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RESEARCH ARTICLE

Optimizing pollen viability and *in vitro* pollen germination potential in foxtail millet genotypes

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Abstract

Foxtail millet is a C4 model crop with very small florets. To improve the yield of any crop, pollen germination and development are important to know, which is still not well-known in foxtail millet. The present investigation was conducted in the Department of Seed Science and Technology, Hemvati Nandan Bahuguna Garhwal University (HNBGU), Srinagar, Garhwal, Uttarakhand, during the*Kharif* seasons of 2023 and 2024 to study flowering phenology and achieve synchronous flowering for the development of a hybrid seed production programme The study involved 14 genotypes, including 12 landraces and 2 varieties as checks. The timing of pollination in foxtail millet germplasm was observed to be approximately 1-2 hrs. Further to understand the pollen viability, *in vitro* pollen germination and development of pollen tube under the influence of different media and time durations at a temperature of 25 °C with 85 % relative humidity. Significantvariation in pollen viability and pollen germination was recorded across the different media tested. The maximum pollen viability, lasting up to 5 hr, was observed in the genotype FMC-1, indicating its potential for crop improvement. A combination of sucrose, boric acid and potassium nitrate (KNO_3) was found to be the best media compared to others, such as 2,3,5-triphenyl tetrazolium chloride (TTC), acetocarmine, boric acid + sucrose, as pollen grains began germinating within 30 min in most genotypes. This study provides valuable insights for designing a crossbreeding programme in foxtail millet.

Keywords: foxtail millet; germination; landraces; pollen; viability

Introduction

Small millets belong to the *Poaceae* family and are widely used as staple food and fodder crops in the semi-arid regions of the world. Foxtail millet (*Setaria italica* L.) is one such ancient crop cultivated for same reason. It is believed to have originated in Central China. It is a C4 diploid species (2n=18) and a self-pollinated crop, making it highly beneficial for breeding purposes aimed at sustainable agriculture (1, 2).

However, the decline in foxtail millet cultivation by farmers, primarily due to its high labor input cost and low yield compared to other staple crops has become a vital concern (3-6). Despite these challenges, foxtail millet holds significant potential as a model bioenergy crop, owing to its small genome size (7).

As a model plant, foxtail millet is recognized for its short growth duration, rapid generation cycle, small genome, plenty of seeds per rachis, easy to grow and wide adaptability, all of which makes it suitable for genetic and physiological studies (8-10). For effective mapping and breeding population development, a model plant should also be easy to cross. However, in the case of *S. italica*, hybridization remains challenging due to its bisexual nature, which complicates controlled crossing (11-13).

Understanding floral morphology and flowering behavior is highly challenging when establishing crosses between desirable parents. The success of pollination and subsequent double fertilization is directly associated with the viability and functionality of pollen, which play a crucial role in achieving effective fertilization and successful seed set. Evaluating pollen health is therefore essential for ensuring reproductive success in both natural reproduction and breeding programs (14). Successful fertilization ultimately leads to higher yield potential and rapid genetic improvement depends on achieving desirable combinations of traits (15).

A lack of knowledge about flowering morphology and pollen biology in foxtail millet may adversely affect seed development. Since crops production depends largely on pollen fertility, which in turn relies on pollen viability and germination, understanding these aspects is fundamental. For successful pollination and seed set during flowering viability of pollen is the prerequisite. Determining fresh pollen viability is a basic yet critical procedure in plant breeding, with pollen germination assay being the most widely used for this purpose.

To understand pollen biology, *in vitro* pollen germination has proven to be an effective approach for determining viability. Various media compositions, along with controlled temperature and humidity, influenced germination success. Staining methods

used to assess pollen viability should be rapid and time efficient. Currently, there is a lack of information about *S. italica* regarding the pollen quality, identification of media for pollen germination.

In sexually reproducing plants, pollen falls on the stigma and germinates to form a pollen tube for successful fertilization (16-19). Viability of pollen is important for dispersion, survival and fitness of the next generation. The ability of pollen to fertilize, germinate and stain effectively is a major determinant of reproductive success in any plant species (20, 21). Previous studies have demonstrated that the composition and concentrations of the germination medium greatly influence pollen viability, germination rate and pollen tube growth (22, 23). *In vitro* pollen germination thus depends on the culture media, which supports the development of pollen tube (24).

Pollen morphology generally remains consistent within a species, although minor variations may occur among genotypes. Advanced microscopy techniques are used to characterize the morphological features of pollen grains (25). Therefore, developing an appropriate protocol to estimate the foxtail millet pollen viability under *in vitro* conditions is essential for enabling controlled pollination, evaluating rapid maturity and supporting crossbreeding programs aimed at developing new varieties.

