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The Potential Impact of Flower Characteristics and Pollen Viability of Four Industrial Hemp (*Cannabis sativa* L.) Grain Varieties on Cross-Pollination

Beatrice N. Dingha * and Louis E. N. Jackai

Department of Natural Resources and Environmental Design, North Carolina A&T State University, Greensboro, NC 27411, USA; lejackai@ncat.edu
* Correspondence: bndingha@ncat.edu

Abstract: Industrial hemp (*Cannabis sativa* L.) is primarily a dioecious plant, and monoecious varieties have been developed for high yield. Production practices vary for each variety, prompting the need for the present study to describe the floral characteristics and evaluate pollen quantity and viability of monoecious and dioecious hemp varieties. All four hemp varieties, Henola, CFX-2, Canda, and Joey, have five lanceolate anthers, basifixed to a threadlike filament. Anther length was significantly different among varieties, but not anther width. The longest length (0.38 ± 0.046 cm) was recorded in Henola, and the shortest (0.34 ± 0.043 cm) in CFX-2. Anther width ranged from 0.088 ± 0.0024 to 0.095 ± 0.0021 cm. Pollen grains were triporate and spheroidal in shape and size and differed significantly, with the largest in Joey (27.83 ± 0.78 µm) and Henola (27.489 ± 0.99 µm), and smallest in Canda (22.04 ± 0.56 µm). The number of pollen grains per flower differed significantly among varieties, ranging from 29,183 in Henola to 104,548 in Joey. Even though Henola recorded the lowest pollen number, it had the highest percentage (69.3%) of viable pollen after 24 h of storage 4 °C and Canda had the lowest (54%). Three weeks after storage at the same temperature, pollen viability decreased for all the hemp varieties and ranged from 52% to 58%. There was a moderate, positive and significant relationship (r = 0.496) between anther length and the number of pollen grains in Joey. The relationship in Henola was moderate and non-significant (r = 0.356), and it was weak and non-significant in Canda (r = 0.188) and in CFX-2 (r = 0.037). The findings from this study provide information for growers and researchers on hemp breeding and cultivation practices that may contribute to the prevention of cross-pollination.

Keywords: industrial hemp; hemp flower; pollen viability; pollen number; dioecious; monoecious

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1. Introduction

Industrial hemp (*Cannabis sativa* L.) is a plant of the Cannabaceae family cultivated globally for its multipurpose applications. North America and Europe are currently leading the market, largely due to favorable regulatory environments and high consumer awareness [1–4]. In the United States, the passage of the 2018 Farm Bill [5,6] propelled the crop's reintroduction into agroecosystems, with 2022 production valued at USD 238 million [7]. This fast-increasing popularity has been attributed to several factors, including the recognition by growers of the crop's diverse uses, such as an alternative cover crop, in addition to its harmony with the environment, and the many economic opportunities, including its potential as a value-added enterprise for entrepreneurs and businesses [8,9].

Hemp is naturally dioecious and most varieties are photoperiod-sensitive, triggering the transition from the vegetative to the flowering growth stage in response to shortening daylight hours [10]. When growing the crop for medicinal purposes, female flowers are highly sought after since they produce higher quantities of cannabinoids, particularly cannabidiol (CBD). Cannabidiol continues to gain popularity globally because of its antioxidative, anxiolytic, and anti-inflammatory effects and its pharmacological potential in managing and treating neurological disorders including epilepsy and Alzheimer's [11–14]. Hemp is also experiencing a resurgence in natural fiber production, with applications in plastics, rubber, paper, and bioenergy, among others [15–20]. The seeds are a valuable source of minerals, macronutrients, and phytonutrients [21,22], and have been reported to contain high quantities of lipids, protein, and carbohydrates [23–25]. The lipid portion is rich in essential fatty acids and contains large amounts of omega-3 and omega-6 [26,27]. Several studies have reported that extracts from leaves, inflorescences, and seeds exhibited antioxidant, antibacterial, and cytotoxic activity against cancer cells [28–31].

