Plant Methods

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Enhancing hybridization potential through standardizing emasculation techniques and molecular marker integration in finger millet (*Eleusine coracana* L.)

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Abstract

Background Finger millet (*Eleusine coracana* L.*)* has been cultivated for thousands of years in semi-arid regions of Asia and Africa. The grains have immense nutritional potential, but the genetic gains needed for high yield are yet to be realized. In the absence of an efficient crossing technique, recombination breeding is still challenging in finger millet genetic improvement programs, owing to its small floret size and highly self-fertilizing nature. In this study, we evaluated three different emasculation methods for finger millet, including a modified hot water technique and different concentrations of two gametocides, trifluoromethanesulfonamide (TFMSA) and gibberellic acid (GA_3) at different plant growth stages.

Results The study revealed a successful emasculation rate of up to 80% using modified hot water treatment at 52 °C for 2 min, when treated on the second day of anthesis in the designated panicles. Further, a similar success rate was noted with a single dose of TFMSA @ 10 mg or two doses of GA_3 @ 1500 ppm applied during the booting stage (7–9 days prior to panicle emergence). To validate the efficiency of these hybridization methods and to test the genetic purity of hybrids generated in this study, a set of 10 single nucleotide polymorphism (SNP) markers were used.

Conclusion These improved emasculation methods (Modified hot water, TFMSA, GA_3) integrated with molecular markers for hybridity testing represent an important step forward in finger millet crop improvement.

Keywords Finger millet, Hybridisation techniques, Chemical hybridization agents, GA_3, TFMSA, SNPs

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Background

Millets are small-seeded cereal crops recognized as climate resilient staples for food and nutritional security in the semi-arid tropics (SAT) worldwide. Small millets include finger millet (*Eleusine coracana* L.), foxtail millet (*Setaria italica* L.), proso millet (*Panicum miliaceum* L.), barnyard millet (*Echinochloa colona* L.), little millet (*Panicum sumatrense*), and kodo millet (*Paspalum scrobiculatum* L.). Among small millets, finger millet is an important millet known for its unparallel nutritional value over major cereals, such as rice and wheat, with high calcium content (344 mg), potassium (408 mg), dietary fibre, minerals, and sulphur containing amino acids [1]. It is a multipurpose crop, which supports the livelihood of millions of smallholder farmers in Asia and Africa. It is the third most important millet globally in terms of both area and production, following sorghum and pearl millet [2]. Globally, it covers an area of 2.2 million hectares with a production and productivity of 3.7 million tonnes and 1.32 tonnes per hectare respectively. In India, finger millet is estimated to cover 1 million hectares, producing 1.76 million tonnes annually with an average productivity of 1.74 tonnes per hectare [3]. Despite its immense agricultural and nutritional value, the cultivated area, production and productivity of finger millet in the last five decades have remained stagnated compared to major cereals, which is mainly attributed to the lack of concerted crop improvement efforts [4, 5].

Though adequate genetic variation was reported in crop germplasm repository [6], breeding efforts in finger millet so far were majorly towards selection of desirable genotypes derived from landraces/germplasm lines which are released directly as a cultivar [5]. Successful hybridization is crucial in finger millet to develop superior cultivars with improved yield that can withstand environmental stresses. However, due to small floral size and self-pollinating nature in finger millet, emasculation and crossing is technically challenging. Floral biology of finger millet is complex due to delicate inflorescences with small florets inside the spikelets. During anther dehiscence, the style elongates along with the filaments, which results in a dense pollen cloud covering the stigma during anthesis, promoting selfing. Moreover, its cleistogamous nature adds to the difficulty in successful crossing [7]. These features ensure high percentage of self-pollination, limiting the spontaneous cross pollination to not more than 2% in finger millet [8]. Hence, improving the ease of emasculation will be a milestone to accelerate the genetic improvement of finger millet through recombination breeding.

A classic example of successful recombination breeding characterized by yield gain is the *Indaf* varieties developed by crossing Indian and African gene pools of finger millet. These cultivars are the preferred choices over traditional landraces among farming communities and seed production agencies due to a potential increase of more 50% productivity in India [9, 10]. However, difficulty in emasculation limited the development of new cultivars at a steady pace.

In general, crossing is achieved by hand emasculation followed by pollination or the contact method. The flowers are less than 1 mm in diameter [10], which makes hand emasculation practically difficult. For this reason, the contact method is the most used crossing method, where male and female designated parents are tied together to generate a cross. In this method, for protection and exclusion of external pollen, the crosses are covered with butter paper bag. Seeds are harvested only from the designated female genotype, which are planted in the next season for identification of hybrid plants. However, very few hybrid seeds (≤ 1%) are recovered through this method. Another suggested method is hot water treatment where the female panicles at an appropriate stage of inflorescence development are dipped in hot water at a temperature of 52 °C for 2 min for effective emasculation [11]. Here, the temperature is a critical factor, as variation in temperature can affect the efficiency of the emasculation and enhance percentage of self-fertilization within the florets.

In several crops, male sterility systems have been well characterized and used as the most effective tools for hybridization. In finger millet, genetic male sterility (GMS) has been reported [12] but its practical utility is limited due to varying degree of sterility across the locations and genetic backgrounds, coupled with maintenance problem in GMS. Therefore, the creation of large scale genetically varied populations of finger millet heavily relies on the availability of effective emasculation methods.

All the crossing methods described above are not completely reliable and strongly require optimisation, coupled with the validation of hybridity of the F_1 offspring. Phenotypic traits with dominant type of inheritance such as anthocyanin pigmentation at the nodes and panicle were used as morphological markers for validation of successful cross [13, 14]. In recent years, molecular markers have emerged as a powerful tool for hybridity testing [15]. Unlike morphological markers, molecular markers provide rapid and reliable results, exhibit minimal environmental sensitivity, are not influenced by epistatic and pleiotropic effects, and can be selected to provide clear differentiation between parental genotypes. Among various molecular makers, single nucleotide polymorphisms (SNPs) are abundant, co-dominant, highly reproducible and distributed across the genome, making them a powerful tool for hybridity confirmation and improving crossing efficiency.

