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MALE FLOWER CHARACTERISTICS OF INDUCED TETRAPLOID, MIXOPLOID, AND DIPLOID BANANA *MUSA ACUMINATA* (AA) CV. "PISANG REJANG"

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SUMMARY

The banana cultivar "Pisang Rejang" (*Musa* AA "Pisang Rejang") is Fusarium wilt resistant and therefore important for banana breeding. Tetraploid (4x) and mixoploid (2x+4x) plants of this cultivar have been induced with oryzalin, and crosses of these two ploidy levels resulted in a triploid hybrid (3x) plant. The availability of these various ploidy levels needs to be accompanied by data on their reproductive biology for their efficient utilization for breeding. This study characterized male flowers concerning qualitative and quantitative morphology, pollen viability, pollen size, nectar volume, nectar Brix index, and acidity level of those different ploidy levels. Observations indicated no differences in qualitative morphological traits, such as, shape and color, but for quantitative morphological traits, such as, size, distinction existed among the different ploidy levels. The triploid plant has huge flower parts in all the quantitative morphological traits, except compound tepal width, followed by the tetraploid, diploid, and mixoploid. Variations also occurred in pollen viability, pollen size, and nectar characters from each level of ploidy. Pollen viability is the highest for the diploid plant, while the tetraploid plant produces bigger pollen than the diploid. The triploid plant produced a low percentage of viable pollen. The study findings indicate that ploidy level affects the flower's quantitative morphological characteristics, pollen viability, and pollen size, but not the flower's qualitative morphological characteristics.

Keywords: Pollen, viability, nectar, morphological character, breeding

Key findings: Sterility is the bottleneck for banana breeding. Here, the study demonstrated the variation of pollen viability as an indication of banana sterility in different ploidy levels. Based on the study, "Pisang Rejang" diploid, tetraploid, and mixoploid (2x + 4x) are potential banana breeding parents due to their fertility level.

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INTRODUCTION

The banana's center of diversity in the world points from South to Southeast Asia and Indonesia. Other Southeast Asian countries are where the banana cultivars evolved from their wild relatives, *Musa acuminata* Colla (A genome) and *Musa balbisiana* Colla (B genome) (Perrier *et al*., 2011). In tropical countries, the banana is an important cash crop and is part of the communities' daily life and local cultures (Kennedy, 2009;

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Sulistyaningsih and Wawo, 2011; Hapsari et al., 2017). The Food and Agriculture Organization reported the largest banana exporting countries in 2021 include Latin America and the Caribbean, with total exports reaching 16,065 t, followed by Asian countries with 3,323 t, and African countries with 684 t (FAO, 2022). Despite its high and notable values, banana production worldwide faces a current threat from one of the deadly banana diseases, the banana Fusarium wilt caused by the *Fusarium odoratissimum* (Maryani *et al*., 2019), previously known as *F. oxysporum* f.sp *cubense* Tropical Race 4 (Ploetz, 2006). The disease attacks almost all types of banana cultivars, and no effective pesticides or sanitation exist to combat this disease (Dita *et al*., 2018; Poerba *et al*., 2019; Lusiyanto *et al*., 2021). Therefore, planting resistant plants are the best way for sustainable banana production.

A few cultivars have demonstrated resistance to banana *Fusarium* wilt disease, and one is "Pisang Rejang" (Sutanto *et al*., 2014; Handayani *et al*., 2017). This cultivar is a diploid 2n=2× (AA) *M. acuminata* species (Poerba *et al*., 2018). In Indonesia this banana has many local names, i.e., in West Sumatera as "Pisang Kalengkeng," in Lampung and Bengkulu as "Pisang Serindit," "Pisang Seringgit" or "Pisang Kepak," and in Java, as "Pisang Rejang," "Pisang Renyang," "Mas Penjalin," and "Mas Beranjut" (Poerba *et al*., 2017). This banana has a sweet flavor like the well-known "Pisang Mas" (sucrier) *Musa acuminata* AA and is usually grown at homestead and not as a commercially cultivated cultivar.