Despite the versatility of hemp, its cultivation is currently facing numerous challenges including the potential for cross-pollination. The plant is anemophilous, and the smooth exine layer of the pollen in conjunction with its light weight facilitates genetic transfer for seed formation [32–34]. Dispersal of pollen during cultivation for CBD could result in pollination, thereby altering the quality and quantity of CBD content [35]. Several tactics have been proposed and the most practiced include indoor cultivation with air filtration systems, planting feminized seeds or vegetatively propagated female clones [36,37], and manual removal of male plants before anthesis, which is laborious and time-consuming. Unfortunately, these tactics have not yielded satisfactory outcomes since pollination by drifting pollen from neighboring grain, fiber, or CBD farms with male plants remains a serious limitation with reports of disputes and lawsuits between farmers [38,39].

Although the morphology of the hemp plant has been described [40–43], there is limited knowledge of floral variation among varieties that could influence and impact successful hemp cultivation and breeding. Most studied varieties in the United States are examined for CBD production. Without focusing on genetic and molecular aspects, this study aims to examine and describe hemp flower and pollen and evaluate the pollen quality (pollen viability) and quantity (number of pollen grains produced) of four hemp grain varieties that are either dioecious or monoecious.

2. Materials and Methods

2.1. Study Site and Plant Varieties

This study was conducted at the North Carolina Agricultural and Technical State University in Greensboro, North Carolina, USA. We used four hemp varieties (Canda, CFX-2, Henola, and Joey) that are among the most popular varieties grown in the United States for grain and fiber and which were available at the time. No seeds of other varieties were available. Seeds were purchased from King's AgiSeeds Inc. (1828 Freedom Road, Lancaster, PA, USA); the seeds of Canda, Joey, and Henola at the time of purchase were described as monoecious and CFX-2 was not described. The seeds of each variety were sown in the greenhouse in 11.35 L plastic plant pots (28 cm wide and 24 cm high) with drainage filled with soil mix (Sunshine® Mix #1, Triangle Chemicals, Kinston, NC, USA). Seedlings were fertilized weekly with fish fertilizer (Alaska fish fertilizer 5-1-1, Lily Miller Brands, Atlanta, GA, USA). After about two weeks, seedlings were thinned to about 4 plants per pot and a total of five pots per variety. Plants were fertilized bi-weekly with nitrogen 34-0-0 and watering was achieved through an automatic watering system regulated to water once daily for 10 min.

2.2. Pollen Viability

For each hemp variety, several unopened flower buds were harvested between 9:00 and 11:00 a.m. Each unopened flower bud was separately transferred into an Eppendorf tube and stored in a refrigerator at 4 °C for pollen grains to be released. After 24 h, the Eppendorf tubes with opened flowers containing released pollen grains were selected, totaling 17 tubes for Canda and Joey and 20 for CFX-2 and Henola. After three weeks, 20 Eppendorf tubes containing pollen from Canda, CFX-2, and Henola and 19 for Joey were selected. In each tube, 2 mL of Ampha buffer #6 was added following the manufacturer's instructions. Each tube served as a replicate. The suspension was hand-shaken to release the pollen grains into the solution. The pollen suspension was filtered using a 50 µm Ampha filter and the pollen grains in the filtrate were counted using the Ampha Z32 Neutec pollen counter (NEUTEC GROUP Inc Farmingdale, New York, NY, USA). The Ampha Z32 uses impedance flow cytometry (IFC) to measure the electrical properties of cells. The IFC system uses a microfluidic chip which permits measurements in the radio frequency range from 0.1 to 30 MHz with alternating current (AC). At a chosen frequency, data of cell size, membrane capacitance, cell concentration, and cytoplasmic conductivity of single cells are simultaneously obtained and related to biological key parameters such as cell viability and membrane permeability [44,45]. The Ampha Z32 data acquisition and processing algorithms display results on a scatterplot as counts of non-viable and viable pollen with the percentage of viable pollen to the top right and non-viable pollen to the top left.