In view of above, the present study focuses on enhancing hybridization by standardizing emasculation techniques and hybridity confirmation using molecular markers in finger millet. We compared the efficiency of different emasculation methods which included the modified hot water treatment and different concentrations of two gametocides or chemical hybridization agents (CHAs) viz.,'trifluoromethanesulfonamide (TFMSA)' and 'gibberellic acid (GA_3)'. Furthermore, SNPs were used as molecular markers to confirm the successful identification of true hybrids. To the best of our knowledge, this is the study evaluating different emasculation techniques combined with the use of molecular markers to verify the hybridity of crosses. By systematically comparing these methodologies, we aim to identify emasculation techniques that not only overcome the challenges posed by finger millet's intricate floral biology and small floret size but also enhance the ease of overall hybridization process.

Materials and methods

The study on emasculation techniques was performed in the glasshouse during 2023 and 2024 at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad (17.3° N, 78.5° E), India. The glasshouse spans 54m^2 (9 m x 6 m) with 16 benches each measuring 1.90 m x 0.95 m. Individual plants were grown in pots (4-inch diameter) filled with a 3:2:1 composition of soil: sand: vermicompost. Nutrients and irrigation were provided as per requirement. The glasshouse was maintained at temperature 25 ± 2 °C with relative humidity around 70% using thermostat and air coolers.

Plant material

The plant material studied under pilot and validation studies constituted germplasm lines and released cultivars. The germplasm lines were procured from gene bank of ICRISAT which houses around 7000 accessions of finger millet. The lines selected were of Asian and African origins from diverse maturity segments. The details are presented in Supplementary Table S1.

Emasculation methods

The selected male and female parents were planted in a glasshouse under controlled conditions (temperature 25 ± 2 °C with relative humidity around 70%). Three staggered sowings of parental lines were done at intervals of 7–8 days to ensure synchronous flowering in both the parental lines to proceed for pollination. The healthy and mature un-emasculated inflorescence (male) was selected for pollination. We evaluated the following three different artificial emasculation methods in finger millet.

A. Modified hot water treatment-based emasculation

- Pilot experiments

a. Determine the optimal temperature and exposure time of hot water treatment for efficient emasculation: The first experiment to optimise the temperature and duration of treatment was done on a set of 15 genotypes at three different temperatures (52 °C, 53 °C, 54 °C) with three different time durations (2 minutes, 3 minutes, 4 minutes). The plants were selected at the onset of anthesis. The entire panicle was treated and then checked for any sign of seed formation after 5-6 weeks post treatment/ at maturity stage of the panicle. The plants were planted in completely randomised design with three biological replications and five technical replications per treatment. The treated panicles were bagged to avoid accidental cross pollination
b. Identifying the target region of treatment on the finger millet panicle: The second experiment was conducted to identify the best target region for hot water treatment on the panicle. This was tested on two genotypes IE 5963 and IE 5965 at 52 °C for 2 min. The same genotypes were utilised to confirm pollen sterility in the identified target region on the panicle over an array of temperatures viz., 48°C for 5 min, 50°C for 4 min, 51°C for 3 min, 52°C, 54°C, and 56°C for 2 min. The treated panicles were bagged to avoid accidental cross pollination.

- Validation experiment
On the second day of anthesis, the self-fertilised spikelets (top 1-2 cm) were removed from the female panicle using forceps. Additionally, the lower spikelets were also removed leaving only up to 20 spikelets spread in the target region on 3-4 fingers in the designated female panicle. This target region spans 1-1.5cm below the self-fertilised spikelets. The clipped panicle was then dipped into a hot water bath (Thermo Fisher Scientific Precision GP 05, USA) containing hot water steadily maintained at 52°C for 2 min to emasculate the flowers. The air-dried hot water treated female panicle was tied with the designated male panicle with a thread and covered with the butter paper bag (pollen proof bag) to eliminate any chance of cross pollination. This method was used to generate 6 cross combinations each for hand emasculation as control, treatments of 51℃ for 3 minutes, and 52°C for 2 minutes. The modified hot water method is illustrated in Fig. 1

Chemical treatment For this approach, two chemicals namely trifluoromethanesulfonamide (TFMSA), and gibberellic acid (GA_3) were utilized.