In breeding programs, "Pisang Rejang" has been used as a parent and provides seeds as a male or a female parent (Poerba *et al*., 2015). Recently, to increase the genetic variability of this banana, a chromosome-doubling induction using oryzalin has resulted in tetraploid (4x) and mixoploid (2x+4x) plants (Poerba *et al*., 2017). Interestingly, the fertility of these polyploids seemed to remain the same since crossing these two-ploidy levels has resulted in triploid (3x) plants (Poerba *et al*., 2015). The presence of tetraploid plants is essential as a 2n gamete producer. Hence, pollination using 2n pollen on an ovum or vice versa seeks to produce sterile triploid plants (Bakry *et al*., 2009).

The availability of tetraploid, mixoploid, triploid, and the natural diploid of "Pisang Rejang" is crucial for further banana breeding. Characterization of male flowers from this various ploidy is needed to assess their potential as breeding parents. This study evaluated morphological characteristics of the male flower that include shape and size of flower parts, pollen size, pollen viability, nectar production, Brix index, and acidity (pH) level of diploid (2x), triploid (3x), tetraploid (4x), and mixoploid (2x+4x) plants "Pisang Rejang".

MATERIALS AND METHODS

Material

The study obtained male flowers of diploid 2x (LIPI-048), triploid 3x (LIPI-078), tetraploid 4x (LIPI-007), and mixoploid 2x+4x (LIPI-016) of "Pisang Rejang" from the banana collection of National Research and Innovation Agency, in Cibinong Science and Technology zone in Bogor, Indonesia during February–March 2020. Researchers conducted sampling in the morning from 08:00 to 10:00 a.m. The male flowers got tested from inflorescences that had lost at least 10 bracts but not more than 30 bracts.

Characterization of male flower morphology

Qualitative and quantitative morphological characters of the three male flowers of each ploidy level were characterized based on the Banana Descriptor (IPGRI, 1996). The male flower was taken from the inflorescences, and its morphological characteristics got measured. The qualitative traits included shape, color, and pattern; the quantitative traits included the lengths of the flower, ovary, stamen, and pistil, and both length and width of the compound and free tepal.

Pollen characterization

The study observed two pollen characters, i.e., viability and diameter. The male flowers from the last bract of male bud were extracted and fixed with 96% ethanol: glacial acetic acid (3:1) for 30 min. Then, they were rinsed with 70% ethanol for a minute three times and stored in 70% ethanol. The fixed male flowers were then stored in the fridge for two months after fixation until the conduct of the viability test. Researchers performed the pollen viability test using Lactophenol Orcein (LPO) staining (Sass, 1964). The study performed three replications of tests for male flowers of each ploidy level. An anther was taken from a fixed and stored male flower and washed by immersing it in distilled water for one minute.

Then, incising the anthers lob using a needle, extracted the pollens from the anthers and placed them on a microscopic slide. Then these were pressed until the pollens came out of that incision. The pollens were bathed in 1-2 drops of sterilized water to keep them from drying out. Then, two drops of LPO were added to the pollens and covered with another microscopic glass slide. After 5 min, they were observed under a light microscope. The viable pollen stained red, while the non-viable pollen stained partially or unstained. At least 200 pollens from each anther were observed to determine the proportion of viable and non-viable pollen. The pollen diameter got measured using ImageJ software (Schneider *et al*., 2012).

The data on the flower measurements were analyzed as Mean and Deviation Standard and presented as histograms. The data on pollen size were presented as frequency distribution, while the proportion of viable and non-viable pollen was presented as a percentage.

Characterization of nectar

Nectar was isolated from male flowers using a pipette and collected in a 1.5 ml tube. The volume of the collected nectar was measured using a 1 ml syringe with an accuracy of 0.01 ml. The flower nectar's average volume got calculated from 10 flowers of the male buds. The nectar total sugar content was measured using the "Atago" pocket refractometer and its pH was measured using the pH paper indicator MColorpHastTM (Merck, Germany). The data obtained got analyzed with Microsoft excel for mean and deviation errors.