The main objective of this research paper was to find out the most suitable media for *in vitro* pollen germination in foxtail millet and to examine the relationship between staining efficiency and germination rate for the rapid and accurate determination of pollen viability.

The present study unfolds the flowering phenology and identifies stages of floral development, while describing the reproductive biology of foxtail millet, including pollen viability, pollen germination and tube growth under the influence of different media and duration at 25 °C. The study highlights the information for crossing in C4 plant species and provides a foundation for advancing crossbreeding and heterosis development in foxtail millet.

Materials and Methods

Study site and material

The present investigation was conducted at the Experimental Farm, Department of Seed Science and Technology, HNBGU, Srinagar, Garhwal, Uttarakhand. The site is situated at Madhi Chauras, Naur, Uttarakhand, India, with a latitude of 30.228483° and longitude of 78.806106°. The germplasm of foxtail millet was collected from different regions of India and the seeds were sown in Uttarakhand. A total of 14 genotypes were studied for flowering morphology.

Identification of pollination time

Ten randomly selected plants from each plot were used to identify the timing of fully visibility of anthers and stigmas at three-time intervals: 6:00, 7:00 and 8:00 AM. This study was conducted in three genotypes only (SIA-3156, PRK-1 and FMC-1).

Flowering phenology

Ten randomly selected plants were selected to study flowering phenology parameters, including total anthesis days (TAD), panicle emergence (PE), days to booting stage (BS) and days taken to fully emerge panicle from flag leaf (FPE). To examine the floral phenology of the *S. italica* L. daily during the early morning hours the observation was recorded when the flower parts are clearly visible.

Anther and pollen collection

Anthers were collected at 7: 00 A.M. when they were fully visible on the tail. The male parts were harvested when pollen is about to dehiscence (7:00 to 8:00 AM.) using transparent polythene bag in the field. After brining in lab, the pollens were placed on slides with cover slips and stored at temperature of 25 °C for viability testing.

In vitro pollen viability test

Pollen viability was assessed using 1 % aqueous acetocarmine solution (26). One drop of the staining solution was placed on a microscopic slide and pollen was stained. Five slides were prepared for each genotype. After incubation of 5 min, pollen grains were observed under a light microscope and the numbers of viable (stained) and non-viable (unstained) pollen grains were recorded. Pollen viability percentage was estimated using the formula

$$\text{Pollen Viability (\%)} = \frac{\text{Number of viable pollen}}{\text{Total number of pollen}} \times 100$$

Pollen germination and tube development

The ability of pollen grain to germinate in artifical germinating media was evaluated using different solutions: acetocarmine (28); 1 % aqueous TTC (29); Boric + sucrose and a mixture of boric + sucrose + KI at varying durations. Five slides were prepared in triplicate for each genotype under the controlled randomized block (CRD). The slides were incubated at 25 °C and 85 % relative humidity and observed under a microscope at different intervals. A slight modification was made to the sucrose and boric acid concentration, using 1 % each dissolved into 100 mL of deionised water (30).

$$\text{Pollen germination (\%)} = \frac{\text{Number of pollen germinated}}{\text{Total pollen}} \times 100$$

Data processing

Mean value and analysis of variance for flowering time, pollen viability and pollen germination were computed using the OPSTAT statistical software. Images were assessed using MagVision image analysis software.

Results and Discussion

The present study focuses on the flowering phenology, floral structure and pollen morphology of 26 genotypes of foxtail millet.

Flowering phenology

Foxtail millet is an annual, short-duration crop with a flowering period of approximately one month, but variation depends on the genotypes. The Flowering time generally starts from the month of June to August. The remarkable difference was observed in flowering period in different stages, including days to head emergence from the flag leaf, days to complete panicle emergence after the booting stage, days to anthesis and TAD (Table 1, 2).

Table 1. Analysis of variance in 26 genotypes during 2023 and 2024 for 4 traits

Source of variation	Degree of freedom	Mean square			
		Days to head emergence	Days to booting stage	Days to complete panicle emergence	Total anthesis days
Genotypes	25	27.902	2.854	3.153	10.439
Error	50	3.097	0.554	0.810	1.209
CV		4.300	17.432	12.534	5.783
P		0.001	0.001	0.001	0.001

