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Flowering Phenology and *SiDREB2*-based SNAP Marker-assisted Hybridity Confirmation for Artificial Hybridization of Indonesian Foxtail Millet (*Setaria italica* L. Beauv) Genotypes

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ABSTRACT

Foxtail millet hybrid development encounters challenges due to its self-pollinating nature, small florets, and tool availability for confirming true hybrid. Our research aimed to assess flowering phenology and explore artificial hybridization employing *SiDREB2* based-SNAP marker. The research consisted of three experiments. The first experiment focused on the panicle phenology of foxtail millet, while the second experiment focused on floret phenology and reproductive organs. The artificial hybridization successfulness was validated with SNAP markers based on the *SiDREB2* gene in the third experiment. Observations on two genotypes (ICERI-5 and ICERI-6) revealed panicle initiation at 49–69 days after planting (DAP), with anthesis occurring 6 days after panicle emergence (DAPE). Florets exhibited the formation of reproductive organs at 3 DAPE, delineating distinct stages of pollen development leading up to anthesis. Our findings emphasized the significance of implementing male-sterile induction treatment at 4 DAPE to regulate pollen development for successful hybridization by 6 DAPE. The hybrid evaluation revealed varied germination rates and confirmed hybrid percentages across different crossing series, validated by *SiDREB2*-based SNAP marker. ICERI-5×Botok-4 exhibited high germination but a low confirmed hybrid percentage, while Botok-4×ICERI-5 and Botok-10×ICERI-6 showed the opposite trend. Reciprocal crossings showed unilateral incompatibility in confirmed hybrid percentage.

1. Introduction

The foxtail millet, recognized taxonomically as *Setaria italica* L. Beauv, holds prominence within the Poaceae family for its nutritional advantages and remarkable adaptability across diverse environmental settings (FAO 2023). The nutritional attributes of foxtail millet encompass various aspects such as its carbohydrate equivalence to rice as indicated by Verma *et al.* (2020), its low glycemic index as highlighted by Ren *et al.* (2022), elevated levels of protein and dietary fiber, and notable antioxidant properties according to Goudar *et al.* (2023). This species has been associated with health benefits, including the prevention of hypertension (Hou *et al.* 2018), reduction of cardiovascular risk factors (Agrawal *et al.* 2023), and potential therapeutic attributes against various cancer types (Shan *et al.* 2022; Gupta *et al.* 2023), as reported in several studies. The adaptability of foxtail millet to adverse environmental conditions like drought (Wang *et al.* 2023), salinity (Ardie *et al.* 2015; Pan *et al.* 2020), and limited nutrient availability (Nadeem *et al.* 2020) underscores its significant agricultural value, particularly in arid and semi-arid regions with limited water resources and inadequate access to agricultural inputs.

Despite its numerous benefits, foxtail millet remains underutilized in Indonesia, with no superior variety released in the country to date. The superior foxtail millet variety 'Jigu32' was developed in China by a conventional hybridization approach (Li *et al.* 2014), while the high-quality foxtail millet cultivar 'GPUF3' was developed in India using a

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selective breeding method focused on pure-line selection (Nagaraja *et al.* 2022). The advancement of a superior variety in foxtail millet via a cross-breeding method faces obstacles attributed to the inherently self-pollinating characteristic of this species (Hariprasanna *et al.* 2017), the small-size and cleistogamous flower (Diao and Jia 2017; Nagaraja *et al.* 2023), and the variation in flowering time between genotypes and between spikelet in the same panicle (Siles and Baltensperger 2001).

Male-sterility induction is a common strategy to develop superior hybrids in self-pollinated plants (Visarada 2021). The manual emasculation method, using a magnifying glass and tweezer, is effective in avoiding self-ing. Still, it is a time-consuming method that requires many workers to do mass work (Siles and Baltensperger 2001). Immersing the panicles in warm water at 48°C for 3-6 minutes (Jiang *et al.* 2013) or treating them with a 500 µM maleic hydrazide solution for 2 minutes (Rizal *et al.* 2015) has previously demonstrated efficacy in inducing sterility in the pollen of green millet (*S. viridis*) and considered to be relatively simpler, faster, and cheaper than manual emasculation. Male-sterility induction needs to be applied at the right flowering stage to avoid self-pollination. Therefore, the flowering phenology study of foxtail millet is the key to determining the precise stage for sterility induction.

Considering the variation in anthesis time between spikelets in the same panicle (Siles and Baltensperger 2001), self-pollination still potentially occurs after male sterility induction (Rizal *et al.* 2015). Foxtail millet produces caryopsis type of fruit with small size, ranging from up to 2 mm in length (Swamy 2023); thus, it is quite challenging to distinguish grains from successful hybridization to those from self-pollination. Molecular markers have been reported to assist hybridity confirmation in several self-pollinating species (Rizal *et al.* 2015; Hangloo *et al.* 2020; Thakur *et al.* 2020; Shaikh *et al.* 2021). A single nucleotide amplified polymorphism (SNAP) marker was devised for the assessment of drought or salt tolerance in foxtail millet. This marker is predicated on a synonymous single nucleotide polymorphism (SNP) found at the 558th base pair of the *SiDREB2* gene, specifically an A/G transition (Lata *et al.* 2011; Widyawan *et al.* 2018). This marker can be used to assist the hybridity confirmation in foxtail millet, particularly if the parental genotypes have different alleles at the 558th base pair. Four Indonesian local foxtail millet genotypes were reported to have different alleles at the 558th base of *SiDREB2*, namely ICERI-5 (A/A), ICERI-6 (A/A), Botok-4 (G/G), and Botok-10 (G/G) (Widyawan *et al.* 2018). Thus, they serve as proper genetic material for evaluating the hybridization efficiency of Indonesian foxtail millet genotypes.