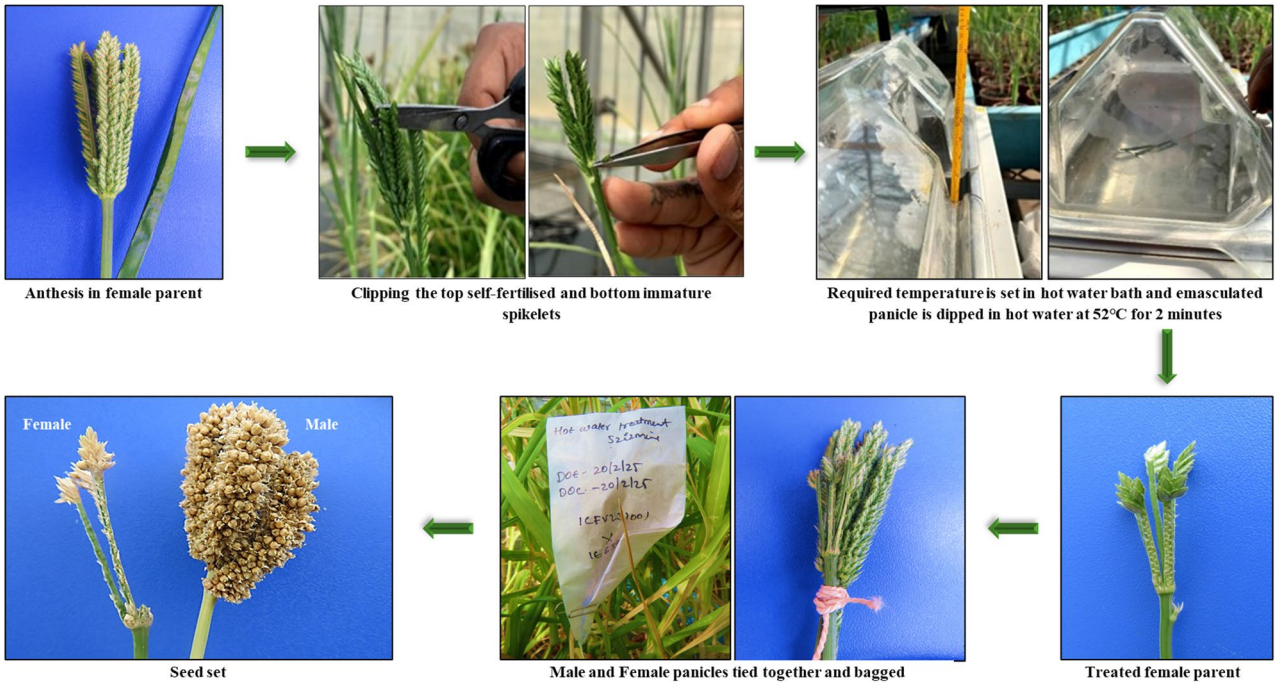


Fig. 1 Illustration of crossing technique following modified hot water emasculation in finger millet

B. TFMSA based emasculation

i. Pilot experiments: The chemical was dissolved in an aqueous solution containing glycerol (5%) and Tween 20 (0.25%). Three different concentrations (5 mg, 10 mg, and 15 mg) of this aqueous solution were utilized for emasculation at two different stages -pre-booting (12–15 days prior to panicle emergence) and booting stages (7–9 days prior to panicle emergence). The pilot was tested on four genotypes in three biological replications with five technical replicates each planted in a completely randomised block design. The chemical was applied @1 ml per plant twice on alternate days using a micropipette to dispense Xml of the solution (conc.) on a cotton plug placed in the whorl of the shoot. A cotton plug was used to help retention of the chemical on the growing terminal tip for a longer time, thus permitting its absorption by the plant. The plants were visually scored as sterile or fertile based on the seed formation 5–6 weeks post-treatment. The TFMSA method is illustrated in Fig. 2.

ii. Validation experiments: Three different concentrations (5 mg, 10 mg, and 15 mg) were tested in booting stage with a single dose @1 ml per plant on the designated female parents (GPU 66, GPU 67, IE 8806) and crossed with the designated male parents (KNE 688, Emeroit) following the method explained earlier (Supplementary Table S2).

C. GA_3 based emasculation

i. Pilot experiments: Ten different concentrations of GA_3, ranging from 300 ppm to 3000 ppm (300 ppm, 600 ppm, 900 ppm, 1200 ppm, 1500 ppm, 1800 ppm, 2100 ppm, 2400 ppm, 2700 ppm, and 3000 ppm), were used as treatments (T1 to T10) in two varieties (GPU 28 and GPU 67). Aqueous solution of GA_3 was applied for two consecutive days @1 ml per main tiller in the whorl of flag leaf using a micropipette. The solution was prepared by stirring 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 mg respectively in 10 ml of ethanol for dissolving followed by addition of 90 ml of distilled water to make up the concentration. The testing was done at three stages: pre-booting (12–15 days prior to panicle emergence), booting stages (7–9 days prior to panicle emergence) and early emergence (1–2 days prior to panicle emergence). To ensure better absorption of the solution by plant tissues, the solution was applied on a cotton ball placed in the whorl of the flag leaf of the main tiller. The mean percentage of pollen sterility was calculated for each GA_3 concentration at each growth stage using acetocarmine staining. To investigate any significant variations between the treatments, analysis of variance (ANOVA) was performed

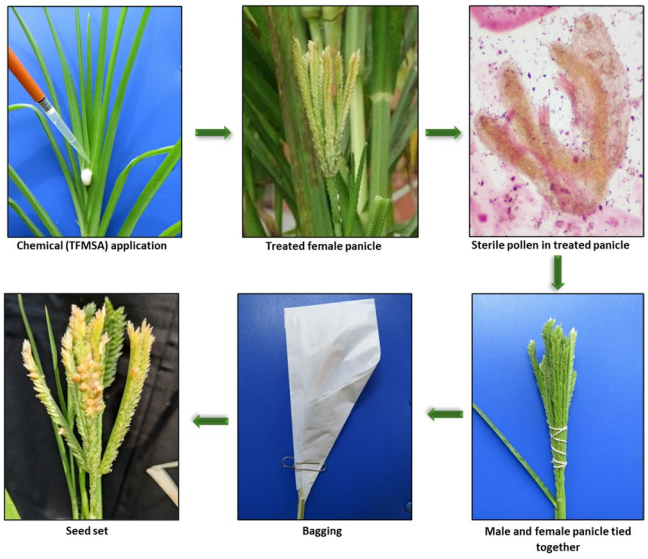


Fig. 2 Illustration of crossing technique following chemical (TFMSA) emasculation in finger millet