Table 2. Mean comparison of measured traits

Genotypes name	Days to head emergence	Days to booting stage	Days to complete panicle emergence	Total anthesis days
SIA-3156	37.667 ± 0.882	3.333 ± 0.333	6.333 ± 0.333	16.667 ± 0.882
S-7	38.000 ± 0.577	3.667 ± 0.333	6.333 ± 0.333	18.000 ± 0.577
PRK-1	35.667 ± 0.333	2.000 ± 0.000	4.333 ± 0.333	15.000 ± 0.577
FMC-1	39.000 ± 0.577	3.000 ± 0.000	5.667 ± 0.333	19.000 ± 0.577
FMC-2	40.333 ± 0.333	3.333 ± 0.333	6.333 ± 0.333	19.667 ± 0.882
FMP-2	40.333 ± 0.882	3.667 ± 0.333	7.000 ± 0.577	19.333 ± 0.333
FMP-1	41.000 ± 0.577	3.667 ± 0.333	6.667 ± 0.882	19.667 ± 0.882
FMP-4	39.667 ± 1.202	4.000 ± 0.000	7.667 ± 0.333	20.667 ± 0.333
FMP-5	37.000 ± 1.528	3.667 ± 0.333	7.667 ± 0.333	17.000 ± 0.577
FMU-1	36.667 ± 1.202	3.667 ± 0.333	7.333 ± 0.667	15.667 ± 0.333
FMA-1	38.000 ± 1.000	4.333 ± 0.333	6.667 ± 0.667	16.667 ± 0.333
FMT-1	48.000 ± 0.577	4.000 ± 0.577	8.333 ± 0.333	18.333 ± 0.667
FMC-3	39.667 ± 0.333	4.667 ± 0.333	7.000 ± 0.577	20.333 ± 0.882
FMP-3	40.000 ± 0.577	4.667 ± 0.667	7.333 ± 0.882	21.333 ± 0.333
FMP-6	41.000 ± 0.577	4.667 ± 0.333	7.000 ± 0.577	21.667 ± 0.333
FMR-1	39.000 ± 0.577	3.333 ± 0.333	5.667 ± 0.333	22.000 ± 0.577
FMR-2	39.667 ± 0.882	3.667 ± 0.333	7.000 ± 0.577	22.333 ± 0.667
FMU-2	44.000 ± 2.082	6.000 ± 0.577	8.333 ± 0.333	18.000 ± 0.577
FMT-2	44.000 ± 0.577	5.000 ± 0.577	7.333 ± 0.333	18.000 ± 1.155
FMU-3	42.333 ± 0.333	4.667 ± 0.882	7.333 ± 0.882	18.667 ± 0.882
FMU-4	43.667 ± 1.856	4.667 ± 0.333	8.333 ± 0.333	20.667 ± 0.667
ER-101	44.000 ± 0.577	5.667 ± 0.333	8.333 ± 0.667	19.000 ± 0.577
SEA 15	43.667 ± 1.856	6.000 ± 0.577	8.667 ± 0.333	19.333 ± 0.333
IC 0263128	42.000 ± 1.732	5.333 ± 0.333	8.333 ± 0.333	19.333 ± 0.333
IC 0263133	46.000 ± 1.000	5.000 ± 0.577	8.333 ± 0.333	18.667 ± 0.667
IC 0355800	43.667 ± 0.882	5.333 ± 0.333	7.333 ± 0.333	19.333 ± 0.333
C.D.	2.895	1.224	1.48	1.808

A significant variation at the 1 % level was observed among the 26 genotypes for days to PE [35.6-48.0], BS [2-6], FPE [4.3-8.6] and TAD [15-22.3]. Germplasms collected from Uttarakhand exhibited a longer anthesis duration compared to the improved varieties. Variations in maturity period were also observed within the same collected germplasm. The different stages from head emergence to pollen shedding after fertilization showed in Fig. 1.

As a member of the grass family, the foxtail millet floret contains only the essential reproductive whorls. These reproductive organs are located at the apex of each spikelet and the flowering type is open. Being a predominately self-pollinated species, the carpels are generally pollinated by pollen grains from the same floret. The flowering process can be divided into three categories (i) panicle emergence to initiation of flowering (ii) the reproduction phase involving anthesis, dehiscence and fertilization (iii) the grain-filling stage.

The reproductive stage started when the anthers became clearly visible at the top of each spike during peak anthesis, while the female was totally covered by the male part. The feather-type stigma was distinctly visible. Flowering began from the uppermost spikelet of the panicle and progressed downwards. The duration of pollen release was found to be highest during the morning hours for effective fertilization. Seed set was noticed in each spikelet.

Each spikelet exhibited three phases: opening the spikelet, pollination and shedding of the anther after fertilization. The total duration from opening to closing of a spikelet was approximately an hour. Each spikelet possessed three yellow anthers and a single ovary. The anthers typically extended the stigma, increasing the chances of self-pollination. Flower opening occurred sequentially from the top to the bottom of the panicle, with maturity following a similar pattern.

Anthers were clearly visible from 7:00 to 9:30 AM, with maximum visibility between 7:00 to 7:30 AM (Fig. 1, 2). The maximum percentage of anthesis occurred during the morning hours. The results taken in five genotypes showed the maximum floret opening occurred between 7:00 to 7:30 AM. Out of the 26 genotypes studied, only 14 were further studied, including 2 varieties and landraces were further analysed for pollen germination and pollen tube growth, as these genotypes exhibited relatively shorter durations for head emergence.