2.3. Pollen Staining and Measurement of Pollen Size

Mature unopened flower buds of each hemp variety were harvested and placed in separate Petri dishes. Petri dishes were manually shaken to release pollen grains, which were then transferred into 1.5 mL centrifuge tubes using a soft camel brush. Tubes were left unsealed to air dry for one hour before sealing and storing in a refrigerator at 4 °C. Using a fresh culture swab, a few pollen grains were deposited on a glass microscope slide (75 mm × 25 mm). Following this, 20 µL of modified Alexander stain [10 mL of 95% alcohol, 1 mL of diluted malachite green, 54.5 mL of distilled water, 25 mL of glycerol, 5 mL of diluted acid fuchsin, 0.5 mL of diluted orange G, and 4 mL of glacial acetic acid [24,25]] was pipetted directly onto the pollen sample. There were ten slides for each hemp variety, for a total of 40 slides. Each slide was slowly heated on an electric burner to near-boiling for about 10 s to allow the stain to set. Slides were allowed to cool for 2 min, and a coverslip was placed on each slide. Each slide was appropriately labeled, dated, and left at room temperature for 24 h before being placed in a microscope slide storage folder. For each variety, pollen size was measured from ten grains, and only undamaged pollen grains were measured. Measurements were obtained from the equatorial plane using an Olympus CX43 compound microscope and recorded. Photos were taken using an Olympus CX43 compound fitted with an Olympus EP50 digital camera.

2.4. Flower and Seed Size

One unopened mature flower of each variety was randomly collected and placed in separate Petri dishes. For each variety, data were collected from 30 anthers. We also randomly selected 30 seeds from each variety and placed them in separate Petri dishes. Measurements and photos were taken of the length and width of anthers and seeds using an Olympus SZX7 stereo microscope fitted with an Olympus EP50 digital camera.

2.5. Statistical Analysis

Data were analyzed using JMP Statistical Discovery software (JMP v.13.0.0 SAS Institute). Pollen size, pollen number and viability, and the length and width of anthers and seeds of all four hemp varieties were subjected to one-way analysis of variance (ANOVA) with alpha at 5%. The Tukey–Kramer HSD test was used to separate means with $p < 0.05$. Graphs were plotted using Microsoft Excel. The relationship between anther width and pollen production for all varieties combined and for each treatment was determined using Pearson's correlation and regression analysis.

3. Results

In this study, the seeds of three hemp varieties (Canda, Joey, and Henola) at the time of purchase were described as monoecious and CFX-2 was not described. However, at the flowering stage, Canda, Joey, and CFX-2 exhibited the characteristics of dioecious hemp plants with the male and female flowers on separate plants (Figure 1A). Figure 1B shows Henola (a monoecious variety) with male and female flowers coexisting on the same plant. At flowering, as shown in Figure 1A, the male plants become slender and taller with fewer leaves than the female plants, which are shorter, with a bunch of leaves associated with the terminal inflorescence. In both the male and female plants, the flowers develop sequentially, with immature flowers located at the upper end of the inflorescence (Figure 1A,B). In general, we observed that the male and female plants do not start flowering at the same time; instead, the male plants flower earlier and die before the female plants.

Figure 1. (**A**) Dioecious and (**B**) monoecious hemp plants; (**a**) unopened flowers and (**b**) opened sepal enclosing tightly packed stamens on a panicle of a male plant.

The male plants and their flowers in dioecious varieties and male flowers in the only monoecious variety (Henola) eventually die after shedding pollen, and the female flowers survive to produce seeds (Figure 2).

Figure 2. (**A**) Dioecious female and (**B**) monoecious hemp plants both show immature seeds and (**C**) mature hemp seeds on the plant.