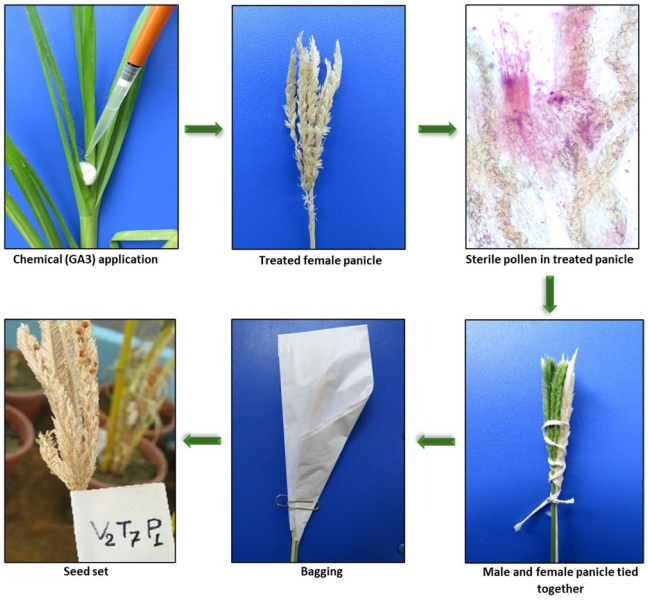


Fig. 3 Illustration of crossing technique following chemical (GA_3) emasculation in finger millet

in ADEL-R software, using a p-value of 0.01 as the critical threshold for determining statistical significance. The GA_3 method is illustrated in Fig. 3.

ii. Validation experiments: Three different concentrations (1500 ppm, 1800 ppm, and 2100 ppm) were tested at booting stage on two consecutive days @1 ml per plant on six designated females (IE 6547, IE 6396, IE 2983, IE 7390, 4842, IE 6537). These females were crossed with two designated males (KNE 688, Emeroit) to generate F_1 seeds.

The validation experiments were grown in three biological replicates and two technical replicates each in a half diallele design for generating the crosses. The chemically treated plants were grown until the seeds matured. When majority of the seeds had turned brown signalling maturity, seeds from the designated treated female parents were meticulously collected separately and labelled. These F1 seeds were used for further molecular studies to confirm their hybridity.

Molecular marker assays

Tissue sampling

Seeds of the designated female parent were harvested 5–6 weeks after crossing. A total of 55 crosses which included 6 from hand emasculation, 12 from modified hot water emasculation, 6 from TFMSA method, and 31 from GA_3 treatment were harvested (Supplementary Table S3). For each cross approximately 15–20 seeds were planted along with their parents in the glasshouse at ICRISAT, Hyderabad, India during post-rainy season of 2024. Young leaf tissues of 20 days old seedling from individual plants were collected according to Intertek-Agritech sampling protocol and dried at 50 °C for 24 h. The dried leaf samples were shipped to the Intertek laboratory (Hyderabad) for DNA isolation and Kompetitive Allele Specific PCR (KASP) genotyping.

Molecular profiling for hybridity verification

From the whole genome re-sequencing information of approximately 170 finger millet lines, around 50 SNP markers were selected for KASP assay development at the Intertek-Agritech laboratory (Alnarp, Sweden, and Hyderabad). These SNPs were carefully selected based on key parameters such as call rate (> 80%), biallelic only, polymorphism information content (PIC- 0.3–0.37), minor allele frequency (0.3–0.4) and observed heterozygosity (0-0.05) etc. The selected markers were subsequently validated using ~ 280 diverse finger millet lines (unpublished data). Of these, a set of 10 most informative SNPs were identified for use in rapid hybridity verification.

The SNP genotyping was verified using allelic information/genetic profiles of the two parental lines of a cross as reference to assess the genetic makeup of their F_1 progeny. By comparing the SNP alleles inherited from each parent, the plants which exhibit ≥ 1 heterozygous loci were confirmed as true F_1s. The following formula was used to estimate the genetic purity percentage:

$$\text{Hybridityconfirmation}\,(\%) = \frac{\text{NumberoftrueF1sobserved}}{\text{TotalnumberofF1seedlingstested}} \times 100$$

Note: Plants with failed DNA extraction or ambiguous marker calls were excluded from percentage calculations or denominator.

Results

i. Pilot studies

A. Modified hot water treatment-based emasculation

i. Determine the optimal temperature and exposure time of hot water treatment for efficient emasculation: Our first experiment demonstrated that 52 °C and 53 °C for 2 min treatment duration gave zero to a few seeds (on the lower portion of the panicle) in majority of the genotypes through self-pollination (confirmed through pollen studies) while the remaining treatments (52 °C, 53 °C at 3 min, 4 min and 54 °C at 2 min, 3 min, 4 min) showed partial to complete burning symptoms. A few lines viz., IE 2983, IE 4646, IE 3077, IE 7390 were sensitive (burnt) at all the tested treatments (Supplementary Fig. 1).

ii. Identifying the target region of treatment on the finger millet panicle: The next experiment aimed to identify target region for emasculation on the panicle when treating with hot water at 52 °C for 2 min on the second day of anthesis. The top 1 cm of self-fertilised region which opened on first day of anthesis showed fertile pollen and initiation of pollen attachment to stigma. The next 1–2 cm region of the panicle which opened on the second day showed a mixture of fertile and sterile pollen in acetocarmine study. The third 1–2 cm region showed complete sterility making it a target region for further study. The remaining spikelets at the bottom of the finger did not take up any stain suggesting immature pollen stages (Fig. 4). This was the reason to find seeds forming at the bottom of the finger in the pilot experiment. This identified target region of 1-2cm below the fertilised spikelets was reconfirmed for pollen sterility using different temperature regimes (48°C for 5 minutes, 50 °C for 4 minutes, 51 °C for 3 minutes, 52 °C, 54°C, and 56 °C for 2 minutes). Pollen studies showed fertile pollen at 48 °C and 50 °C, partial to complete sterility at 51 °C and complete sterility at 52 °C. The spikelets tested at 54°C and 56 °C showed burning symptoms (Fig. 5). These pilot studies confirmed the success of 51 °C for 3 minutes and 52 °C for 2 minutes at 1-2 cm below the fertilised spikelets inducing complete male sterility. These treatments were further validated by hybridisation followed by hybridity testing with molecular markers.

