Viability of sugarcane and *Erianthus arundinaceus* pollen under marcotting treatment

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**Abstract.** Most of the sugarcane cultivated today is the hybrid between *Saccharum officinarum* and its relative, *S. spontaneum*. The breeding relies mainly on the crossing of male and female parents, which needs the excellent viability of the pollen from the male parent. Marcotting or air layer is a technique to facilitate parents to be crossed outside of the sugarcane fields. The technique allows sugarcane roots to grow from stalk internodes wrapped with soil to maintain the viability of the male and female gametes during the crossing. We investigated the effect of marcotted stalks on pollen viability. The cultivars observed in the study were PS-862, PS-865, 88-1762, and 89-2143, along with the *Saccharum*’s wild relative, *Erianthus arundinaceus*. Pollens from marcotted and control stalks were taken and stained with Lugol’s or IKI solution daily to confirm their viability and diameter measurement. Additional parameters observed were root weight, male and female organ morphometry. The results showed that pollen viability was not affected by marcotting or air layering treatment. In addition, the treatment could maintain flower anthesis in panicles or arrows within more than two weeks. The pollen sizes varied among cultivar and genus, while the viable pollen tended to be bigger than the non-viable ones.

**Keywords:** crossing, stain, Lugol’s solution

1. Introduction

Sugarcane breeding programs rely on the hybridization between female and male parents. The progenitors of most sugarcane varieties cultivated all over the world today are *Saccharum officinarum* and *S. spontaneum*. The high sucrose content of the first species is desired for high sugar production, while the latter species has several beneficial characteristics related to the resistance against biotic and abiotic stress. In addition, *S. spontaneum* is also remarkable in agronomic characters such as profound tillering ability, which is essential for high biomass production and ratooning ability [1].

The modern sugarcane cultivars today have a narrow genetic-based due to limited progenitors involved in their hybridization. We can trace back to the early breeding programs in the 1900s, which only utilized 19 genotypes of *S. officinarum* and two genotypes of *S. spontaneum* in the hybridization [2]. The narrow genetic base may contribute to yield stagnations and vulnerability against biotic and abiotic stresses [2, 3], including pests, and diseases. Therefore, broadening the sugarcane genetic-based is very urgent, which can be made through hybridization between sugarcane with its wild relatives within Andropogoneae such as *S. spontaneum* and *S. robustum, Erianthus, and Miscanthus.*

Sugarcane tasseling is affected by the physiological status of the stalks as well as the environmental conditions such as temperature, day length, and precipitation [4]. Meanwhile, genetics, nutrients, and environmental factors such as photoperiod, temperature, and relative humidity influence pollen viability
The pollen viability is vital in hybridization or crossing, and usually, it is expressed as the proportion of viable pollen grains based on the staining and direct count method [10]. The pollen viability should be at least 60% for the male parent to achieve a good crossing [8].

The quality of parental gametes and the reproductive organs are essential in the sugarcane breeding program; thus, we must maintain them during the crossing. Sugarcane crossing is commonly made in-situ or ex-situ methods, both need transporting stalks either or both parents from the initial area to the crossing facility. Thus, we need to cut stalks one or both parents. The stalk cutting facilitates breeders to emasculate male parents and to set up female and male parents in the lantern. However, this stalk cutting potentially disrupts nutrient and water supply from the roots to the panicle, which may affect the stamen and pistil during the crossing. Therefore, we should maintain the physiological process of stalks for ensuring anthesis, pollination, ovaries development, fruit formation, and fuzz ripening. Two techniques are common in maintaining stalk's physiological process, namely, stalk marcotting and weak acid dipping [11]. We investigated the impact of stalk cutting and marcotting on the pollen grains viability due to the lack of information on marcotting.

2. Materials and Methods

We experimented with the research station and laboratory of the Indonesian Sweetener and Fiber Crops Research Institute in Malang, Indonesia, during the sugarcane flowering season in May-July 2020. We utilized survey and experimental methods in this research.

2.1. Preliminary observations

Several sugarcane cultivars were observed, such as Bululawang, PS 862, PS 865, 88-1762, 89-2143, and its relative S. spontaneum, Erianthus arundinaceus, and E. bengalense to determine the cultivars or clones to be treated and observed. The purposes of this stage were to determine cultivars or clones used for the marcotting.

2.2. Marcotting and pollen viability observation

Five sugarcane cultivars, namely PS 862, PS 865, 88-1762, 89-2143, and E. arundinaceus were utilized in this experiment. Two basal nodes of each stalk were marcotted with wet soil and wrapped with polyethylene plastic around 8-10 weeks before panicles or arrows emergence for roots to grow from the internodes.