RESULTS

Qualitative morphological characteristics of male flowers among diploid, triploid, tetraploid, and mixoploid plants were similar (Figure 1). In this study, the color of the male flower is

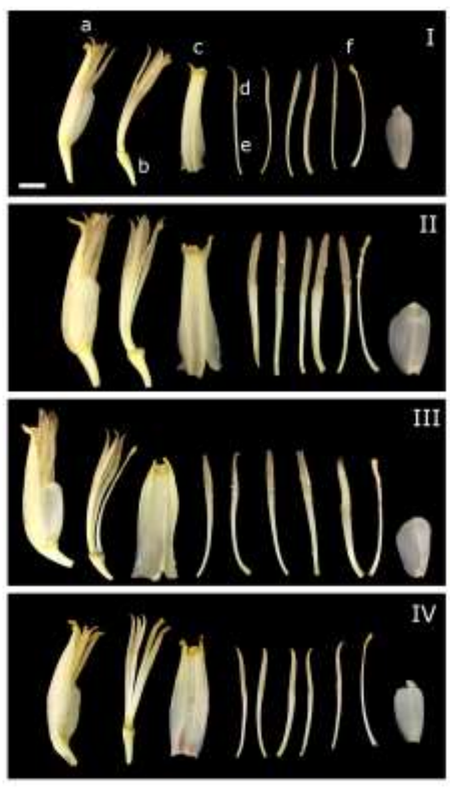

Figure 1. The morphology of male flowers of "Pisang Rejang" with different ploidy levels, diploid 2x (I), triploid 3x (II), tetraploid 4x (III), and mixoploid 2x+4x (IV) are similar but differ in size. The male flower part (a) A male flower at anthesis; (b) Reduced ovary; (c) Compound tepal; (d) Pollen bearing-anther; (e) Filament; (f) Slender style and stigma; (g) Free tepal. The triploid flower is the biggest, followed by tetraploid and both diploid and mixoploid, which are similar. The scale on the image is equivalent to 1 cm for all images.

creamy, with all flowers having five filaments, one compound tepal, and one free tepal. The compound tepal is creamy and has no pigmentation. The lobes of compound tepal are orange with very developed lobes. Free tepal is transparent white with a rectangular to an oval shape and creases under the tip. The tip of the free tepal is triangular, and the filament is creamy. The filament position is of the same height and parallel to the pistil. The pollen sac is brown or rustic brown. The style is straight and creamy, while the stigma is orange. The ovary is straight, creamy and has no pigmentation (Figure 1).

However, variations occurred in the quantitative characters (Figure 2). The triploid flowers showed the highest values for all measured parameters, except for the compound tepal width. The tetraploid plant showed a smaller dimension compared with the triploid plant. However, it is large compared with the diploid or mixoploid plants for all parts of the flowers, except for the ovary, which is shorter, and the compound tepal is wider. The diploid and mixoploid plants were similar but measured the smallest in size of flower parts compared with the triploid and tetraploid.

The viable pollen was round and stained red or dark red, while the non-viable pollen was round or wrinkled but with no stain or colorless (Figure 3). The percentage of viable pollen was highest at 90% for diploid plants, followed by tetraploid, mixoploid, and triploid with 80%, 73%, and 43%, respectively (Figure 4). Pollen diameter, both viable and non-viable, vary with the ploidy of the plants, in which the diameter increases significantly with ploidy. Pollen from the mixoploid plant had a nearly similar diameter to that of triploid plants, and both were somewhat close to the average diameter of diploid and tetraploid. The viable pollen has a significantly larger diameter than the non-viable pollen (Table 1).