B. TFSMA-based emasculation

In pilot experiment 1, three concentrations, viz., 5 mg, 10 mg, and 15 mg at the stages of pre-booting and booting before panicle emergence were used. They were applied in two doses on alternate days. Among these treatments, 5 mg resulted in a partially sterile panicle with lower phytotoxicity. Slightly phytotoxic and deformed panicles were observed

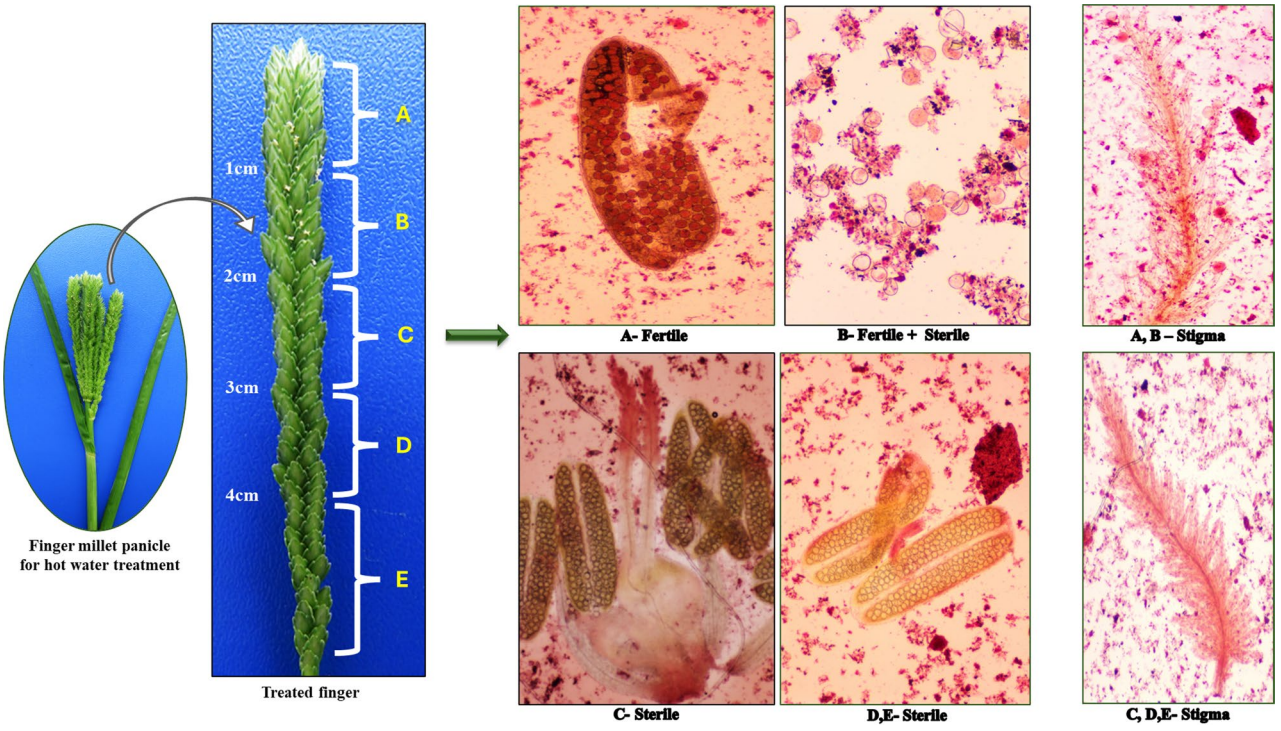


Fig. 4 Optimising the region of emasculation on the finger millet panicle

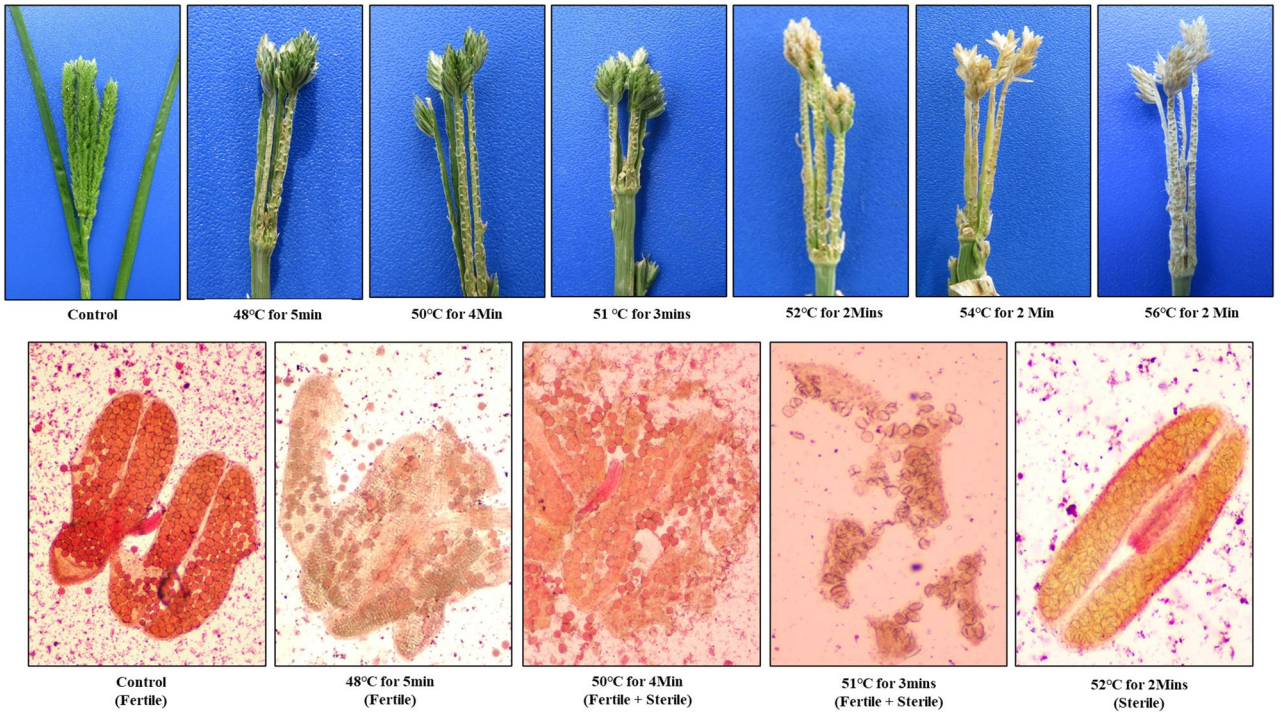


Fig. 5 Standardising the modified hot water treatment in finger millet

at 10 mg with reduced seed set. Plants treated with 15 mg resulted in the non-emergence of panicles and severe phytotoxicity, leading to the death of the plants. The effect was predominant in pre-booting stage as the phytotoxicity symptoms were evident from the vegetative stage onwards, resulting in the deterioration of plant health. Additionally, two doses on alternate days could have promoted phytotoxicity

owing to its minimal time interval between the doses.

Thus, the final experiment was conducted with only one dose and one time of application, i.e., at booting stage with 5 mg, 10 mg, and 15 mg concentrations. At 5 mg, panicles showed partial sterility with very low seed set and no deformation. Panicles treated with 10 mg were found to be completely sterile with no seed formation in some genotypes. At 15 mg, panicles emerged and were found to be completely sterile but with a mild deformation (Fig. 6).

C. GA3-based emasculation

The findings of our study demonstrated the impact of gibberellic acid (GA_3) application (10 treatments) in two varieties (GPU 28 and GPU 67) of finger millet as a male gametocide at three different stages of panicle emergence namely, pre-booting (12–15 days prior to panicle emergence), booting (7–9 days prior to panicle emergence) and early emergence (1–2 days prior to panicle emergence) stages. Both the varieties showed the highest pollen sterility when treated at the booting stage (52%) followed by pre-booting (40%) and early emergence stages (28%) across all treatments. The highest pollen sterility was observed in T5 (1500 ppm), T6 (1800 ppm) and T7 (2100 ppm) at booting