We used the two factorial of completely randomized design (CRD). The first factor was the treatments namely marcotting and control and the second factor was the day of observation. The replication was three for each treatment. On the first day of anthesis, stalks were cut right below the marcotted internodes and transported to the crossing facility. We trimmed the leaves until only 10 cm left to reduce water transpiration. Watering was given for stalks every two days to maintain the vigor of stalks and panicles. On the other hand, the control stalks remained in the field without marcotting and leaf trimming.

Pollen grains were collected daily started on the first day of anthesis and the following days until the last flower's anthesis. Opened flowers from every panicle were cut with scissors and collected in Petri dishes in the early morning at 06.00 am - 07.30 am then exposed to the sunlight to stimulate dehiscence. The pollen released from anthers in each petri dish were collected gently with a fine brush. We added a few drops of Lugol’s or iodine kalium iodide (IKI) solution onto pollen grains then covered them with cover glass. The solution would stain black the starch within pollen grains. The fully stained pollens were the viable ones while the partly stained and unstained pollens as the non-viable ones. We used the Lugol’s for pollen staining due to its stability [12].

We examined the pollen grains under Carl Zeis stereo zoom microscope at 32x magnifications equipped with Axioo ERc 5S digital camera to capture pollen pictures. Thus, the viability and morphometry were observed based on these pictures. We used the AxioVision Re. 4.8 software for the morphometry measurements.
Along with the pollen viability examination, floral morphometry and morphology such as stigma, stylus, and ovary length were also observed under the microscope, as explained previously. The roots from marcotted or air-layered stalks were harvested and washed before the drying at 75°C for 72 hours and weighted. The data of reproductive organ morphometry, flower anthesis, root dry weight, and pollen viability were analyzed with the analysis of variance (ANOVA) and followed by the post hoc test. The analysis was performed with JMP 13.0 from the SAS Institute.

3. Results and discussions

3.1. Flower characters

The hermaphrodite flowers or spikelets of sugarcane and its wild relatives were consisted of sessile and pedicel and were arranged in a long panicle. Normally, each flower had three stamens comprised of two lobes anther and a long hyaline filament, while the pistil consisted of two stigmas, two styli, and one ovary.

Sugarcane anthers were yellow at flower pre-anthesis, which turned into violet, reddish, or brownish after flower anthesis. In some cases, the color of some cultivars remained yellow after anthesis, such as Bululawang, which remained unopen; thus anthers did not release any pollen grain. This character indicated the sterility of the Bululawang cultivar. We could use it as a female parent to reduce or eliminate the risk of self-pollination or selfing.

The appearance of anthers hanging on each spikelet indicated flower anthesis, which lasted for around 1-2 weeks for each panicle. As the sun rose and the temperature increased, the relative humidity started declining, and evapotranspiration occurred. Finally, the dehydration triggered anthers dehiscence [13], and anthers released pollen grains. The anther dehiscence of sugarcane occurred along the longitudinal slit or latero-longitudinal dehiscence. The opening started from the apical and might extend to the basal of anther with various lengths, from partial to fully opened. However, anthers of some cultivars remained unopen during anthesis or indehiscent such as in the Bululawang.

![Figure 1. Female and male organs of sugarcane spikelet](image)

3.2. Pollen viability

Stigmas of the sugarcane and *E. arundinaceus*, as shown by Figure 1, were dark red plumose at anthesis and have sticky papillae that enable pollen grains to catch and stuck on them when falling. Morphometry and characters female and male organs of sugarcane flowers are presented in Table 1. *E. arundinaceus* stigma, ovary, and pollen grains were significantly smaller than the sugarcane cultivars. The tiny pollen
size of this species supported the previous observation and potentially influenced the compatibility of the crossing [14, 15].

**Table 1.** Morphometry and qualitative characters of female and male reproductive organs.

| Cultivar/Clone | Stigma (µm) | Stylus (µm) | Ovary (µm) | Anther color | Pollen size (µm) |
|----------------|-------------|-------------|------------|--------------|-----------------|
| PS 862         | 1584.7 b    | 902.7 c     | 614.1 bc   | yellow, red  | 42.14 c         |
| PS 865         | 1807.2 c    | 879.0 bc    | 594.3 b    | red, brown   | 43.90 c         |
| 88-1762        | 1776.6 c    | 790.7 b     | 685.5 d    | red, brown   | 40.24 b         |
| 89-2143        | 1820.5 c    | 652.8 a     | 639.3 bcd  | yellow, red  | 42.85 bc        |
| E. arundinaceus | 887.9 a     | 918.8 c     | 345.1 a    | purple       | 33.22 a         |

Numbers within a column followed by the same letters were statistically not different based on Tukey test at α=0.05.