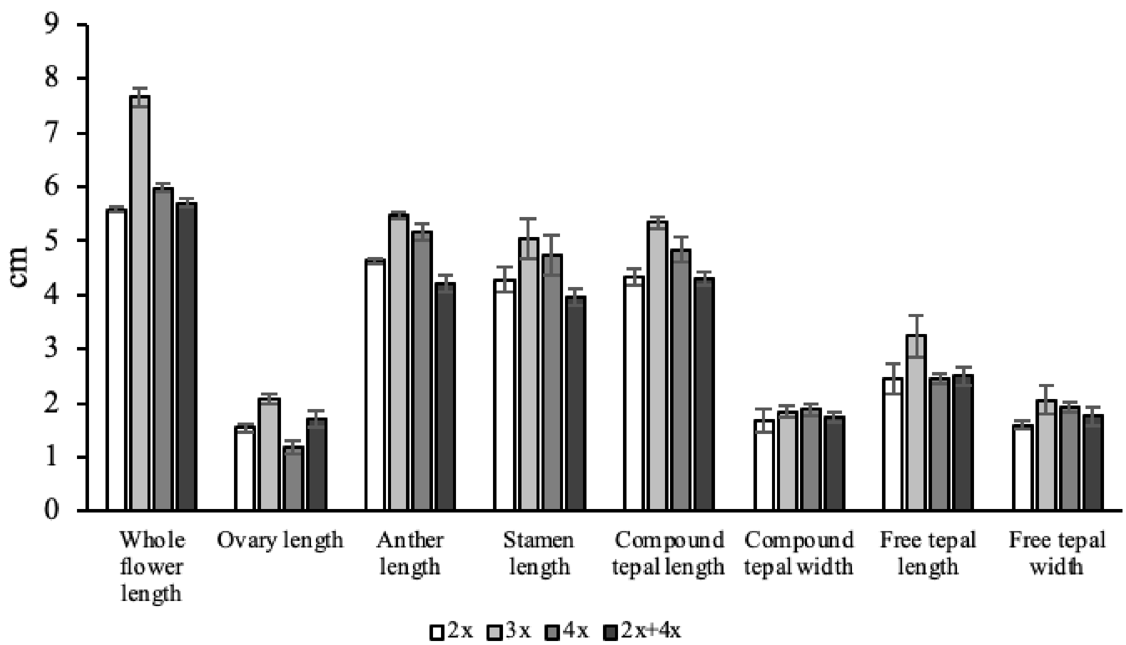

Figure 2. The size of various qualitative characteristics of "Pisang Rejang" male flowers (2x, 3x, 4x, and 2x+4x).