stage. Statistically significant variation in the pollen sterility of finger millet was observed at different concentrations of GA_3 for all three stages. However, there is no significant difference in inducing pollen sterility in booting stage by applying GA_3 in these three treatments (T5, T6, T7) in both the varieties of finger millet (Fig. 7a and b; Supplementary Table S4). This suggests that these concentrations of GA_3 are ideal for achieving the desired outcome in terms of pollen sterility. Our findings indicated that the booting stage of panicle emergence was the most suitable stage for inducing male sterility in finger millet.

ii. Validation studies

A. Modified hot water treatment-based emasculation: We applied hand emasculation, and hot water treatments 51℃ for 3 min and 52°C for 2 min to develop six cross combinations each. The resultant seed set observed across all cross combinations ranged from 8 to 40, 5–27 and 7–22 seeds per crossed panicle under hand emasculation, 51°C for 3 min, and 52°C for 2 min, respectively. Hybridity confirmation studies showed 16.6% hybrids for the 51°C for 3 min and 83% in 52°C for 2 min treatments, while no true hybrids were obtained through hand emasculation.

B. TFSMA-based emasculation: In the present study, successful seed set was observed in all the 6 crosses developed using TFMSA, with a range varied from 10-90 seeds, 44-69 seeds, and 15 seeds in 5 mg, in 10 mg, and 15 mg concentrations of TFMSA treatment, respectively. The 10 mg concentration of TFMSA at booting stage (7-9 days prior to panicle emergence) generated 80% true F1s followed by 15 mg with 73%, and 5mg with 50% success rate.

C. GA3-based emasculation: In our study, 31 crosses were attemptedthrough applications of three concentrations (1500 ppm, 1800 ppm, and 2100 ppm) of GA_3 treatment at booting stage (7–9 days

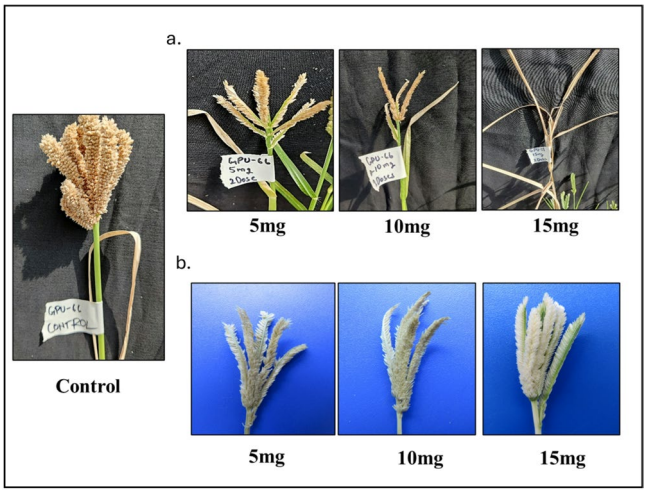


Fig. 6 Standardising the TFMSA treatment in finger millet. **a** Effect of different concentrations (5 mg, 10 mg and 15 mg) of TFMSA treatments when applied at 12–15 days and 7–9 days before panicle emergence stage in 2 doses; **b** Effect of different concentrations (5 mg, 10 mg and 15 mg) of TFMSA treatments when applied in 8–10 days before panicle emergence