Having anther indehiscent type in the Bululawang cultivar and the rooting difficulty when marcotted of *E. bengalense*, they were not included in the marcotting treatment. In those cultivars tested, the marcotting could stimulate roots to grow from nodes of stalks. Therefore, the rooting could support flower anthesis for up to two-week periods (Table 2). It showed that there was no water supply disruption from the root to the panicle after stalk cutting and transporting to the crossing facility.

Flower or spikelet anthesis started from the most apical part of the panicle and its branches. Thus, flowers on the top opened first and followed by the below positions. The anthesis pattern and spikelet positions reflected the developmental stage and maturity of spikelets. The earlier the anthesis showed the more advances of their developmental stage. When the stalks were cut, spikelets on the top position were already mature, while the spikelets below them were in the developmental stage and needed water and nutrition supplies. Therefore, roots produce within marcotted stalk had to support their development and maturity.

**Table 2.** Flower anthesis period and root dry weight of marcotted stalk.

| Cultivar/Clone | Anthesis period (day) | Root dry weight (g) |
|----------------|------------------------|---------------------|
| PS 862         | 10.8 a                 | 7.9043 ab           |
| PS 865         | 17.0 b                 | 10.636 ab           |
| 88-1762        | 14.5 ab                | 6.533 ab            |
| 89-2143        | 13.2 a                 | 12.687 b            |
| E. arundinaceus | 13.8 ab                | 4.287 a             |

Numbers in a column followed by the same letters were statistically not different based on Tukey test at α=0.05.

The high carbohydrate content is the character of sugarcane pollen, mainly starch and sugar as the energy reserves and callus builder material [16, 17]. These substances are essential in pollen germination, tube growth, and signaling during fertilization [18]. Consequently, starch is an indicator of pollen viability. Starch is an energy reserve converted into soluble sugars to facilitate pollen germination on the stigma and affected sugar content in pollen [19]. A low starch level may cause premature termination of maturation during pollen development [20].

Sugarcane and its relatives belong to the graminaceous plant with starch-rich pollen and depend on wind for pollination [17, 21, 22, 23]. The starch-rich pollen property is valuable as an indicator of its viability. In apricot and sweet cherry, the Lugol’s staining had a strong positive correlation with the pollen germination test as pollen viability indicator [24]. The Lugol’s solution stained the starch of pollen into dark violet or black, while the non-viable ones were only partly stained or remain unstained.
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(Figure 2). The fully and partly stained pollen grains were common among those cultivars and clones observed in this experiment. Besides, staining might differ in its intensity which could be light or dark violet/black, and only the dark violet/black-stained pollen is considered viable. Starch in viable pollen was higher than in non-viable pollen [25], likewise affected the darkness intensity and staining coverage. Kim [23] showed a strong correlation between pollen viability carbohydrate structure as well as lipid reserves.

In addition to staining, round shape plum pollen was also a good indicator of viability. Otherwise, the non-viable or infertile pollens were often shrunk and shriveled in their shape due to collapsing of the pollen grain walls, which could be observed in 88-1762 and 89-2143 cultivars. The shrinking walls were caused by a lack of or less cytoplasmic content [26].

Despite of Lugol’s staining, the morphology of anther lobes was also an indicator of sterility. The Bululawang anthers were wholly yellow and did not dehiscence, similarly to PS 862, 88-1762, and 89-2143, which produced some yellow anthers. Dunckelman & Legendre [11] has noted that the yellow anther was an indicator of sterileness of the pollen while the purple color anther was fertile.

![Figure 2. Pollen viability test by Lugol’s staining a). viable predominant and b). non-viable predominant.](image)

The ANOVA table showed that the main factors of variety and day had significant effects on pollen viability, as in Table 3. Additionally, the interactions of variety x treatment, as well as interaction of variety x day on pollen viability affected pollen viability as well. Therefore, the mean comparisons of pollen viability should be made for those interactions.