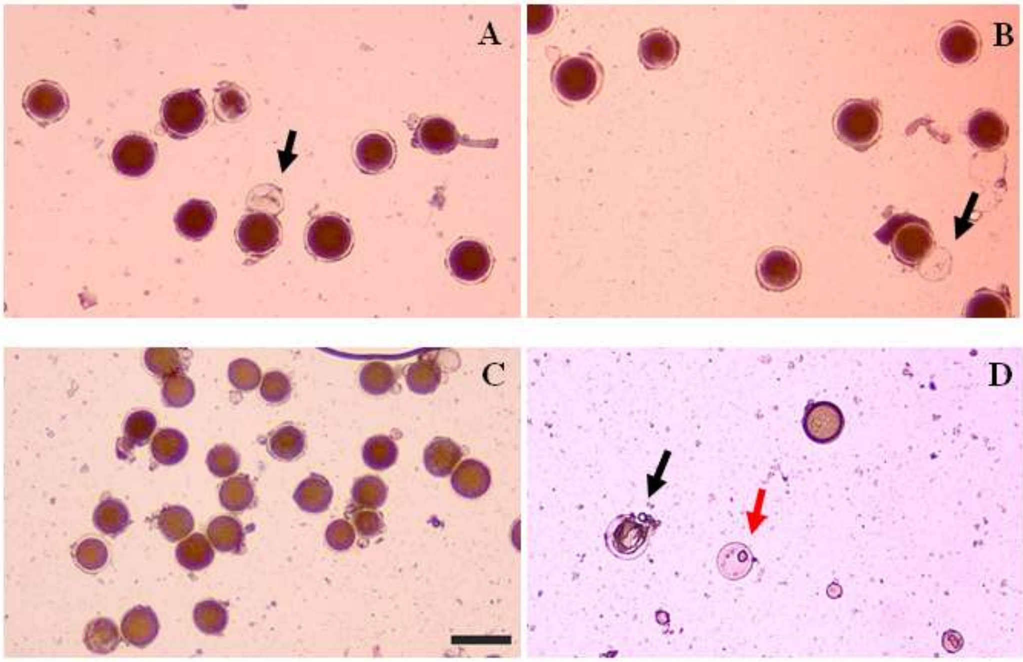

Figure 3. Pollen of "Pisang Rejang" 2x (A), 3x (B), 4x (C), 2x+4x (D). The viable pollens are red or dark red, while the non-viable pollen looks transparent (red arrow) and some of them are wrinkled (black arrows). The bars indicate 200 μm for all images.

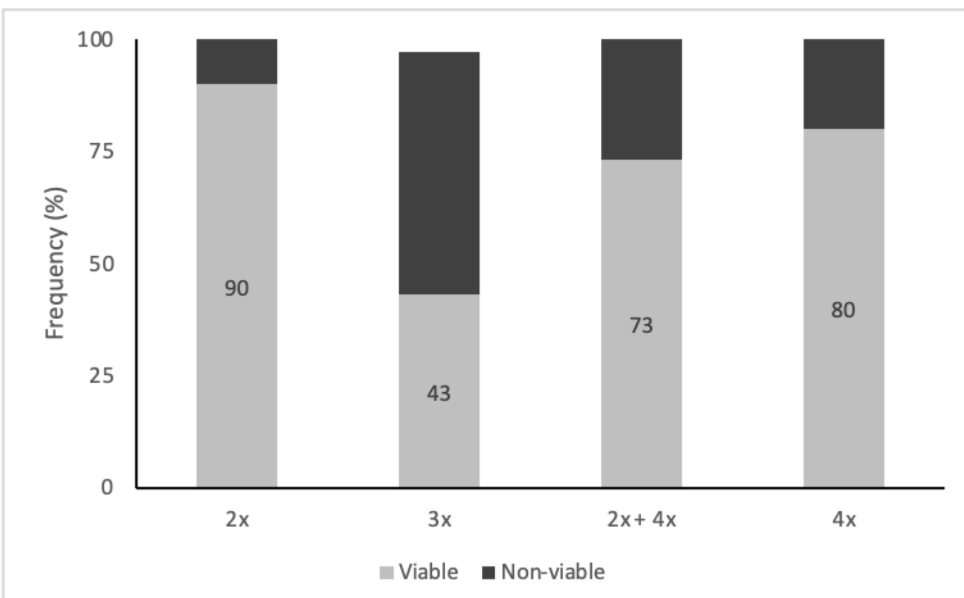

Figure 4. The percentage of viable and non-viable pollen of "Pisang Rejang" 2x, 3x, 4x, and 2x+4x.

Table 1. Average of pollen diameter of "Pisang Rejang" 2x, 3x, 4x, and 2x+4x (μm)

Pollen	2x	3x	4x	2x+4x
Viable	104.8 ±3.1	123.9±9.3	139.2±7.3	121.6±4.8
Non-viable	91.1±1.7	96.2±7.3	116.3±4.3	98.8±5.1

