravnak* 1 exhibited the lowest variability (SD= 1.32), suggesting more stable embryological development. The results of ANOVA analysis confirmed statistically significant differences among the studied genotypes. According to Tukey's HSD test, genotypes marked with different letter groups showed significant differences at $P < 0.05$, whereas genotypes sharing the same letters did not differ significantly (Table 2). Embryological analysis provides an important basis for evaluating reproductive potential in cotton genotypes. A higher proportion of ovules penetrated by pollen tubes is associated with improved fertilization efficiency and a lower probability of empty boll formation. Therefore, genotypes such as Ravnak 1 and Namangan 77, which demonstrated both high ovule penetration rates and relatively low variability, may be considered promising candidates for breeding programs aimed at improving cotton productivity.

Figure 1. Cyto-embryological stages of the fertilization process: black arrow-mechanical isolation of ovules from the ovary; red arrow-unsuccessful pollen tube penetration into the ovule; blue arrow-successful pollen tube entry into the micropyle; green arrow-post-fertilization developmental stage (4×magnification)

Pollen Fertility in the Studied Genotypes

The analysis of pollen fertility revealed clear differences in male gametophyte stability among the studied cotton genotypes. The highest pollen fertility was recorded in Ravnak 1, reaching 96.78 ± 0.94%, which indicates a high level of male gametophyte stability. Other cultivated varieties, including *Ravnak* 2 (90.78 ± 1.49%) and *Namangan* 77 (90.08 ± 0.72%), also demonstrated relatively high fertility levels. In contrast, the semi-wild genotype subsp. *purpurascens* showed the lowest pollen fertility among the studied samples (85.43 ± 0.89%). The remaining genotypes, such as subsp. *glabrum* var. *marie-galante* (89.32%) and *Genofond* 2 (89.45%), showed intermediate fertility levels (Fig. 2).

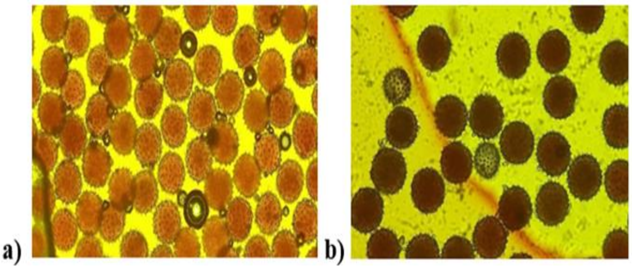

Figure 2. Pollen fertility analysis: a) Namangan 77 cultivar (90.08±0.72%); b) subsp.*purpurascens* (85.43±0.89%) (10 × magnification)

Overall, the comparative analysis of pollen fertility among the studied genotypes demonstrated significant differences in reproductive capacity, confirming the importance of pollen viability as an indicator of reproductive stability in cotton (Table 3).

Cytogenetic Stability During Microsporogenesis

Cytogenetic analysis of microsporogenesis revealed important differences in meiotic stability among the studied genotypes. In particular, the tetrad stage of meiosis in subsp. *punctatum* var. *gambia* was examined in detail (Fig. 3).

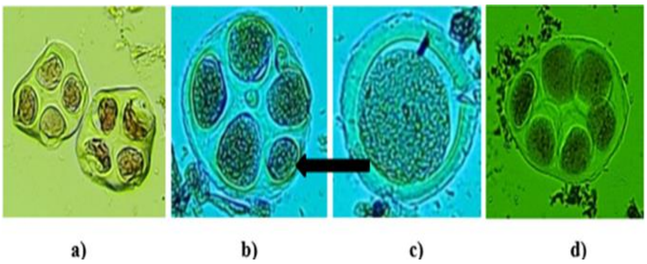

Figure 3. Microscopic images of tetrads in subsp.*punctatum* var.*gambia*: a) normal tetrads; b) micronucleated tetrads (arrows indicate micronuclei); c) monads; d) hexads (at 40 ×magnification)

The meiotic index in this genotype was 90.39 ± 1.19%, indicating relatively stable microsporogenesis. However, several meiotic abnormalities were also detected, including micronucleated tetrads (3.14 ± 0.78%) and polyads (6.47 ± 0.95%). The presence of micronucleated tetrads suggests irregular chromosome segregation during meiosis. These tetrads contained between one and seven micronuclei, which may lead to the formation of genetically unbalanced gametes. In addition, polyads consisting of abnormal sporads such as pentads, hexads, and heptads were also observed. These abnormalities indicate disturbances in the normal meiotic process and may partially explain the reduced fertility observed in some semi-wild genotypes.