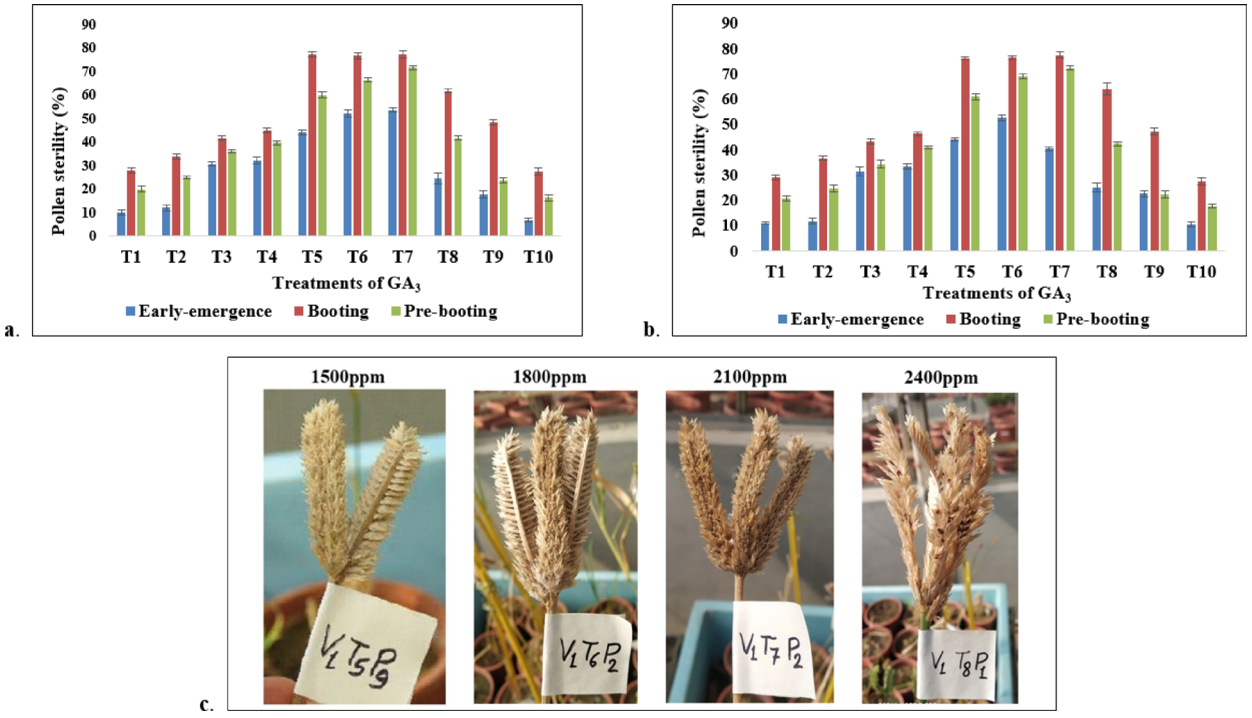


Fig. 7 Standardising GA3 application on finger millet genotypes. **a**, **b** Male sterility percentage in different concentration of GA_3 in three different crop growth stages in V_1 (GPU 28) and V_2 (GPU 67) respectively; **c** Effect of GA3 treatments on finger millet panicle: T5-1500PPM, T6-1800ppm, T7- 2100ppm, T8- 2400ppm

prior to panicle emergence). Successful seed set was observed in all the crosses that ranged from 33 to 300 seeds, 50–280 seeds, and 150–300 in 1500 ppm, 1800 ppm and 2100 ppm, respectively. Among the three concentrations, 1500 ppm yielded approximately 80% true F_1s followed by 1800 ppm and 2100 ppm with 25% true F_1s as revealed by molecular markers.

iii. Molecular marker-based hybridity confirmation: The set of 10 informative SNP markers used for the study clearly distinguished the parental lines selected for the study. The KASP assays were selected based on low missing data, low heterogeneity, and very high scoring (>95%) success rate in the parental lines tested. The allelic profiles of parental lines and their F1s were correlated to determine true hybrids in the study. Integrating validation experiments with molecular marker-based confirmation displayed significant differences among the treatments under each technique tested (Supplementary Table S5) and provided a high success rate of 80–83%.

Discussion

The complex floral biology, small floret size and self-pollinating nature of finger millet are the biggest bottlenecks for recombination breeding in finger millet. Till date, the contact method/hand emasculation remains the method of choice, but with a success rate of less than two per cent [16]. However, this approach demands substantial investments in terms of resources, time, space, and labour thereby demanding development of more efficient alternatives [17]. Addressing these challenges, our study focussed on a comprehensive evaluation of three distinct emasculation methods including a modified hot water treatment and two chemical hybridising agents (TFMSA and GA3).

Modified hot water treatment-based emasculation

The application of hot water has proven to be a quick method for generating segregating populations for crop improvement especially in crops such as small millets where floral morphology, tiny florets, and anthesis behaviour are major hindrances for crossing [7, 18–20]. Previous attempts with hot water treatment in finger millet noted the temperature of 52 °C sustained for a duration of 2–5 min to be successful [11, 21–23]. Based on these experiments, we initiated experiments by treating the finger millet panicles at 52 °C, 53 °C and 54 °C for 2, 3 and 4 min. The primary objective of our experiment is to identify the effective temperature-time combinations which can induce male sterility. Thus, we focused on the specific temperature-duration treatment rather than inferences on an absolute temperature threshold. Future studies can focus on full factorial design experiments with

temperature and exposure time treatments to understand their independent effects.

In this study, we deviated from the standard protocol by administering it on the second day of anthesis instead of targeting panicles anticipated to flower in the next 2 to 3 days as depicted in the literature. This adjustment has proven to be effective in achieving higher levels of emasculation. While 52 °C for 2 min was comparatively promising, the pollen studies revealed a mixture of partial to complete sterility across the finger length which showed the need to identify a targeted region for treatment.