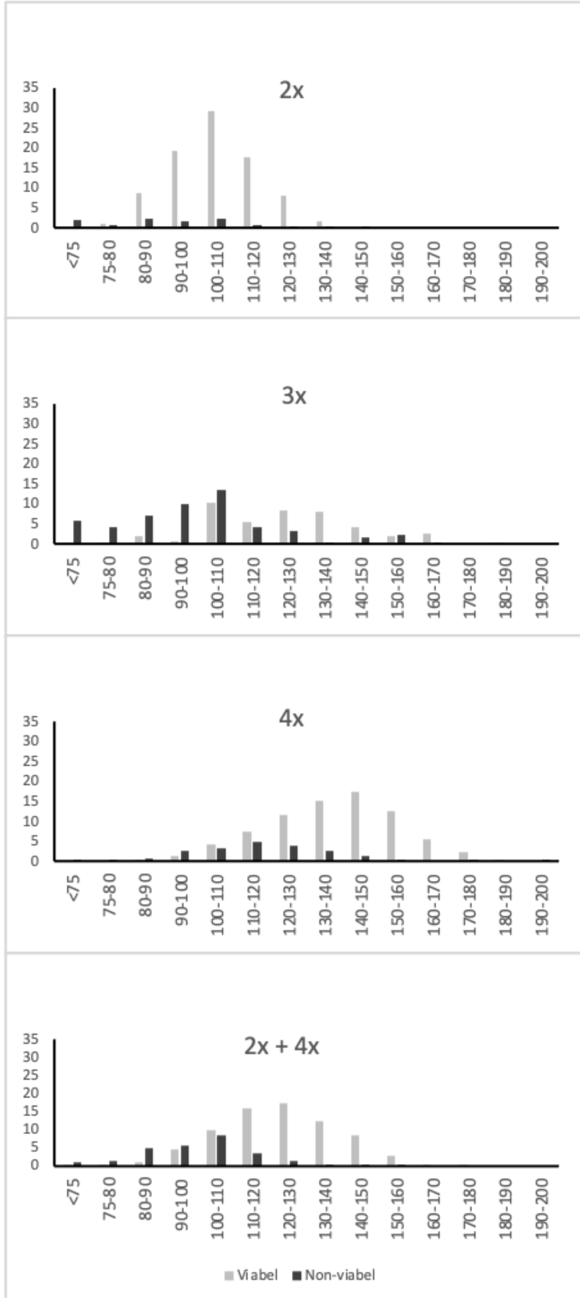

Figure 5. Frequency distribution of diameter of viable and non-viable pollen of "Pisang Rejang" at various ploidy levels, 2x, 3x, 4x, and 2x+4x. Axis X: Diameter range (μm); Axis Y: Frequency.

Figure 5 presents the frequency distribution pattern of pollen diameter. Frequency distribution of pollen diameter indicated that viable pollen diameter had a unimodal, simple distribution pattern for diploid, tetraploid, and mixoploid. However, in triploids, the distribution of pollen diameter seemed to be bimodal. The study noted the pollen diameter range in the diploid plant was narrow, while in the triploid and tetraploid plants were large. Likewise, the mixoploid plant produces pollen with a diameter range between the diploid and tetraploid. The frequency distribution of the non-viable pollen is similar to that of the viable pollen for triploid, tetraploid, and mixoploid. But for the diploid, the pattern was unnoticeable, probably due to its low frequency.