Establishment of pollen germination and pollen tube growth in different solutions

Pollen viability varied significantly among the different genotypes, both in fresh and stored samples. The viability of freshly collected pollen was above 90 % for all genotypes. A slight reduction in viability was observed after 2 hrs of collection, ranging from 91.00 ± 0.577 % to 96 ± 1.528 %. In acetocarmine solution, pollen bursting was observed after 2 hrs of incubation. After 30 min of incubation, freshly collected pollen did not exhibit germination in acetocarmine, TTC or boric acid + sucrose; however, germination occurred within 30 min in nutrient media. Anther structure was also examined, which had not been previously studied in foxtail millet, revealing distinct cellular characteristics (Fig. 3).

Acetocarmine and TTC stains were used to visualize pollen grain, which appeared pink under the light microscope. Fresh pollen collection was a critical step for this study. Pollen grains were stained using different solutions, such as acetocarmine, TTC and boric acid + sucrose and nutrient media (Sucrose + boric acid +

Fig. 1. Flowering process (A- initiation of panicle from flag leaf; B-fully panicle emergence without flower development; C- visibility of anther; D- during pollination the feathery type stigma visible after pollen burst; E- pollen shrink after fertilization; F- pollen shrinkage from top to bottom of panicle).

Fig. 2. Visibility of anther and stigma (A- anther ; B- feathery white stigma).

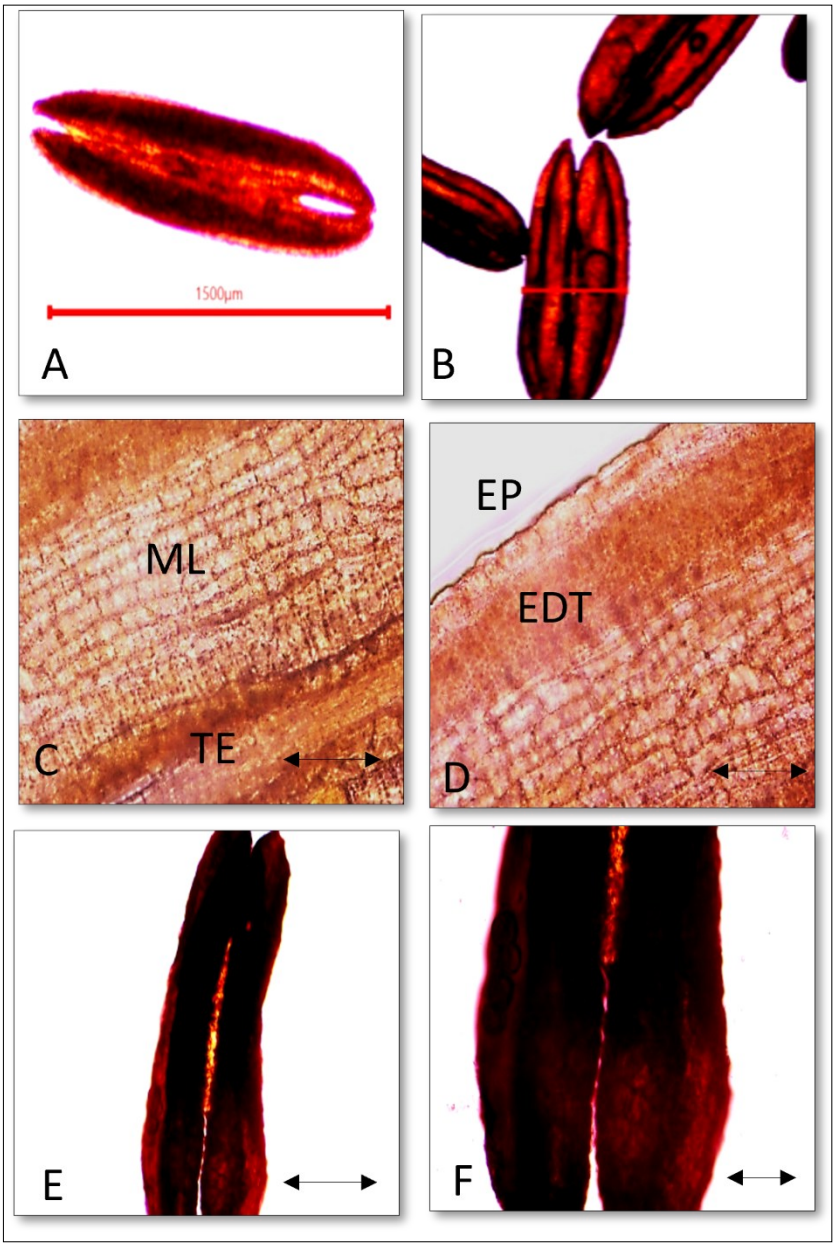


Fig. 3. Anther structure (A & B- anther length and width; C & D- presence of layers in anther cell ML- middle layer, TE- tapetum, EP- epidermis, EDT- endothecium; E & F- connective layer in between anther lobes).