Another reason for selecting ICERI-5, ICERI-6, Botok-4, and Botok-10 as parental line candidates is their potential performance, which may be inherited by their progeny, as demonstrated by the benefits of crossing outlined by Brown *et al.* (2014). Genotypes ICERI-5 and ICERI-6 exhibit traits such as short posture, early maturation, and drought tolerance but with low yield. Conversely, Botok-4 and Botok-10 represent foxtail millet with tall posture, late maturation, and susceptibility to drought but with high productivity (Ardie *et al.* 2015; Lapuimakuni *et al.* 2018; Widyawan *et al.* 2018). Thus, the ultimate goal of this breeding program is to obtain pure foxtail millet lines with traits of early maturity, medium posture, drought tolerance, and high productivity after selecting some generations from ICERI and Botok crossing. The objective of this research was twofold: first, to ascertain the optimal timing for male-sterile induction by assessing flowering phenology, and second, to appraise the efficacy of hybridization utilizing *SiDREB2*-based SNAP marker in foxtail millet.

2. Materials and Methods

This study consisted of three consecutive experiments. The first experiment focused on panicle phenology, while the second on floret and reproductive organs phenology of two foxtail millet genotypes. The third experiment evaluated the artificial hybridization successfulness using several series of foxtail millet crosses utilizing *SiDREB2*-based SNAP markers.

Two genotypes of foxtail millet from the Indonesian Cereals Research Institute (ICERI), ICERI–5 and ICERI–6, were used in this study since these two genotypes possess relative tolerance to salinity (Ardie *et al.* 2015) and drought stress (Lapuimakuni *et al.* 2018; Widyawan *et al.* 2018). These two genotypes also have short stature (<100 cm) and early harvest time (64 days after planting/DAP); thus, they are ideal

for studying flower phenology in contrast to Botok genotypes, which have very small florets that are difficult to analyze, particularly in the early stages of flowering phenology. Flower phenology was observed at individual panicles in the first experiment and at individual floret, reproductive organ, and pollen development in the second experiment.

Additional genetic materials, i.e., Botok-4 and Botok-10 originating from East Nusa Tenggara (Ratnawati *et al.* 2024), were used in the artificial hybridization experiment. These genotypes exhibit tall stature (>170 cm), drought susceptibility, late maturity, and high productivity. The mating design adopted a line-by-tester approach, encompassing the crossing series: ICERI-5×Botok-4, ICERI-5×Botok-10, ICERI-6×Botok-4, and ICERI-6×Botok-10, along with their reciprocal crosses. The hybridization outcomes were validated using the *SiDREB2* gene-based SNAP markers, where a true hybrid will possess the A/G allele constitution at base 558th of the *SiDREB2* gene.

2.1. Panicle Characteristic and Phenology on Foxtail Millet

This experiment was arranged in a randomized complete block design with a single factor of genotypes and three replications, conducted at Cikabayan Experimental Station, IPB University. Each plot of 1.25 m × 2.0 m consisted of 72 plants with a 25 cm × 10 cm planting distance. Fertilizers were applied twice, i.e., before planting at rates of 150–150–75 kg ha^{-1} of urea–SP36–KCl and one month after planting 150 kg ha^{-1} of urea. Ten plants were sampled from each plot. The observation was conducted on (1) the time of panicle emergence (DAP), (2) the time of anthesis (the day after panicle emergence/DAPE), (3) the pollination period (minutes), and (4) the anthesis period (days). The time of panicle emergence was determined when the panicle appeared between the flag leaf of the sample plant. Anthesis time was determined when >50% of the floret in a panicle showed the appearance of anthers. The pollination period was determined from the time of anthesis start until the anther shed in most of the florets for each plant sample. The anthesis period was determined from the starting day of anthesis until it ends for each plant sample.

2.2. Floret, Reproductive Organs, and Pollen Development on Foxtail Millet

The second experiment was arranged in a split-plot randomized complete block design for repeated measurement over time (Gomez and Gomez 1984) with genotype as the main plot and day of observation as a subplot, with three replications, conducted at Cikabayan Greenhouse, IPB University. Planting was done in a pot with a mixture of soil and sand in a 1:1 (v/v) ratio. The pH condition was 6.5. Fertilizers were applied two times before planting at rates of 1–0.75–1 g per pot of urea–SP36–KCl and one month after planting 1 g per pot of urea. Observation was conducted daily on two florets in the pollination zone (Figure 1A) from the first time they appeared until anthesis. Variables observed include floret length, floret width, floret area, filament length, anther length, anther width, style length, ovary length, and ovary width (Figure 1B). Observations on floret and reproductive organs were carried out using a binocular microscope (Olympus) with 2× magnification. Pollen staining was done with I2KI 1% (v/v) and observed under a light microscope (Olympus) with 40× objective magnification. Those observations were conducted at the Seed Biology and Biophysics Laboratory at IPB University. Dark blue, round pollen was classified as fertile pollen, while pale or wrinkled pollen was classified as sterile pollen, according to Talukder *et al.* (2022). Data collection was assisted with the CoolingTech software 2.0.0.0 version connected to a portable microscope device. ImageJ software was used to measure the floret area.

2.3. Artificial Hybridization on Foxtail Millet Utilizing *SiDREB2*-based SNAP Marker

The experiment began with the cultivation of parental plants for crossing, organized based on a randomized complete block design single factor with 16 replications of four genotypes as the parent crosses. Planting and crossing were conducted at Cikabayan Greenhouse, IPB University. The treatment factor consisted of eight cross combinations: ICERI-5×Botok-4, ICERI-5×Botok-10, ICERI-6×Botok-4, ICERI-6×Botok-10, Botok-4×ICERI-5, Botok-4×ICERI-6, Botok-10×ICERI-5, Botok-10×ICERI-6. Each experimental unit comprised one polybag with one female parent plant. The male parent plants were planted at different intervals to avoid pollen source depletion.