Finger millet panicles usually measure 4–6 cm long, with anthesis progressing top down for 3–6 days covering 1–2 cm a day based on the weather parameters and genotype. This forms the basis to test the 1–2 cm region containing mature pollen just below the self-fertilised spikelets on the second day of anthesis for consistent results.

The temperature requirement for hot water emasculation may vary with the geographical location and genotype sensitivity [17]. For example, 51 °C for 1.5 min (in case of sensitive genotypes) to 54 °C for 2 min (in case of genotypes exhibiting partial sterility at 52 °C for 2 min) can be utilised as an alternative to the recommended treatment of 52 °C for 2 min. The results of our study at 52 °C for 2 min demonstrated a favourable seed set in all cross combinations ranging from 7 to 27 seeds per crossed panicle treated at 52 °C for 2 min. A study noted ~ 30–90 seeds per panicle with a 52–80% successful sterilisation rate by following hot-water treatment technique in finger millet [24]. Present findings align with the results of their study.

Compared to the traditional method of manual anther removal, the modified hot water treatment proved to be a more efficient alternative by significantly reducing the labour and time required. This method capitalizes on the idea that pollen is more open to deactivation than that required to inactivate the ovary, thus ensuring successful emasculation. Nevertheless, it is essential to address certain limitations inherent to the hot water treatment method. This method necessitates specific equipment such as hot water bath, to maintain the precise temperature, which could potentially limit its application. The use of excessively high temperature during treatment can adversely affect the stigma; resulting in a relatively small amount of seed set. Therefore, it is imperative to strike a balance between temperature effectiveness and avoiding detrimental effects on the reproductive structures. Our research suggests that while the modified hot water method offers a practical and efficient approach to finger millet crossing, its variability across genotypes remains a challenge. Hence, it is necessary to investigate potential genetic factors contributing to this variability, enabling better control and understanding.

TFMSA-based emasculation

TFMSA has demonstrated its capacity to induce male sterility in plants, specifically, without detectable effects on other plant functions. Notably, the first utilization of TFMSA for inducing male sterility was observed in the maize crop [25], subsequently followed by its application in sorghum to induce male sterility [26]. Another study reported a minimum dosage threshold (between 10 and 15 mg) resulting in male sterility of panicles in sorghum [27]. These studies claimed that depending on the dosage, timing of treatment, and sorghum genotype, TFMSA caused different degrees of male sterility under greenhouse conditions. In the present study, higher success was observed in all the six crosses developed using TFMSA at booting stage (7–9 days prior to panicle emergence) treated with 10 mg of TFMSA, which fits the need for efficient emasculation and generating breeding populations.

Proline is known to play important roles during the reproductive stage of plant growth and TFMSA is known to decrease proline transport in plants [25, 28]. Report suggest TFMSA impacts phenotypic features in sorghum, such as slight plant height reduction and blooming delay [29], although these effects were not prominent in the finger millet genotypes tested. Future studies should investigate the impact of TFMSA on the functionality of female plant organs.

GA_3-based emasculation

Initial studies on the use of gibberellic acid to induce male sterility were conducted in common millet (*Panicum milliaceum* L.) and little millet (*Panicum miliare* L.) [30] followed by in safflower (*Carthamus tinctorius*) [31]. Notably, our study revealed that the concentrations of GA_3 played a pivotal role, with the most notable success (80% true F_1s) observed with a 1500 ppm concentration at booting stage in finger millet. The booting stage, being a critical point in reproductive organ development, offers an ideal window for emasculation. 1500 ppm GA_3 disrupts pollen development, thereby facilitating successful cross-fertilization while minimizing the risk of self-pollination. Our findings also suggest that further research is needed to unravel the intricate interplay between GA_3 concentrations, developmental stages, and successful hybridization in finger millet.

Molecular marker-based hybridity confirmation

The present study also highlights the significance of SNP markers as a robust tool for quality control. For routine QC involving a small number of markers, the KASP platform is a user friendly and cost-effective SNP genotyping solution [32]. This not only broadens the toolkit available for finger millet breeders but also provides for broader applications in other millet and cereal crops, offering

new opportunities for confirmed hybridization and crop improvement especially for small millets. This strategy holds substantial promise for achieving genetic gain through targeted crop improvement efforts.

Conclusion

In this comprehensive study, we evaluated the effectiveness of three different emasculation and crossing methods in finger millet. Among the methods explored, the modified hot water treatment approach at 52 °C for 2 min emerged as a practical, fast, and easy method for effective crossing producing successful true F_1s (> 80%) based on the tested genetic backgrounds. Chemical (TFMSA/ GA_3) treatment can enhance recovery of true F_1s up to 80%, which is a significant increase. We found that TFMSA/ GA_3 chemical treatment techniques are good for large-scale crossing initiatives and potentially the commercial production of hybrid seeds. Best results were achieved when TFMSA @ 10 mg and GA_3 @ 1500 ppm were applied in a single dose, and two doses, respectively, during the booting stage (7–9 days prior to panicle emergence). One of the most important factors in the crop improvement process in self-pollinated crops such as finger millet is the confirmation of hybridity. The current study introduced the use of SNP markers for hybridity testing in finger millet. These crossing methods integrated with molecular markers can be utilized in finger millet breeding programs to enhance genetic variability and crop improvement.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13007-026-01522-9.

Supplementary Material 1.

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Author contributions

JP, PJ, YJ, SS, HPI, SP: Planning and pilot experimentation of emasculation techniques. MV, SS: Planning and selection of material for experiments. JP, PJ, KR, SAM, SS: Validation experimentation of emasculation techniques. HK, MS: Molecular analysis for hybridity testing. AKV: Data analysis. SS, JP, PJ: Manuscript development. HK, SKG, SM, KS: Reviewing of Manuscript.

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Data availability

Data is provided within the supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Authors declare consent for publication.

Competing interests

The authors declare no competing interests.

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