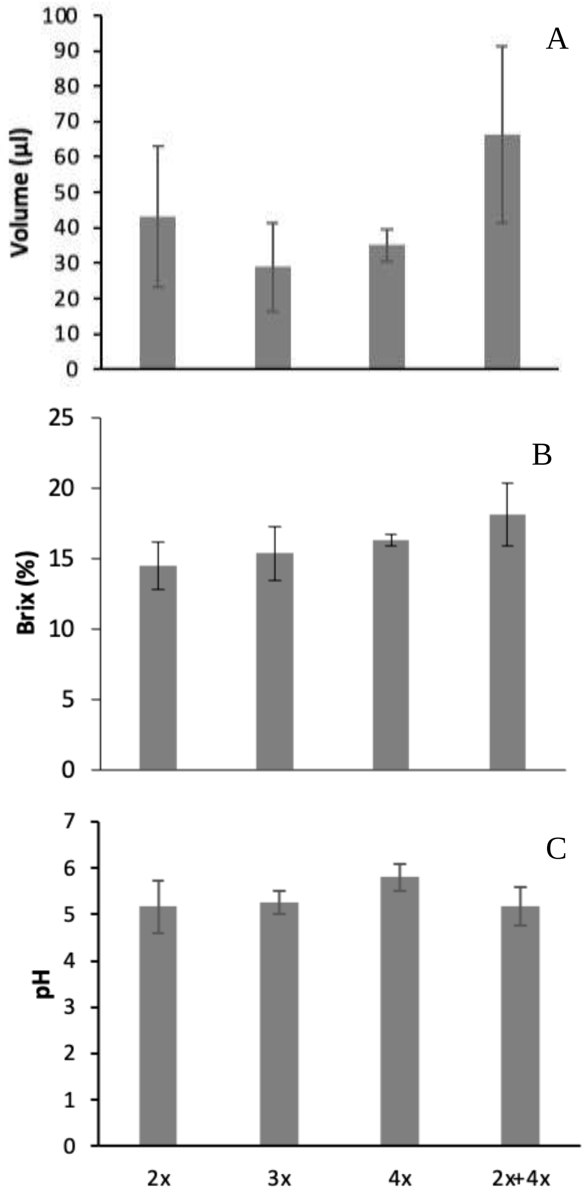

Figure 6. Nectar volume (A), Brix (B), and pH (C) of "Pisang Rejang" at various ploidy levels (2x, 3x, 4x, and 2x+4x).

The volume of nectar collected from the male flower tends to differ with different ploidy levels (Figure 6a). The study noticed that the male flower of the mixoploid plant produced the highest average volume of nectar compared with other ploidy levels. There is no significant difference among ploidy levels regarding the Brix value and pH of the nectar (Figure 6b, c).

DISCUSSION

Different ploidy level in plants incurs various plant morphological characteristics, i.e., the plant size increases with ploidy to a certain level and then decreases with increasing ploidy level (Niu *et al.*, 2016). Observation of male flower morphology from four ploidy levels of "Pisang Rejang", *M. acuminata,* indicated that ploidy level greatly influenced its quantitative traits, in which the triploid had the highest value, followed by the tetraploid and the mixoploid. The diploid produced the smallest size of the flower. This result agrees with the previous observation (Poerba *et al*, 2017) regarding plant stature and fruit size, which were larger in triploid and tetraploid plants than in their diploid counterpart. The ploidy levels also affect the flower size, for instance, the increasing ploidy levels increase the sepal length of Arabidopsis (Robinson *et al*., 2018).

Pollen viability from diploid, tetraploid, and mixoploid of "Pisang Rejang" remain high at above 70%, whereas, for the triploid, the pollen viability decreases significantly (40%). The triploid banana cultivar has been shown to have low pollen viability, for example, the Indian cultivar had pollen viability of 21%–29%, while the Australian cultivar has pollen viability of 6%–10% (Fortescue and Turner, 2004). In triploid plants, the division orientation of the homolog chromosomes during Anaphase I tends to be unbalanced, which later affects its fertility (Dodds, 1943; Wilson, 1946; Shepherd, 1999). The high pollen viability for tetraploid bananas is also uncommon since Fortescue and Turner (2004) showed that tetraploids of Indian and Australian origin had pollen viability of 28% and 29%, respectively. The high level of pollen viability of diploid Rejang has similar observations by Fortescue and Turner (2004) that indicated pollen viability of 84% of diploid *M. acuminata* and confirmed by Ahmad (2021) that demonstrated pollen viability of 77.7% from Rejang diploid. Since the frequency of viable pollen in a viability test indicates the fertility level in bananas (Fortescue and Turner, 2004), this result shows that the diploid natural cultivar of "Pisang Rejang" and its tetraploid and mixoploid derivative is amenable for a breeding parent. Further, polyploid induction is unnecessarily to complete to reach tetraploid. Instead, mixoploid is sufficient for the breeding parent.