Plants designated as potential female parents received male sterility induction treatment applied on the 2nd day at the large floret or 4 DAPE stage using warm water immersion at 48°C for 3 minutes after prior trimming at the lower panicle zone due to its ability to eliminate viable pollen effectively (>90%) while simultaneously preserving pistil receptivity,

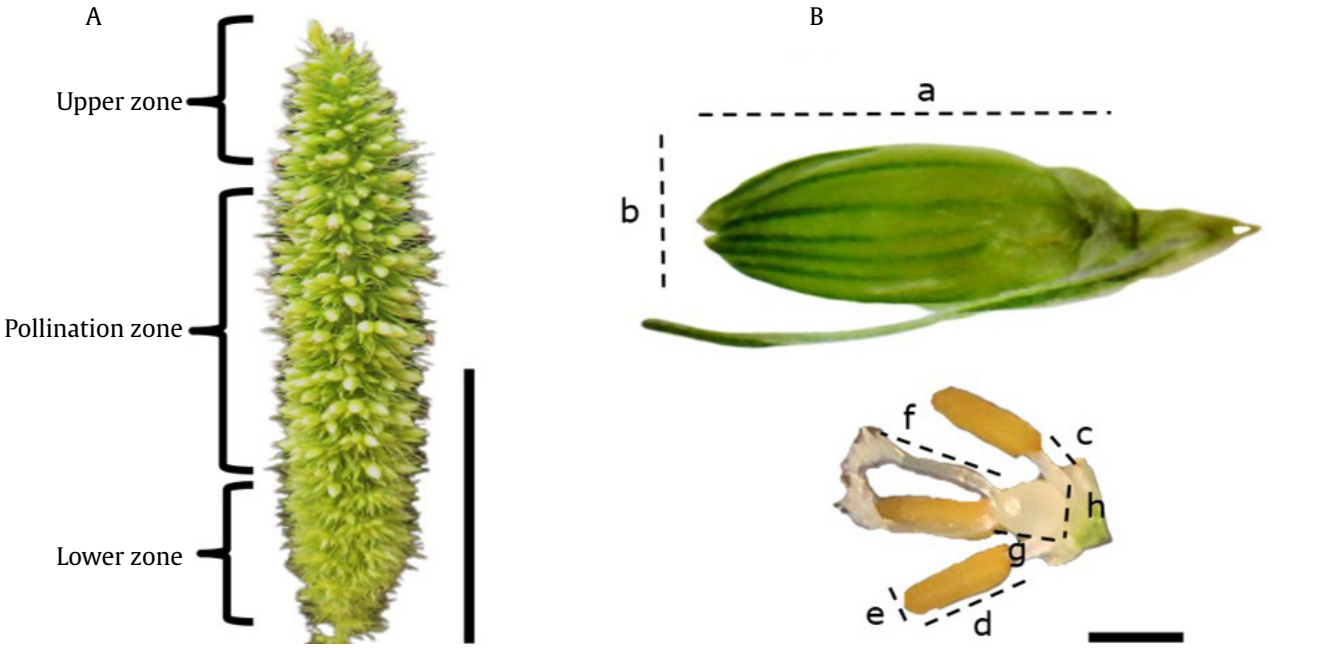

Figure 1. (A) Panicle zone in foxtail millet, (B) floret and reproductive organs in foxtail millet; a. floret length, b. floret width, c. filament length, d. anther length, e. anther width, f. style length, g. ovary length, h. ovary width; bar Figure A =10 cm; bar Figure B =1 mm

as reported by Jiang *et al.* (2013) and Nugroho *et al.* (2020), then covered with an envelope to prevent unintended pollination. Artificial hybridization was performed when the panicle showed post-treatment anthesis symptoms. Pollen donors came from the panicle of male parent plants that had undergone anthesis. The artificial hybridization was conducted using the panicle-to-panicle method (Jiang *et al.* 2013). The panicle of male parent plants that had anthesis was immediately harvested before the anthers dried out (typically harvested before 7:00 am Western Indonesia Time), then gently tapped onto the panicle of female parent plants also undergoing anthesis simultaneously. One male parent panicle was used to pollinate one female parent panicle to enhance pollination success. Subsequently, the panicles were enveloped and labeled until harvest. Evaluation of pollination success was carried out on the 3rd day after artificial hybridization. Artificial hybridization succeeded if the majority of florets showed a yellowish color. Still, if, on the 3rd day after artificial hybridization, the majority of florets remained green, the artificial hybridization was repeated. Harvested hybridized panicles were then cut along with their envelopes and labeled. Seeds were promptly harvested, stored in glass bottles, and kept at 5°C.

Due to the abundance of fruit sets resulting from each artificially hybridized panicle, we refrained from quantifying their quantity. Instead, we randomly selected 40 F1 seeds from the entire fruit set formed on a single hybridized panicle. If a panicle had fewer than 40 seeds, all seeds were planted. Additionally, ICERI-5, ICERI-6, Botok-4, and Botok-10 plants were self-pollinated, with three plants each. Planting was conducted in pot trays using a substrate mix of charcoal and manure at a 1:1 (v/v) ratio, with daily watering. Leaf isolation was carried out after the juvenile plants developed five leaves. DNA isolation was performed from approximately 0.05 g of young leaves using a modified CTAB method (Aboul-Maaty and Oraby 2019). DNA concentration quantification was done using a spectrophotometer at a wavelength of 260 nm.

DNA amplification was conducted in a 10 µL PCR mix comprising 2.5 µL of genomic DNA (12 ng.µL^{-1}), 2.5 µL of forward and reverse primer (10 pmol each), and 5.0 µL of 2× PCR mix (KAPA2G Fast HotStart ReadyMix, Sigma-Aldrich, Germany). Each sample was reacted in two separate tubes. The first tube used primer SD2-558-SNP-A, and the other used primer SD2-558-SNP-G to distinguish between homozygous A and G alleles as well as their heterozygotes (Ratnawati *et al.* 2024). The PCR profile included an initial denaturation for 5

minutes at 94°C, followed by 25 cycles consisting of denaturation for 5 seconds at 94°C, annealing for 1 minute at 55°C, and extension for 30 seconds at 72°C. The PCR reaction ended with a final extension for 10 minutes at 72°C. The amplified PCR products were analyzed via electrophoresis on a 1.5% (w/v) agarose gel at 90 V for 30 minutes in 1x TAE buffer stained with 0.5 $\mu g.ml^{-1}$ ethidium bromide (EtBr) solution. Gel visualization was performed using a UV transilluminator (AlphaImager® Mini).

Widyawan *et al.* (2018) reported that genotypes ICERI-5 and ICERI-6 are drought and salinity-tolerant genotypes with homozygous A/A alleles, while genotypes Botok-4 and Botok-10 are drought and salinity-sensitive genotypes with homozygous G/G alleles. The expected F1 progeny resulting from the second gamete fusion of parents should have a heterozygous genetic constitution (A/G) to produce bands in both primers. F1 progeny showing only one band indicates no artificial pollination occurred. Observations comprised (1) germination rate percentage, which is calculated by dividing the total germinated seeds by the total planted seeds and multiplying by 100%, and (2) the confirmed hybrid percentage, which is calculated by dividing the total confirmed hybrids by the total plants and multiplying by 100%.