Table 2. Analysis of the growth of pollen tubes into the ovule

Plant Samples	Total number of nodes	Total number of ovules	Ovule penetrated by pollen tube	Mean ± SE	SD
subsp. *glabrum* var. *marie-galante*	10	287	254	88,45 ± 0,78 BCD	2,48
subsp. *purpurascens*	10	269	225	83,72 ± 1,22 D	1,23
Ravnak 1	10	329	311	94,52 ± 0,42 A	1,32
Namangan 77	10	328	303	92,42 ± 0,73 AB	2,32
subsp. *punctatum* var. *gambia*	10	285	244	85,63 ± 1,04 CD	3,29
Genofond 2	10	296	266	90,12 ± 1,54 ABC	4,88
Ravnak 2	10	320	289	90,58 ± 2,59 ABC	8,2

Table 3. Development of cotton pollens in genotypes

Plant Samples	Total number of pollens	Pollen fertility, % Mean ± SE	SD
subsp. *glabrum* var. *marie-galante*	788	89,32 ± 2,16 BC	2,48
subsp. *purpurascens*	932	85,43 ± 0,89 C	2,82
Ravnak 1	836	96,78 ± 0,94 A	2,96
Namangan 77	913	90,08 ± 0,72 BC	2,26
subsp. *punctatum* var. *gambia*	985	87,56 ± 0,95 BC	3,01
Genofond 2	876	89,45 ± 1,82 BC	5,76
Ravnak 2	925	90,78 ± 1,49 B	4,73

Note: Within each column, means labeled with different uppercase or lowercase letters indicate significant differences at $P < 0.05$, as determined by Tukey's HSD test. Corresponding ANOVA outcomes (F and P values) are presented in the main text

Table 4. Seed index

№	Plant Samples	1000 seed weigh			
		Mean ± SE	Range	SD	V
1	subsp. *punctatum* var. *gambia*	81,78 ± 1,25	78,70-84,81	2,49	3,05
2	subsp. *purpurascens*	91,70 ± 1,29	89,4-95,2	2,57	2,81
3	subsp. *glabrum* var. *marie-galante*	108,62 ± 1,02	105,8-110,5	2,04	1,88
4	*Namangan* 77	94,33 ± 1,29	92,0-98,0	2,58	2,74
5	*Ravnak* 1	99,39 ± 1,74	96,0-104,0	3,49	3,51
6	*Genofond 2*	117,14 ± 0,63	115,56-118,6	1,25	1,07
7	*Ravnak 2*	133,89 ± 1,16	130,5-135,7	2,32	1,73

Analysis of the 1000-Seed Weight (Seed Index)

The analysis of 1000-seed weight (seed index) revealed considerable genotypic variability among the studied cotton samples. The lowest seed index was observed in subsp. *punctatum* var. *gambia* (81.78 ± 1.25 g), whereas the highest value was recorded in the cultivar *Ravnak* 2 (133.89 ± 1.16 g). Among the semi-wild forms, subsp. *glabrum* var. *marie-galante* demonstrated a relatively high seed weight (108.62 ± 1.02 g), indicating good seed development potential. The ultivar *Genofond* 2 showed particularly high genetic stability, as reflected by its low coefficient of variation (V=1.07). Such low variability indicates high population uniformity and is considered an important trait for mechanized agriculture and large-scale seed production. In contrast, *Ravnak* 1 exhibited a higher coefficient of variation (V=3.51), suggesting greater sensitivity of this genotype to environmental conditions during seed formation. Overall, the obtained results demonstrate that both embryological parameters and seed index characteristics vary significantly among cotton genotypes and may serve as important criteria for selecting promising breeding material.