Sugarcane cultivars responded differently against cutting and marcotting treatment that as shown in Table 4. Only 88-1762 cultivar was affected by marcotting treatment while the rest were not affected. According to [8], a fertile male parent should have pollen viability of at least 60%, while a partial fertile should have a 30-60% viability. Thus, based on these criteria, only the marcotted stalks of PS-865 and E. arundinaceus in Table 4 met the requirement for male parents according to Z-test with $P<0.0001$ and $P=0.0193$, respectively. Even though the rests had low pollen viabilities, adding more panicles during the crossing may compensate for the low pollen viability. The plumose and sticky stigma of sugarcane might accommodate a big pollen load size; thus it might increase the chance of pollen deposition and pollination. Eventually, fertilization would be higher, and more seed sets would be produced.
Table 3. ANOVA of pollen viability across cultivar, treatment, and day.

| Source                      | df | Sum of Squares | F Ratio  | Prob>F |
|-----------------------------|----|----------------|----------|--------|
| Variety                    | 4  | 62443          | 88.4767  | <.0001*|
| Treatment                   | 1  | 269            | 1.5287   | 0.2176 |
| Day                         | 10 | 8552           | 4.8474   | <.0001*|
| Variety x Treatment         | 4  | 1959           | 2.7759   | 0.0280*|
| Variety x Day              | 40 | 12978          | 1.8390   | 0.0032*|
| Treatment x Day            | 10 | 3196           | 1.8116   | 0.0599 |
| Variety x Treatment x Day  | 40 | 9844           | 1.3949   | 0.0704 |

Table 4 also showed that PS 862, PS 865, and *E. arundinaceus* pollen viabilities on the second week were as good as the first week. However, this situation has a risk when they were used as male parents due to the potential of seed contamination from the male parent. During two weeks of crossing, self-pollination may occur in male parents because they were not emasculated and might produce viable seeds. Preventing seed contamination, the male parents need to be replaced before the seed formation and maturation.

Table 4. Pollen viabilities across days and treatments among sugarcane cultivars.

| Pollen viability (%)          | PS-862 | PS-865 | 88-1762 | 89-2143 | *E. arundinaceus* | Pooled |
|-------------------------------|--------|--------|---------|---------|-------------------|--------|
| Marcots                       | 54.6 a | 81.4 a | 33.4 a  | 52.7 a  | 65.4 a            | 59.3 a |
| Control                       | 53.9 a | 79.7 a | 44.5 b  | 54.7 a  | 63.7 a            | 57.4 a |
| Pollen viability of marcotted stalk (%) |        |        |         |         |                   |        |
| 1st week                      |        |        |         |         |                   |        |
| PS-862                        | 54.2 a | 81.3 a | 37.3 b  | 53.1 b  | 66.7 a            | 58.7 b |
| PS-865                        | 49.3 a | 80.3 a | 23.8 a  | 40.7 a  | 62.6 a            | 51.3 a |

Pairs of numbers in a column followed by the same letters were statistically not different based on contrast t-test at α=0.05.

Subramanyam and Andal [27] noted that male sterility in sugarcane is supposed to be due to anthers non-dehiscence, defective pollen formation, pollen grain agglutination, pollen sterility in protogyny, non-emergence anthers in protogyne. We found the non-dehiscence and defective pollen formation in this experiment.

Table 5 shows the size of viable pollens was statistically larger than the non-viable one, except for *E. arundinaceus*, which was only a slight difference. The pollen size as a viability indicator was shown in blue-eyed mary and seep monkeyflower [10], and the smaller diameter would have less nutrition content such as starch [14, 17, 28].
Table 5. Pollen size of viable and non-viable pollen among sugarcane cultivars.

|                | PS-862 | PS-865 | 88-1762 | 89-2143 | E. arundinaceus | Pooled |
|----------------|--------|--------|---------|---------|-----------------|--------|
| Viable         | 41.2 b | 41.1 b | 39.5 b  | 42.9 b  | 32.4 a          | 39.4 b |
| Non-viable     | 34.5 a | 35.6 a | 32.6 a  | 35.7 a  | 29.1 a          | 33.5 a |

Pairs of numbers in a column followed by the same letters were statistically not different based on contrast t-test at α=0.05

We discovered an unknown species of bees and two thrips from sugarcane spikelets. Even though sugarcane is known to be anemophilous, the starchy-rich pollen grains might slightly attract pollinators and pollen feeders to visit. Less attractiveness of mature pollen grains to pollinators due to its starchy but less in lipids content [29].

4. Conclusion

High pollen viability is very critical in a sugarcane crossing. The stalk marcotting can maintain pollen viability; therefore, it can be utilized in broadening genetic-based sugarcane through hybridization.

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