KNO_3) at 25 °C for varying durations, such as 30, 60, and 120 min, to find the pollen dehiscence and germination structure.

In acetocarmine, very low germination was observed after one hr of incubation (Fig. 4). The genotypes FMP-1 and FMR-1 exhibited the lowest germination percentage (0.33 % and 2.3 % respectively), while the maximum was recorded in FMC-1 (6.66 %). A sudden increase in germination was noted after 2 hrs of incubation with the same solution, with most genotypes recorded more than 50 % germination, including both varieties and landraces, while 2 landraces FMA-1 and FMT-1 exhibited less than 50 %.

The average pollen germination in TTC varied among genotypes and incubation durations. Staining with TTC produced a higher degree of pollen bursting compared to acetocarmine at both time intervals. The range differences in the germplasm were 41.33 ± 1.66 (FMT-1) to 59 ± 10.26 (PRK-1) for 60 min and 69.33 ± 13.69 (FMC-1) to 82.66 ± 5.04 (FMU-2) for 120 min (Fig. 5). The varieties showed better interaction with TTC as germination reached 80 %.

A mixture of sucrose and boric acid results in higher pollen germination percentage in 2 hrs compared to 1 hr. The genotype FMP-1 (69.33 ± 8.95) showed germination below 70 % even after 2 hrs; others showed 70 % above germination. Beyond 2 hrs of incubation, germination either ceased or was negligible and thus not considered for further analysis.

However, the germination under the nutrient media occurred much earlier, within 30 min. All genotypes exhibited germination rates between 45 % to 60.66 %, except FMC-1, which showed slightly lower germination during the same duration. After 1 hr, all genotypes recorded germination above 80 %.

Overall, the comparative germination efficiency of different solutions followed the order: Acetocarmine < TTC < sucrose + boric acid < nutrient media, when tested at 25 °C for 30, 60 and 120 min. Only a slight reduction in pollen viability was observed to freshly collected pollen. Among all genotypes, FMP-1 (collected from Pauri district) exhibited the lowest pollen germination percentage.

The flowering phenology at different developmental stages, such as the emergence of head from the flag leaf, booting stage completion, days to complete panicle initiation