Using stereo and compound microscopy, observations were made and recorded on the anther and pollen shape, size, and the presence of apertures (pores) in pollen grains. Our findings reveal that anther size varied among varieties, with a significant difference ($F_{3,232}$ = 9.6 $p < 0.0001$) in length (Figure 3). The longest length was recorded in Henola (0.38 ± 0.046 cm), Joey (0.37 ± 0.054 cm), and Canda (0.37 ± 0.046 cm), and the shortest in CFX-2 (0.34 ± 0.043 cm). Anther width was wider for Canda (0.095 ± 0.0021 cm) and CFX-2 (0.094 ± 0.0022 cm); however, this was not significantly different ($F_{3,232}$ = 1.9 $p < 0.1503$) from Henola and Joey. with a much more slender width of 0.088 ± 0.0024 cm and 0.089 ± 0.0029, respectively.

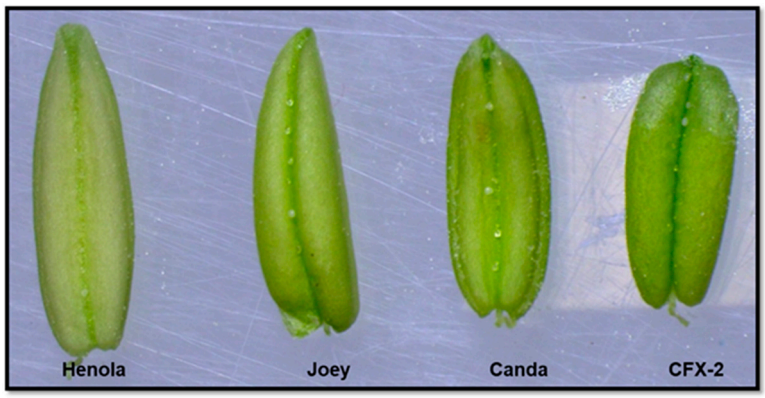

Figure 3. Single isolated anthers of the four hemp varieties showing differences in size.

All hemp varieties have five lanceolate anthers, basifixed to a threadlike filament, and dehiscence was observed to be latrorse, with the anthers splitting open towards the sides to release pollen (Figure 4). The immature male flowers of all four hemp varieties are greenish and enclosed in a simple calyx consisting of five green sepals (Figure 4a,b). At maturity, the calyx turns greenish-yellow and opens to expose the stamens (Figure 4c,d).

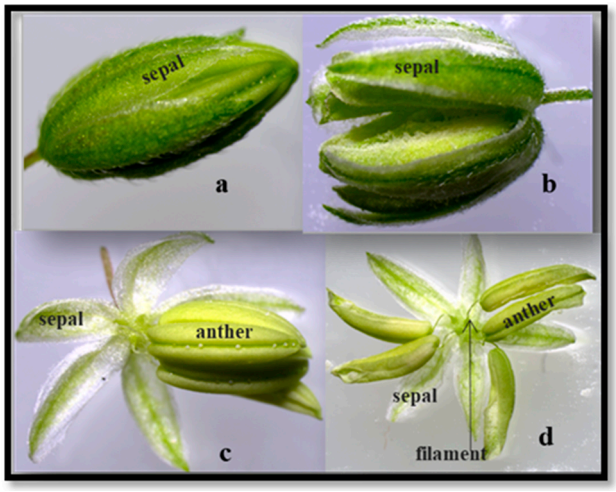

Figure 4. The inflorescence of the male hemp plant shows (**a**) an immature flower bud, (**b**) a partially opened flower, (**c**) a flower with exposed anthers and sepals, and (**d**) a flower with visible filaments and dehisced anthers.

All pollen from the four hemp varieties were predominantly spheroidal in shape, with three circular apertures (triporate) (Figure 5). Pollen size was significantly different ($F_{3,39}$ = 13.4, $p < 0.0001$), with the largest size recorded in Joey (27.83 ± 0.78 µm) and Henola (27.489 ± 0.99 µm), followed by CFX-2 (26.75 ± 0.53 µm). The smallest pollen size was in Canda (22.04 ± 0.56 µm).