2.4. Data Analysis

All collected data were subjected to variance analysis (ANOVA) using the Statistical Tool for Agricultural Research (STAR) ver. 2.0.1 (Gulles *et al.* 2014). Correlation analysis in the second experiment was conducted using Minitab ver 17.1.0, while contrast tests in the third experiment were analyzed using SAS ver. 9.00 software.

3. Results

3.1. Panicle Characteristic and Phenology on Foxtail Millet

Flowering on foxtail millet begins with the appearance of panicles from the flag leaves. Panicle emerges in the late vegetative stage and continuously develops until the seeds mature. Flowering development on ICERI–5 and ICERI–6 is shown in Figure 2. The results from the first experiment indicated that the time of panicle emergence of ICERI–5 and ICERI–6 genotypes was not significantly different, i.e., from 49 to 69 DAP ($\bar{x}$ = 55.20±7.16 SD). The appearance of rachises containing florets accompanied panicle emergence. In general, flowering started from the upper panicle downward and required three days for a complete panicle for ICERI–5 and ICERI–6. Anthesis began from 55 to 75 DAP ($\bar{x}$ = 60.95±7.32 SD), followed by pollination period from 88 to 104 minutes after anthesis ($\bar{x}$ = 97.08±6.24 SD). ICERI–5 had an anthesis period from 2.5 to 2.9 days ($\bar{x}$ = 2.70±0.20 SD), which was longer than ICERI–6 with an anthesis period of 1.3 to 1.9 days after anthesis ($\bar{x}$ = 1.60±0.30 SD) ($P<0.01$).

ICERI–5 and ICERI–6 genotypes have slightly similar panicle characteristics and phenology. From the first experiment, we divided the panicle stages prior to anthesis into three, i.e., panicle initiation stage, characterized by the emerging of panicle tip from flag leaves (day 0 of panicle emergence); incomplete panicle stage, characterized by panicle not yet appear completely (1 to 3 DAPE); and complete panicle stage, characterized by panicle appear completely (4 to 6 DAPE) as shown in Figure 2. The outcome of this research is shown in Table 1.

3.2. Floret, Reproductive Organs, and Pollen Development on Foxtail Millet

Floret on the pollination zone appeared on day 1 after panicle emergence and significantly increased in length, width, and area on day 3 after panicle emergence in both genotypes (Figure 3, $p<0.01$). Three floret stages in the pollination zone can be classified as (1) small floret stage, characterized by floret width less than 1.20 mm (1–2 DAPE); (2) large floret stage, characterized by floret width more than 1.20 mm (3–5 DAPE); and (3) anthesis stage, characterized by the emerging of anther and stigma from floret (6 DAPE) as shown in Figure 2.

In order to identify the time of reproductive organs formation, observation was conducted on the internal organs of sampled florets. Reproductive organs (stamen and pistil) were formed on 3 DAPE and were gradually increased in size until anthesis (Figures 2 and 4). The genotype main effect and genotype × day interaction effects were significant on the floret area ($P<0.01$). The floret width on day 4 after panicle emergence had a significant increase due to the maturation of the reproductive organ.

Reproductive organs were formed on day 3 after panicle emergence and were steadily developed until anthesis (Figure 4). They increased significantly on days 4 and 6 after panicle emergence, except for anther length. A significant increase in days 4 and 6