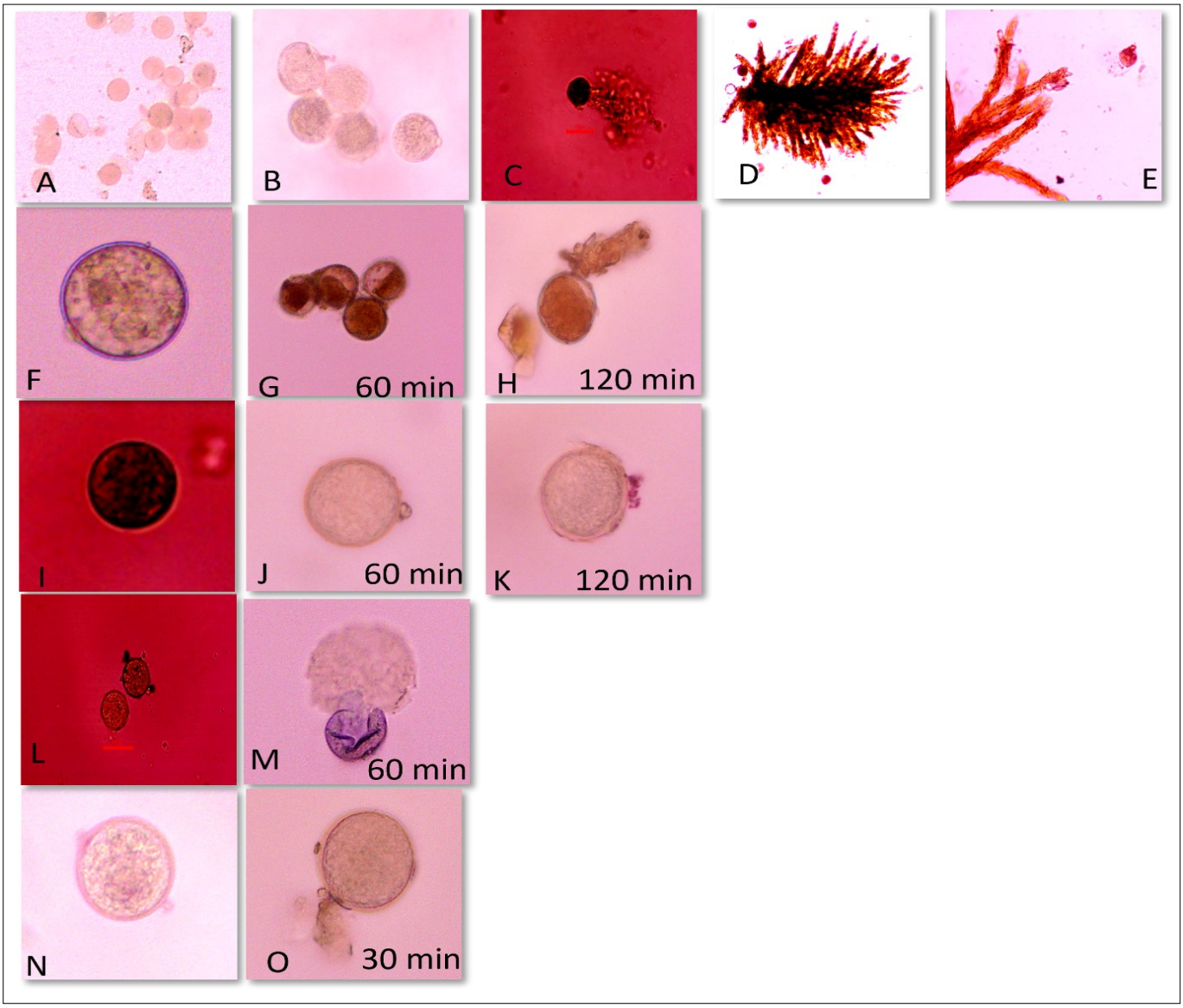


Fig. 4. Pollen viability and pollen germination (A- viable pollen with acetocarmine; B- pollen is ready to burst; C- pollen; D & E- pollen tube development; F, G, H- TTC staining; I, J, K- Acetocarmine; L & M- boric acid + sucrose; N & O- nutrient media 30 min).

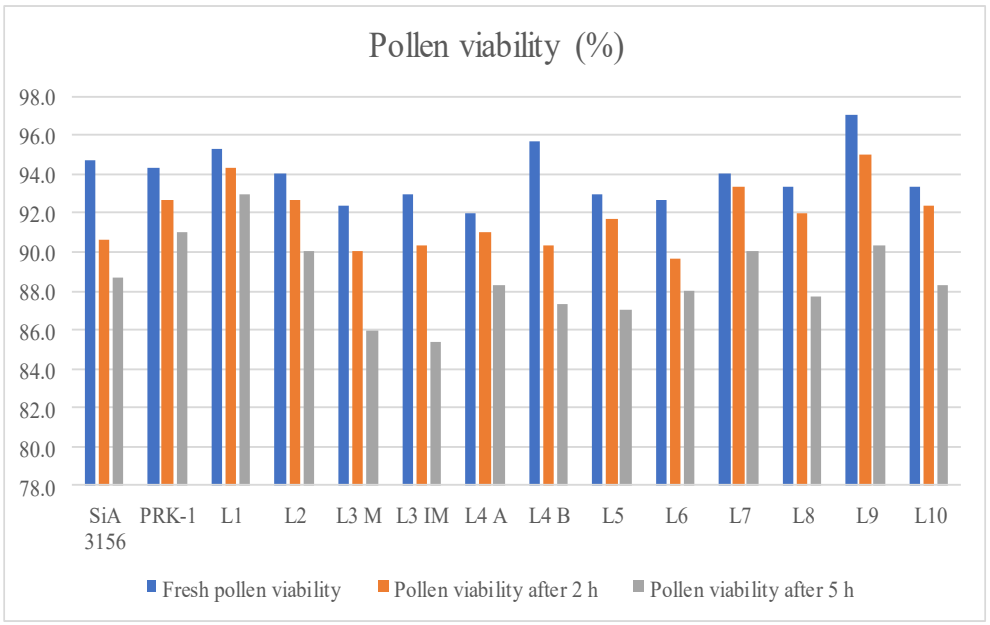
and TAD. The study of flowering phenology is important for selecting parental lines with synchronous maturity, as synchronous flowering remains a major constraint in controlled pollination of foxtail millets. Knowledge of pollen viability and germination is also crucial for developing hybrid breeding programme in foxtail millet, especially due to the lack of work in this area.