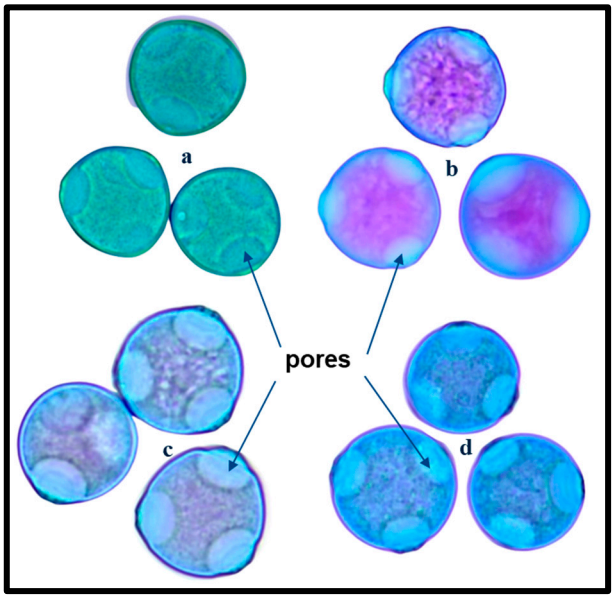

Figure 5. Stained pollen grains: (**a**) Joey, (**b**) Henola, (**c**) CFX-2, and (**d**) Canda observed under light microscopy at a magnification of 400X.

Although there were no differences in the number of anthers, there was a significant difference in anther size, and in the total number of pollen grains (viable and non-viable) produced ($F_{3,70}$ = 20.9 p < 0.0001) among the four hemp varieties. The lowest number was recorded in Henola (Figure 6).

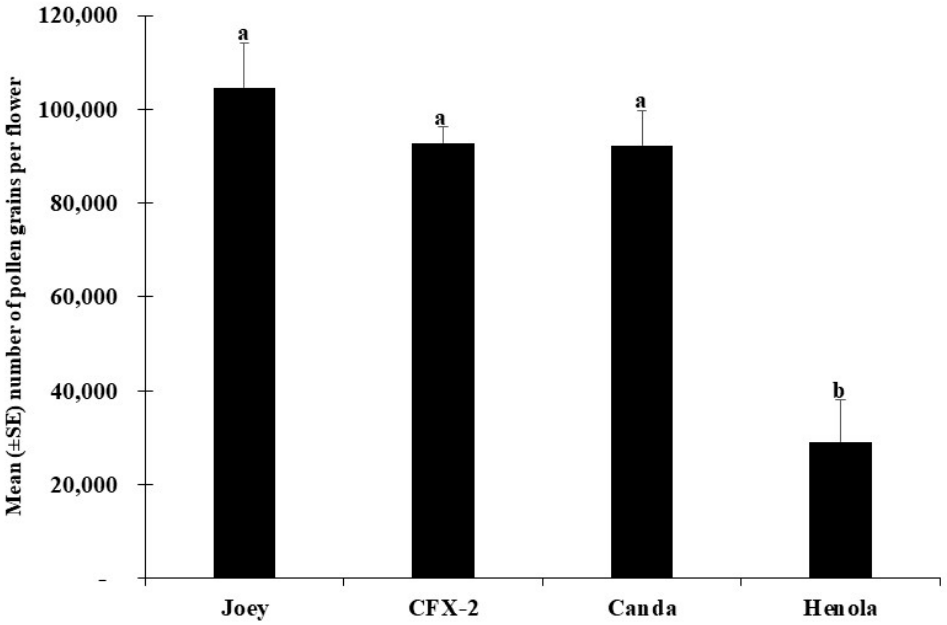

Figure 6. Mean total number of pollen grains (viable and non-viable) of the four hemp varieties. Means with the same letters are not significantly different (p > 0.05).