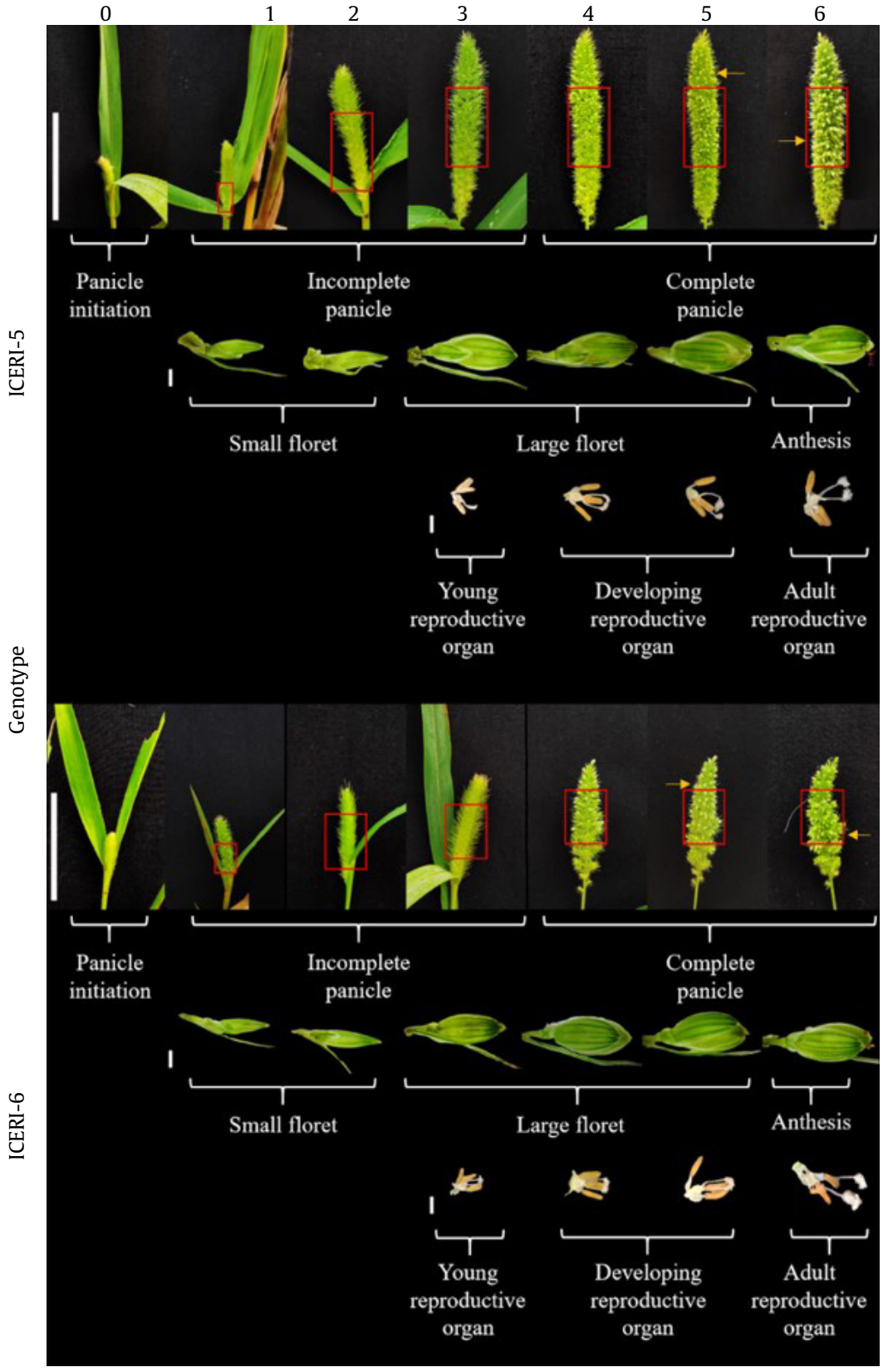

Figure 2. Development of panicle, floret, and reproductive organs from panicle emergence to anthesis in pollination zone; square = pollination zone; arrow = the lowest anthesis floret; panicle bar = 10 cm; floret and reproductive organs; bar = 1 mm

Table 1. Summary of flowering phenology of ICERI–5 and ICERI–6 genotypes

DAPE	Panicle stage	Range of floret width (mm)	Floret stage	Reproductive organ stage	Pollen stage	Action
0	Panicle initiation	-	-			
1	Incomplete panicle	0.85-1.20	Small floret	-	-	
2						
3				Young reproductive organ		
4	Complete panicle	1.21–2.65	Large floret	Developing reproductive organ	Vacuolated pollen	Male-sterile induction
5					Starch accumulation	
6			Anthesis	Adult reproductive organ	Mature pollen	Hybridization

DAPE: days after panicle emergence

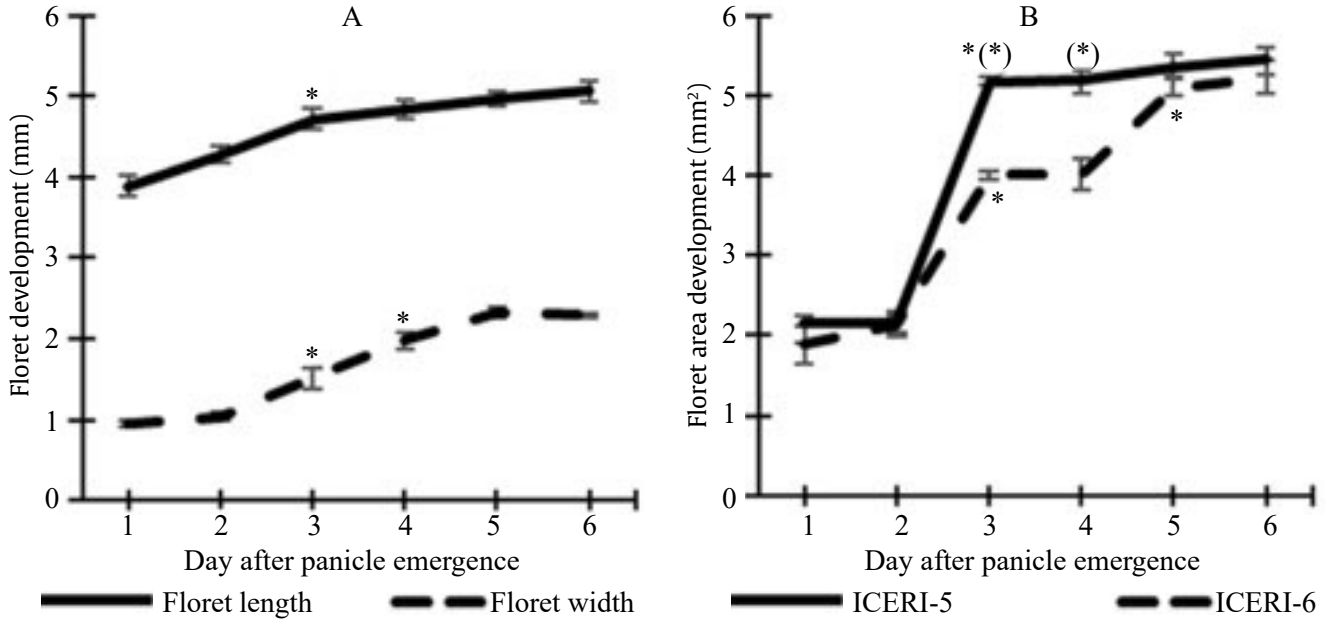

Figure 3. Development of floret: (A) development of floret length and width (B) development of floret area; error bars indicate ± standard error (SE); *: significantly different from the previous day; (*): significantly different among genotypes; n: 12 florets/day

after panicle emergence was caused by maturation and anthesis, respectively. Anther and stigma were discharged from the floret during anthesis. ICERI-5 and ICERI-6 have no significant differences between each other, neither between genotype and day, in the aspect of the development of reproductive organs. In the aspect of phenology study, there were three stages of the development of reproductive organs in the pollination zone, i.e., young reproductive organs stage, characterized by fragile organs with pale color (day 3 after panicle emergence); developing reproductive organs stage, characterized by increasing size, stronger color, and more hardened (day 4 and day 5 after panicle emergence); and adult reproductive organs stage, characterized by anther and stigma discharged and ready to shed pollen (6 DAPE).