In vitro pollen germination using culture medium is a specialized technique that replicates the natural conditions of the style and stigma under controlled environment, thereby facilitating the germination of pollen grains and the growth of pollen tubes. This method plays a crucial role in research on plant reproduction, genetics and breeding. Each species requires a specific culture media composition to optimize pollen grain germination and tube development. The media provides essential nutrients and environmental conditions to stimulate the natural reproductive process. Pollen germination and pollen tube growth are the most important physiological activities in sexually reproducing plants (31). The lack of knowledge about the pollen biology, unlike other major cereal crops was the major plus point of this study.

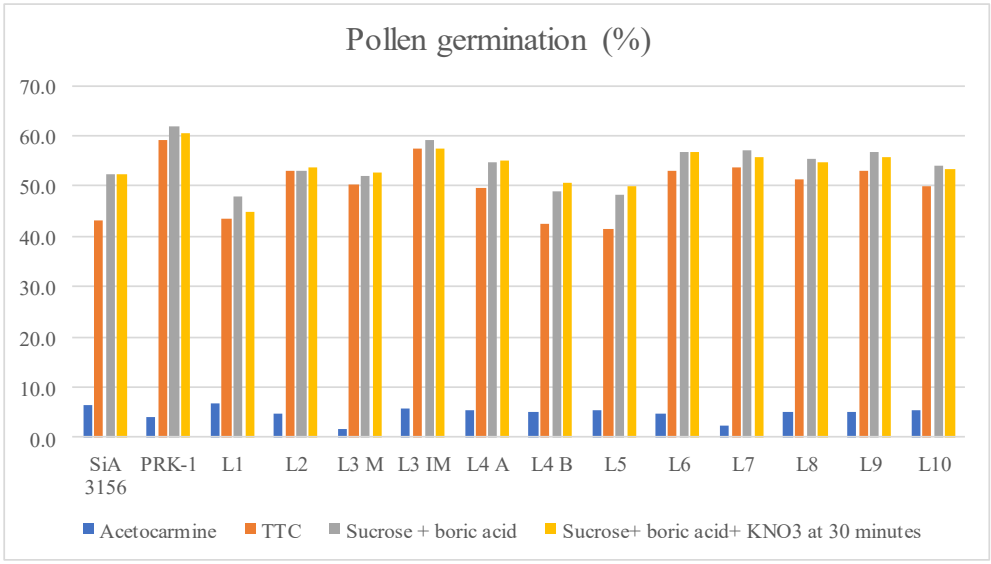
Different *in vitro* germination media such as acetocarmine, TTC, boric acid + sucrose and a combination of boric acid, sucrose and KNO_3, were tested across 14 genotypes for comparison. Accurate timing of pollen collection is vital for successful germination, as flowering time varies between species and genotypes (32). In foxtail millet, the maximum floret opens at 7:00 AM to 8 :00 AM.

Acetocarmine was used for staining pollen to assess viability (33), while sucrose served as the primary source of energy (14, 34). Both acetocarmine and TTC are commonly used to evaluate the pollen viability (35, 36). It allows evaluating assessment of fertility and identification of factors influencing pollen development and tube growth-important parameters for breeding programs. Acetocarmine successfully differentiated between the live and dead pollen in foxtail millet. Therefore, the staining percentage varied from 92 %-97 % in fresh pollen, where very low decline in the percentage of pollen viability when kept at 25 ℃ for 2 hrs. The viable pollen turns pink in colour, while the dead pollen stained white in colour. Similarly, TTC can be used to differentiate the pollen viability but due to high pollen germination percentage, it was considered as a germinating agent.

Boric acid crucially influenced the growth and germination



A



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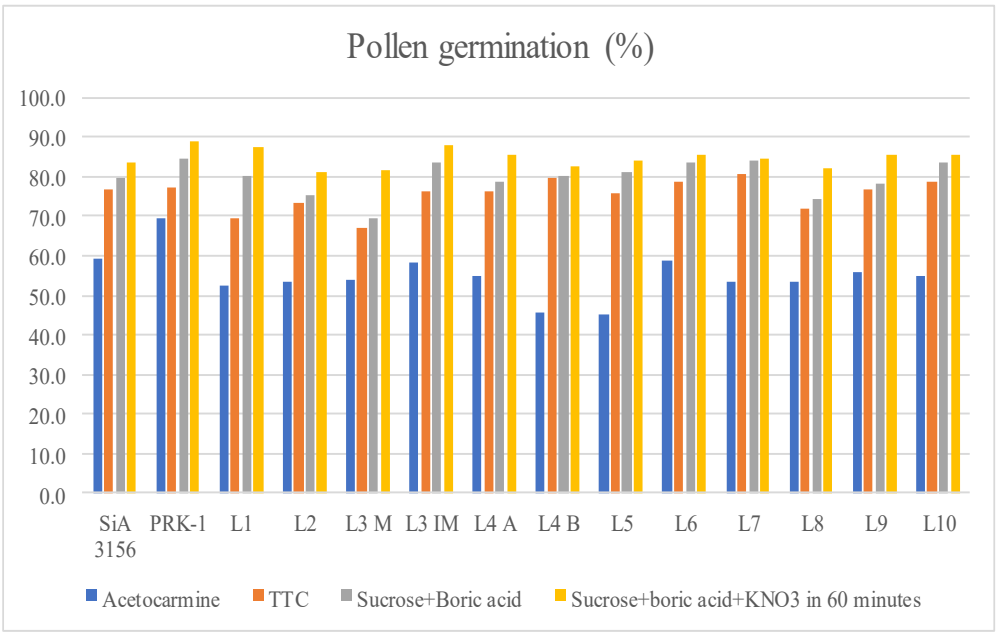