In this study, data from all hemp varieties combined showed a weak and non-significant correlation between anther length (r = 0.19, p = 0.098) and the number of pollen grains produced (Figure 7a). Similarly, there was a non-significant correlation between anther width (r = 0.16, p = 0.175) and the amount of pollen produced (Figure 7b). However, analysis of individual varieties showed a moderate albeit positive and significant correlation (r = 0.496; p = 0.043) between anther length and the number of pollen grains in Joey (Figure 8d). All the other three varieties showed a non-significant relationship:

(r = 0.356; p = 0.124) in Henola, (r = 0.188; p = 0.47) in Canda, and (r = 0.037; p = 0.877) in CFX-2 (Figure 8a–c).

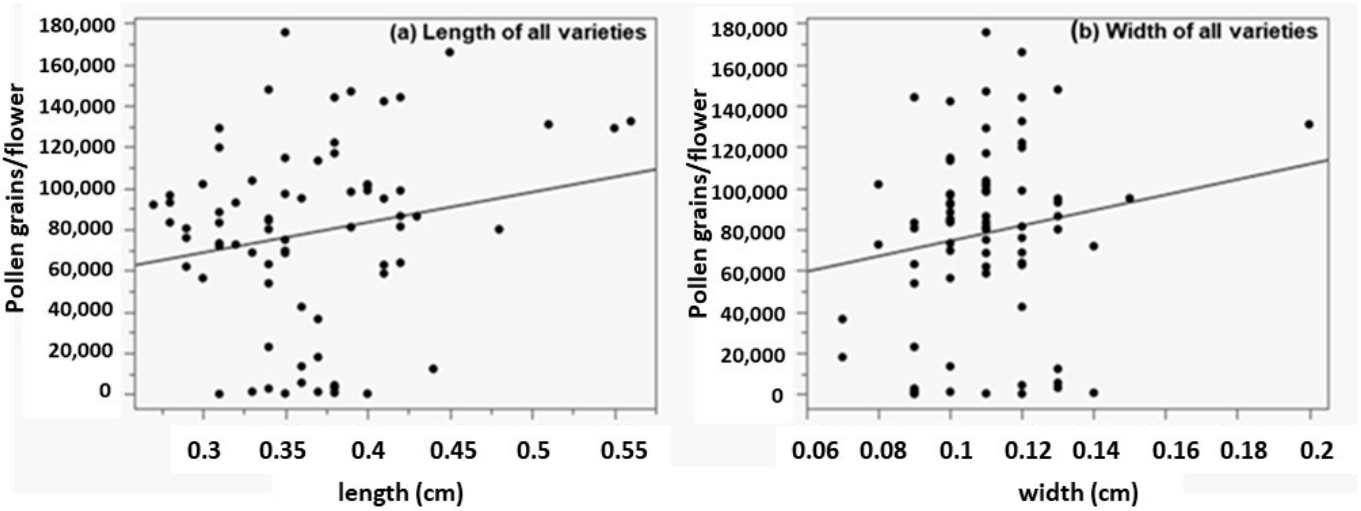

Figure 7. Linear regression from all four hemp varieties showing the relationship between anther length and pollen production: (**a**) anther length (y = 24,652.906 + 146,776.29x; n = 74; r^2 = 0.039; p = 0.0977), (**b**) anther width (y = 37,238.184 + 371,801.39x; n = 74; r^2 = 0.025; p = 0.175).