Pollens were formed on day 4 after panicle emergence. The viability of pollen was identified by the iodine (I2KI 1%) staining method that verified the

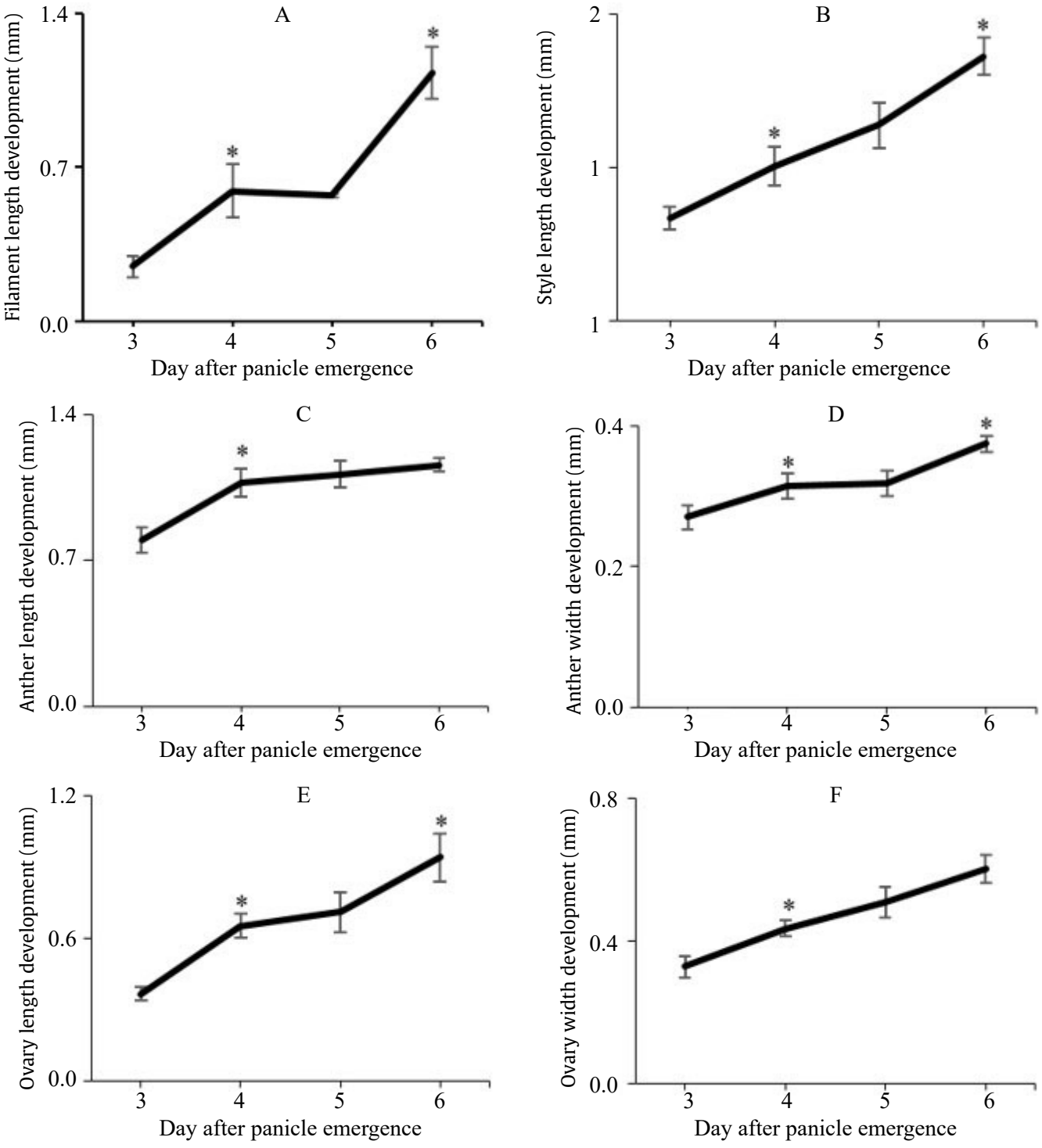

Figure 4. Development of reproductive organ: (A) development of filament length (B) development of style length (C) development of anther length (D) development of anther width (E) development of ovary length (F) development of ovary width; error bars indicate ±SE; *: significantly different from the previous day; n:12 florets/day

viability of pollen in relation to the accumulation of starch levels (Figure 5A). The phenology of pollen, based on the I2KI staining method, can be classified into three stages, namely vacuolated pollen grain stage (day 4 after panicle emergence) when there is no starch in pollen but exine and intine have been formed, characterized by transparent pollen; starch accumulation stage (day 5 after panicle emergence), characterized by pale brown pollen; and mature pollen stage (day 6 after panicle emergence) when starch accumulation in pollen was optimal, characterized by dark blue color in pollen. The increase of pollen maturity per day (Figure 5B) between the two genotypes was not significantly different, and there was no genotype × day interaction.

Floret area, anther, and style length were positively correlated with each other ($P<0.01$). On the other hand, the percentage of pollen maturity did not correlate with other variables (Table 2), which means that the developmental stage of the reproductive organs could be identified by observing the floret developmental stage. This can be used to determine the precise time for the induction of male sterility in foxtail millets.

3.3. Artificial Hybridization on Foxtail Millet Utilizing *SiDREB2*-based SNAP Marker

Artificial hybridization conducted in foxtail millet after male-sterile induction resulted in only a few florets exhibiting signs of pollination, indicated by green color in many florets, while pollinated florets typically represent browning three days after artificial hybridization (Figure 6). Furthermore, a subsequent observation revealed a low germination rate across all crossing series, particularly in the Botok-10×ICERI-5 series, with only 7.19% of the planted seeds showing germination ability compared to their parental lines. The highest germination rate was observed in the ICERI-5×Botok-4 series at 37.88% (Table 3 and Figure 7).

Based on the validation results utilizing a *SiDREB2*-based SNAP marker, the ICERI-5×Botok-4 resulted in a remarkably low percentage of confirmed hybrids, with only 8.35% of the seeds capable of germination, the electrophoresis results depicted in Figure 8. The highest confirmed hybrids were found in the Botok-10×ICERI-6 series, with 91.50% of the 10.75% artificially pollinated seeds exhibiting to germinate (Table 3).