As expected, the induced tetraploid "Pisang Rejang" plants produced significantly larger pollen the diploid plant, with distinct frequency distribution. Higher pollen size distribution of other tetraploid plants compared with its diploid relatives has been observed in Betula (Karlsdóttir *et al*., 2008) and Arabidopsis (De Storme *et al*., 2007). The tetraploid plant consequently produced pollen of 2n gamete that commonly has a bigger size than the pollen of n gamete has been known for some species, such as, *Solanum tuberosum* (Carputo *et al.*, 2000), *Lilium auratum* × *L. henryi* (Chung *et al.*, 2013), *Agave tequilana* and *A. angustifolia* (Gomez-Rodriguez *et al.*, 2012), *Cymbidium* (Zeng *et al.*, 2020), and many more. However, the diploid banana "Pisang Lilin" was reported to produce 2n gamete pollen as indicated by the frequency distribution of pollen diameters (Ortiz 1997). It means the pollen is a giant size than the average diameter of n gamete pollen.

The study emphasized that the triploid plant produced bimodal pollen size distribution with a range as wide as the tetraploid, while diploid, tetraploid, and mixoploid had a unimodal distribution. The bimodal indicated that there are two kinds of pollen population, presumably the diploid and haploid pollen. Karlsdóttir *et al*. (2008) also showed bimodal frequency distribution of pollen size from the natural triploid hybrid of *Betula sp*. Kovalsky and Neffa (2012) analyzed the frequency of non-reduced microspores, the size range of pollen, and the constitution of the spores of *Turnera sidoides* to understand the mode of polyploid formation with a bimodal distribution of 2n pollen size producer. A study suggested that bimodal pollen size distribution from a diploid plant indicates the production of non-reduced gamete (diploid pollen) (Jones *et al*., 2007).

This study showed variation in the level of volume, sugar content, and pH. Rutikanga *et al*. (2016) reported variation in sugar content, pH, and nectar volume in various banana cultivars of different genomic compositions. Nectar is a sugar-rich product of flowers that attracts several pollinators like insects, birds, and mammals (Itino *et al*., 1991). In Itino's *et al*. (1991) research, the pollinators that come to the banana flower of *M. acuminata* var. *halabanensis* and *M. salaccensis* are bats, squirrels, honey-sucking birds, and insects from ordo Hymenoptera, Lepidoptera, Dermaptera, and Blattaria. The presence of these pollinators can be either beneficial or detrimental to the plants visited. The one thing that can be beneficial for the flower is help pollination. Otherwise, pollinators in banana cultivation are not essential because the cultivated bananas are parthenocarpy. This phenomenon occurs in fruit formation without pollination (Simmonds, 1962). Pollinators, especially insects in banana cultivation, are more often detrimental because they can be the disease vector of bacterial or viral diseases. Examples are the blood disease caused by bacteria with the vector from bees and fruit flies (Blomme *et al*., 2017) and the viruses distributed by aphids *Pentalonia nigronervosa* (Dale, 1987; Qazi, 2016). Here, we did not study the relationship between nectar production and insects. Therefore, further research on these needs attention to mitigate the disease spread by the insects.

Banana breeding requires early information on the level of fertility of potential parents for crossing. Therefore, cytological studies concerning pollen viability and their genetic composition are vital. Here, the study demonstrated that diploid, triploid, tetraploid, and mixoploid bananas have different levels of pollen viability, indicating their level of fertility. Hence, the observation that "Pisang Rejang" diploid, tetraploid, and mixoploid are all potential banana breeding parents.

CONCLUSIONS

The qualitative morphology of male flowers, Brix value, and pH of the nectar among diploid, triploid, tetraploid, and mixoploid "Pisang Rejang" bananas are similar. However, there was a variation in the quantitative characteristics of its flower. Diploid plants showed the highest percentage of viable pollen at 90%, while tetraploid, mixoploid, and triploid plants showed 80%, 73%, and 43% viable pollen, respectively. This level of pollen viability indicates that "Pisang Rejang" diploid, tetraploid, and mixoploid are all potential for parents in banana breeding.

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