C

Fig. 5. A) Fresh pollen viability and at after 5 hrs of collection, B) pollen germination after 1 hr staining with acetocarmine; pollen germination in TTC in 1 hr; boric acid + sucrose in different duration and nutrient media in ½ hr in 14 genotypes, C) pollen germination after 2 hrs of staining in acetocarmine; pollen germination in TTC in 2 hrs; boric acid + sucrose in different duration and nutrient media in 1 hr in 14 genotypes.

of pollen (37, 38). Boron supports pollen germination and the rapid growth of the pollen tube toward the ovary, which is essential for fertilization and seed formation. A deficiency in boron can impair this process, leading to issues such as anther and filament atrophy, which can result in unsuccessful pollination and poor seed set. Boron promotes the germination and growth of pollen, but the concentration needs to optimum (39). Both a deficiency and an excess can hinder pollen development.

Similarly, the appropriate sucrose concentration helps maintain a balance between internal and external osmotic pressures in pollen grains. This osmotic balance is essential to preserve pollen vitality and ensure that the cells remain functional and capable of germination (40). The *in vitro* germination conditions closely mimic the natural conditions of pollen germination on a stigma (28, 41). A combination of both compounds' influences growth and germination of pollen (42). This similarity makes *in vitro* methods a reliable approach for studying pollen viability, as they yield results comparable to natural processes.

Here, the concentration of boric acid and sucrose at appropriate concentrations effectively promoted the pollen germination (34). The addition of 1 % KNO_3 with boric and sucrose further improved the germination percentage in foxtail millet genotypes, with no negative impact on pollen health. A concentration of 100 mg of KNO_3 influenced pollen germination and pollen tube rupture, facilitating tube development (43). The shorter time required for pollen germination-achieving around 50 % germination within 30 min in most genotypes-demonstrated the efficiency of the optimized *in vitro* medium under room temperature conditions.

Temperature plays a great role in floral biology and yield determination. Environmental stress, particularly heat, significantly affects reproductive behaviour in plants (44-46). Higher temperature can influence the reproductive stage such as pollen germination, leading to reduced seed set, impaired grain filling and lower yield (47, 48).

The use of male sterility in foxtail millet to produce cross-breeding genotypes (3, 49) can help identify the superior lines based on pollen size and short germination duration contributing to the development of high yielding, abiotic and biotic stress varieties through morphogenetic variations. Successful pollen germination leads to double fertilization and seed formation (50). Pollen viability indicates the presence of various enzymes during germination. Comparing landraces with developed varieties in this study provided valuable insights for utilizing landraces as genetic resources to enhance yield, stress tolerance and novel trait identification (7).

Additionally, viability and *in vitro* germination of foxtail millet under proper temperature and period are of prime importance (14). The pollen possesses high level of viability at the time of anthesis (15). In this study, the variation in the flowering phenology to solve the synchronise problem in foxtail millet germplasm and further, some randomly selected genotypes for pollen viability under different staining chemicals, which has not been researched in foxtail millet till yet. *In vitro* pollen germination and pollen tube development involve many factors, such as media, duration and temperature were analysed and combination of boric acid + sucrose + KNO_3 concentration influenced better pollen germination in 14 genotypes compared to acetocarmine, TTC and boric acid + sucrose alone.

Conclusion

Foxtail millet exhibits distinct reproductive behaviour that must be understood for successful breeding aimed at yield improvement. The appropriate temperature that maintained pollen viability for 2 hr was 25 °C. A prolonged pollen shedding period, similar to that observed in maize, can be highly beneficial for breeding purposes. Therefore, genotypes with extended pollen shedding duration should be utilized in crop improvement programs. For *in vitro* pollen germination, nutrient media proved effective in inducing pollen germinate within 30 min at 25 °C.

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Authors' contributions

AR and JSC carried out the conceptualization, methodology, investigation and performed data curation and analysis. AR carried out the experimental analysis. AR wrote the original draft of the manuscript. JSC, DP, ST carried out the editing and reviewing of the study. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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