Table 2. Correlation between floret, reproductive organ, and percentage of pollen maturity

Floret area				
Anther length	0.99**			
Style length	0.94**	0.94**		
% Pollen maturity	0.55	0.53	0.77	
	Floret area	Anther length	Style length	% Pollen maturity

N: 3; **: significant α: 1%; *: significant α: 5%

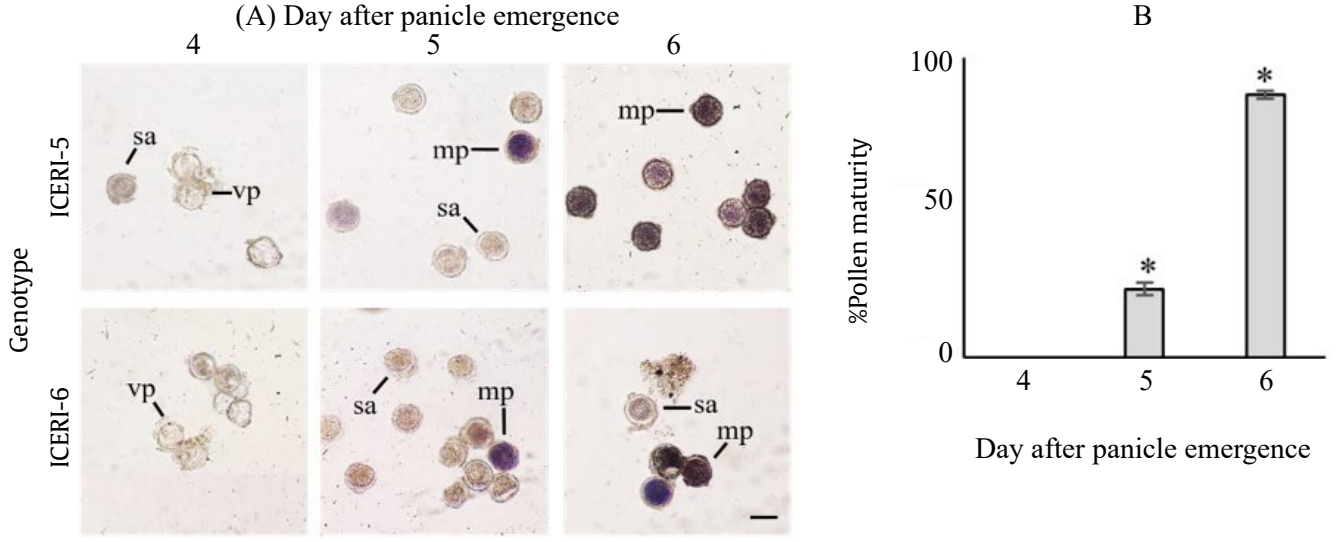

Figure 5. (A) Development of pollen in foxtail millet (B) Bar chart showing the pollen maturation; vp: vacuolated pollen; sa: starch accumulation; mp: mature pollen; error bars=±SE; *: significantly different from the previous day; bar Figure A: 20 µm; n: pollen from 12 florets/day

Figure 6. Floret condition in panicles three days after artificial hybridization; bar: 1 cm

Table 3. Percentage of germination rate and confirmed hybrid percentage

Crossing series	% Germination rate	% Confirmed hybrid
ICERI-5×Botok-4	37.88^{a}	8.35^{d}
ICERI-5×Botok-10	29.17^{ab}	33.39^{abcd}
ICERI-6×Botok-4	19.74^{abc}	18.78^{bcd}
ICERI-6×Botok-10	11.57^{abc}	15.71^{cd}
Botok-4×ICERI-5	14.82^{bc}	78.81^{a}
Botok-4×ICERI-6	7.88^{c}	44.63^{abc}
Botok-10×ICERI-5	7.19^{c}	68.75^{ab}
Botok-10×ICERI-6	10.75^{c}	91.50^{a}

Numbers with the same letter in the same column are not significantly different based on the HSD test at α = 5%

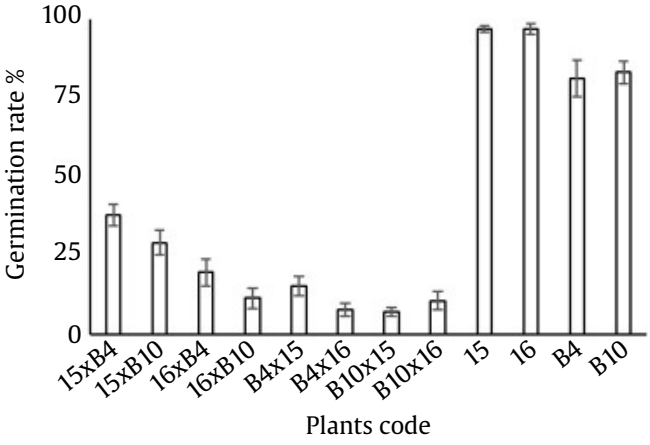

Figure 7. Germination rate graph for each crossing series with parents; I5: ICERI-5; I6: ICERI-6; B4: Botok-4; B10: Botok-10; n=40 seeds per plant code per replication; error bars= ±SE

There was a phenomenon that closely resembles unilateral incompatibility, where a cross can be made but not its reciprocal cross. This was supported by the contrast test values (Table 4), where the total hybrids originating from the female parent ICERI-5 or ICERI-6 significantly differ from the total hybrids originating from the female parent Botok-4 or Botok-10.

4. Discussion

From our findings, the development of reproductive organs can be observed from the age of florets as it has a positive and significant correlation. Specifically, in the pollination zone, which is the widest zone with florets of similar condition, by the 3rd days after panicle emergence (DAPE), reproductive organs have formed, but no pollen appears. Vacuolated pollen becomes visible on the 4th DAPE, followed by starch accumulation (mainly) on the 5th DAPE. However, by 5 DAPE, at least 20% mature pollen had already been found. This