4. Discussion

The experimental results demonstrate a clear physiological and genetic divergence in the reproductive efficiency of *Gossypium hirsutum* genotypes. The embryological analysis revealed that Ravnak 1 exhibits superior reproductive potential, with a peak ovule penetration rate of 94.52±0.42, significantly outperforming semi-wild forms such *as* subsp. *purpurascens* (83.72±1.22). This heightened efficiency in pollen tube navigation to the micropyle is inherently linked to the stability of the male gametophyte. Statistical analysis of our data reveals a significant positive correlation between pollen viability and pollen tube growth ($r=0.583$, $p<0.001$), supporting the link between high fertility and successful fertilization processes. Among the studied genotypes, Ravnak 1 exhibited the highest performance, recording the top fertility rate (96.82±2.87%) and superior pollen tube growth (94.52±1.29%). The moderate-to-strong nature of this correlation ($R^2=0.34$) suggests that while pollen viability is a primary determinant of fertilization success, the efficiency of pollen tube penetration also plays a critical role in the overall reproductive stability of these cotton varieties. Recent studies have also emphasized the importance of pollen fertility and pollen tube growth for cotton productivity. For instance, Zhang et al. (2024) reported that environmental stress factors, particularly elevated temperature and drought, significantly reduce pollen fertility and consequently affect boll formation and yield components in cotton [16]. Similarly, Luqman et al. (2025) demonstrated that disruptions in pollen germination and pollen tube elongation are closely associated with metabolic disturbances in anthers and may lead to decreased reproductive efficiency in *Gossypium hirsutum* under stress conditions [15]. These findings confirm that reproductive biological parameters such as pollen viability and pollen tube growth play a key role in determining cotton yield potential and should be considered in breeding programs aimed at improving stress tolerance and productivity. These findings suggest that directional pollen tube growth, governed by the mechanisms of chemotropism and durotropism discussed in classical literature, is more optimized in modern local cultivars compared to semi-wild accessions. The cytogenetic investigation into subsp. *punctatum* var. *gambia* provides a critical perspective on the chromosomal basis of fertility. Although the meiotic index (90.39±1.19) indicates a relatively stable microsporogenesis, the presence of micronucleated tetrads and polyads (up to 6.47%) suggests occasional aneuploid sporads and irregular chromosomal segregation. Such meiotic irregularities explain the intermediate fertility levels observed in wild-type genotypes. Furthermore, the variability in the 1000-seed weight (seed index) highlights the impact of targeted selection. The exceptional seed mass in Ravnak 2 (133.89±1.16 g) and the high population uniformity of Genofond 2 (V=1.07) demonstrate that breeding for embryological stability directly translates into superior agronomic traits. The statistical significance established by Tukey's HSD test confirms that genotypes with higher embryological performance (Group A) are the most suitable candidates for high-yield breeding and hybridization programs.

5. Conclusions

The present study demonstrated that significant differences exist among *Gossypium hirsutum* genotypes in terms of their reproductive efficiency, cytogenetic stability, and seed productivity. The results of embryological analysis showed that modern cotton cultivars generally possess higher fertilization efficiency compared with semi-wild forms. Among the studied genotypes, the cultivar *Ravnak* 1 exhibited the highest reproductive performance, characterized by a high rate of pollen tube penetration into ovules and superior pollen fertility. These parameters indicate strong stability of the male gametophyte and efficient interaction between pollen tubes and maternal reproductive tissues. Similarly, *Namangan* 77 also demonstrated relatively high fertilization efficiency, suggesting its potential value as a promising genotype for breeding programs. In contrast, semi-wild forms such as subsp. *purpurascens* and subsp. *punctatum* var. *gambia* showed lower reproductive indices, which may be associated with observed meiotic irregularities, including the formation of micronucleated tetrads and polyads. Such cytogenetic abnormalities can negatively affect gamete viability and may reduce overall fertilization success. The analysis of yield-related traits revealed substantial variability in 1000-seed weight (seed index) among the investigated genotypes. The cultivar *Ravnak* 2 showed the highest seed weight, whereas *Genofond* 2 exhibited exceptional population uniformity with a very low coefficient of variation. These characteristics indicate that targeted breeding and selection have contributed to improving seed productivity and genetic stability in modern cotton cultivars.

Overall, the results of this study highlight the importance of integrating embryological and cytogenetic indicators into cotton breeding strategies. Parameters such as pollen fertility, pollen tube penetration efficiency, meiotic stability, and seed index variability can serve as reliable criteria for identifying promising genetic resources. The genotypes identified in this study may therefore represent valuable material for future cotton breeding programs aimed at developing high-yielding and genetically stable cultivars.

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