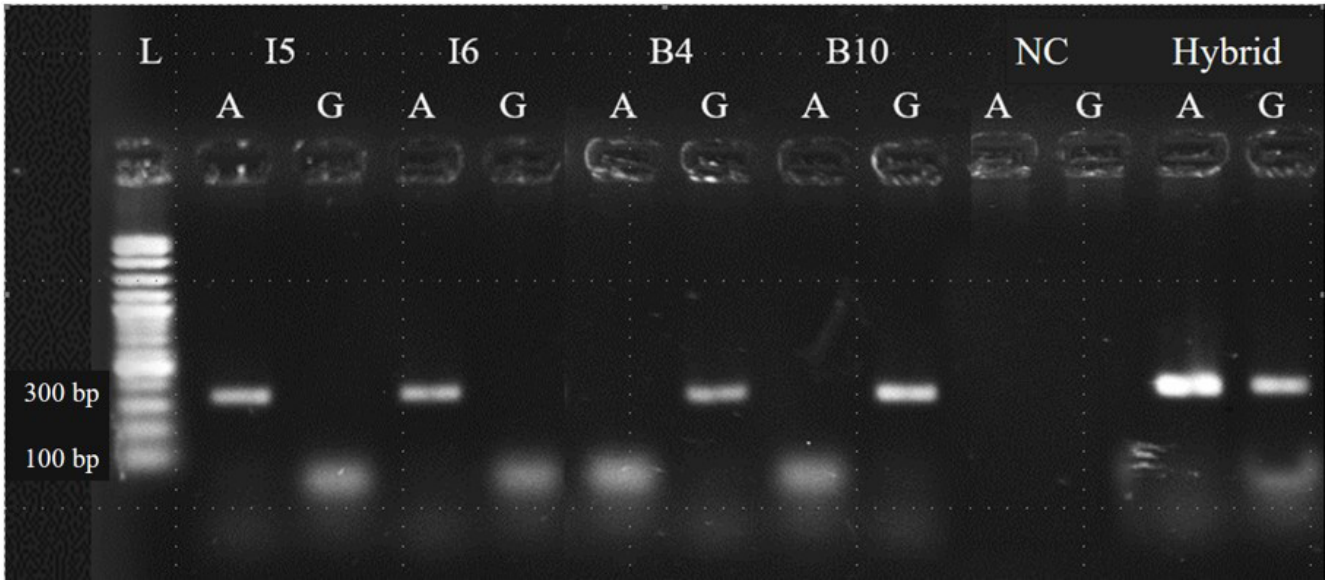

Figure 8. Electrophoresis results for positive control, negative control, and hybrids; L=1,000 bp Ladder; I5=ICERI-5; I6=ICERI-6; B4=Botok-4; B10=Botok-10; NC=negative control; A=PCR reaction using SD2-558-SNP-A and SD2-558-SNP-rev primer pair; G= PCR reaction using SD2-558-SNP-G SD2-558-SNP-rev primer pair

Table 4. Contrast values of the crossing series with their reciprocal series

Contrast of hybrid numbers	Mean difference (%)
ICERI-5 and 6 vs. Botok-4 and 10 (parental lines)	-212.55**
ICERI-5×Botok-4 vs Botok-4×ICERI-5	-69.77**
ICERI-5×Botok-10 vs Botok-10×ICERI-5	-45.99**
ICERI-6×Botok-4 vs Botok-4×ICERI-6	-36.22**
ICERI-6×Botok-10 vs Botok-10×ICERI-6	-60.56**

**=significant at α = 1%

could disrupt the male sterility induction process. Therefore, we suggest conducting induction on the 4th DAPE.

Differences in the use of chemical solutions can alter the decision regarding the timing of male sterility induction. For instance, (1) induction using SQ-1, targeting the failure in premeiotic male process in foxtail millet plants of genotypes ICERI-5 and ICERI-6, is recommended to be administered no later than 3 DAPE as reproductive organs have formed but pollen has not yet developed (Saxena and Hingane 2015; Zhang *et al.* 2016); (2) induction using maleic hydrazide, which disrupts the enzymatic process of pollen development, and induction through warm water soaking aimed at a starch deposition in pollen, can be performed at 4 DAPE as pollen is present but not yet mature (Harsant *et al.* 2013; Jiang *et al.* 2013; Rizal *et al.* 2015). In simpler terms, we attempt to illustrate Table 1, summarizing the flowering phenology of ICERI-5 and ICERI-6, accompanied by suggested actions for plant breeders.

Artificial hybridization is certainly carried out during anthesis when floret conditions are open in the female parent and pollen is mature in the male parent (Brown *et al.* 2014). It's not an easy task, and thus, we conducted numerous repetitions. Several obstacles are highly likely to occur during crosses between varieties, especially in genotypes with a considerable genetic distance, such as between ICERI genotypes from the Indonesia Cereal Research Institute and Botok from East Nusa Tenggara. The first obstacle is the pre-fertilization barrier, where pollen does not easily interact with the stigma of the female parent (Wang *et al.* 2022a). We encountered such difficulties; therefore, even though at times we used three panicles to pollinate one female panicle, conditions often occurred, as depicted in Figure 6. Low pollination success resulted in low hybrid seed yields.

The second obstacle is the low germination capacity of hybrid seeds. According to He *et al.* (2023), low germination is a common phenomenon in crosses between plants with a significant genetic distance, caused by (1) failure of embryo development into viable seeds, (2) mechanisms hindering zygote development even at the first cell division, (3) specific gene actions, leading to

mismatch between nuclear genes and cytoplasmic genes or between the embryo and endosperm.

The third obstacle encountered is the difficulty in obtaining confirmed hybrids. This is highly likely, especially as the optimized male sterility induction technique still leaves 7% viable pollen, making selfing highly probable in cleistogamous pollination patterns (Nugroho *et al.* 2020). Confirming hybrids in crosses of drought-tolerant and drought-sensitive foxtail millet can utilize SNAP markers based on the *SiDREB2* gene. This technique can confirm the banding pattern of the drought-tolerant and drought-sensitive parentage as well as their hybrids. Allele A, associated with drought tolerance, is referred to as the alternate allele. In contrast, allele G, which is associated with drought sensitivity, is referred to as the reference, and hybrids should exhibit both alleles (Figure 8). Bands from DNA amplification appear at ~300 bp (Widyawan *et al.* 2018). Additionally, a phenomenon resembling almost unilateral incompatibility is suspected, where a cross can be performed but not its reciprocal cross (Wang *et al.* 2022b). This is supported by contrast test values (Table 4), where the total hybrids originating from the female parent ICERI-5 or ICERI-6 significantly differ from the total hybrids originating from the female parent Botok-4 or Botok-10.

Despite encountering numerous obstacles, we obtained a considerable number of hybrids, thanks to the numerous repetitions used. We are still conducting several optimizations for male sterility, crossing strategies, and the development of other selection markers in this plant to enhance the chances of successful foxtail millet crosses in the future.

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