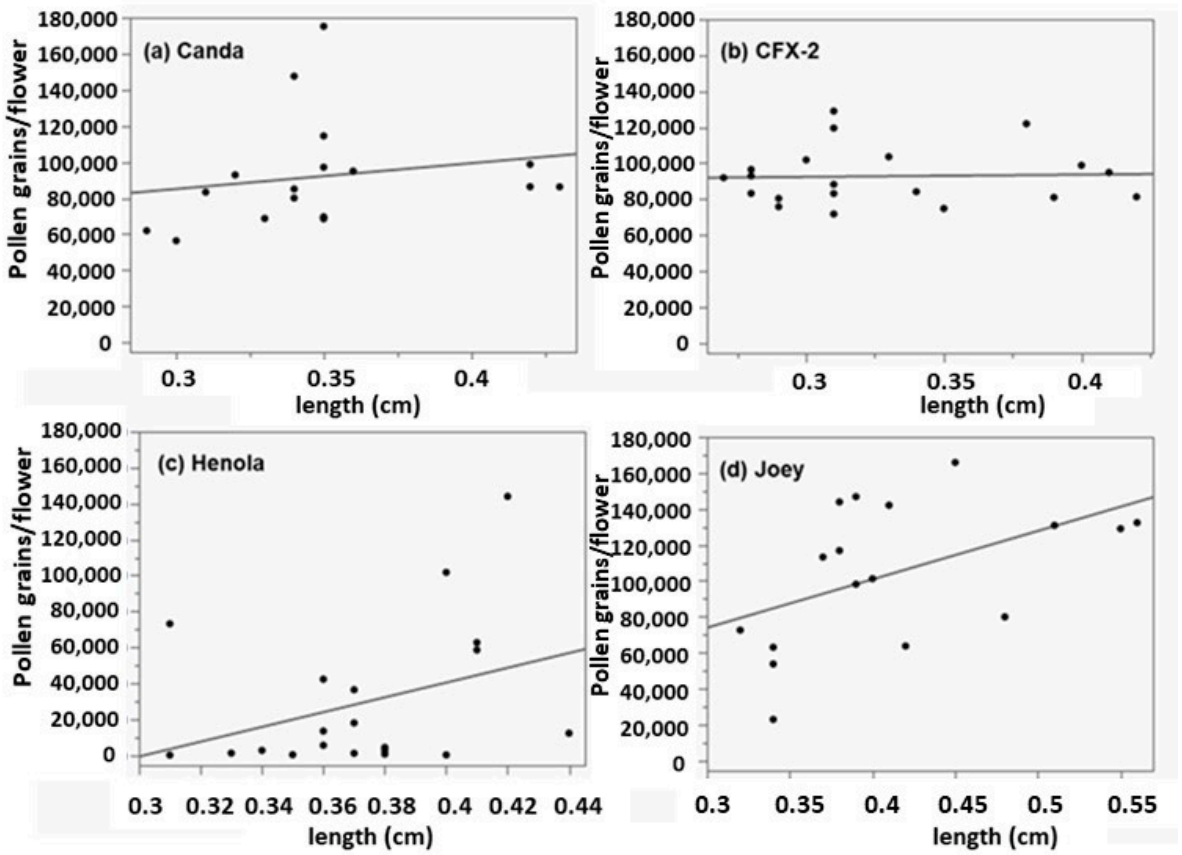

Figure 8. Linear regression showing the relationship between anther length and pollen production: (**a**) Canda (y = 42,228.932 + 142,950.78x; n = 17; r^2 = 0.035; p = 0.47), (**b**) CFX-2 (y = 88,729.339 + 12,468.785x; n = 20; r^2 = 0.0014; p = 0.877), (**c**) Henola (y = −124,002.9 + 411,238.3x; n = 20; r^2 = 0.127; p = 0.124), and (**d**) Joey (y = −6658.993 + 268,920.47x; n = 17; r^2 = 0.247; p = 0.043).

The relationship between anther width and the number of pollen grains was negative and non-significant (r = −0.312, p = 0.18) in Henola (Figure 9c). The relationship was

slightly positive and non-significant in Canda (r = 0.1940, p = 0.456), and CFX-2 (r = 0.081, p = 0.734) (Figure 9a,b) and moderate positive and non-significant in Joey (r^2 = 0.403, p = 0.109) (Figure 9d).

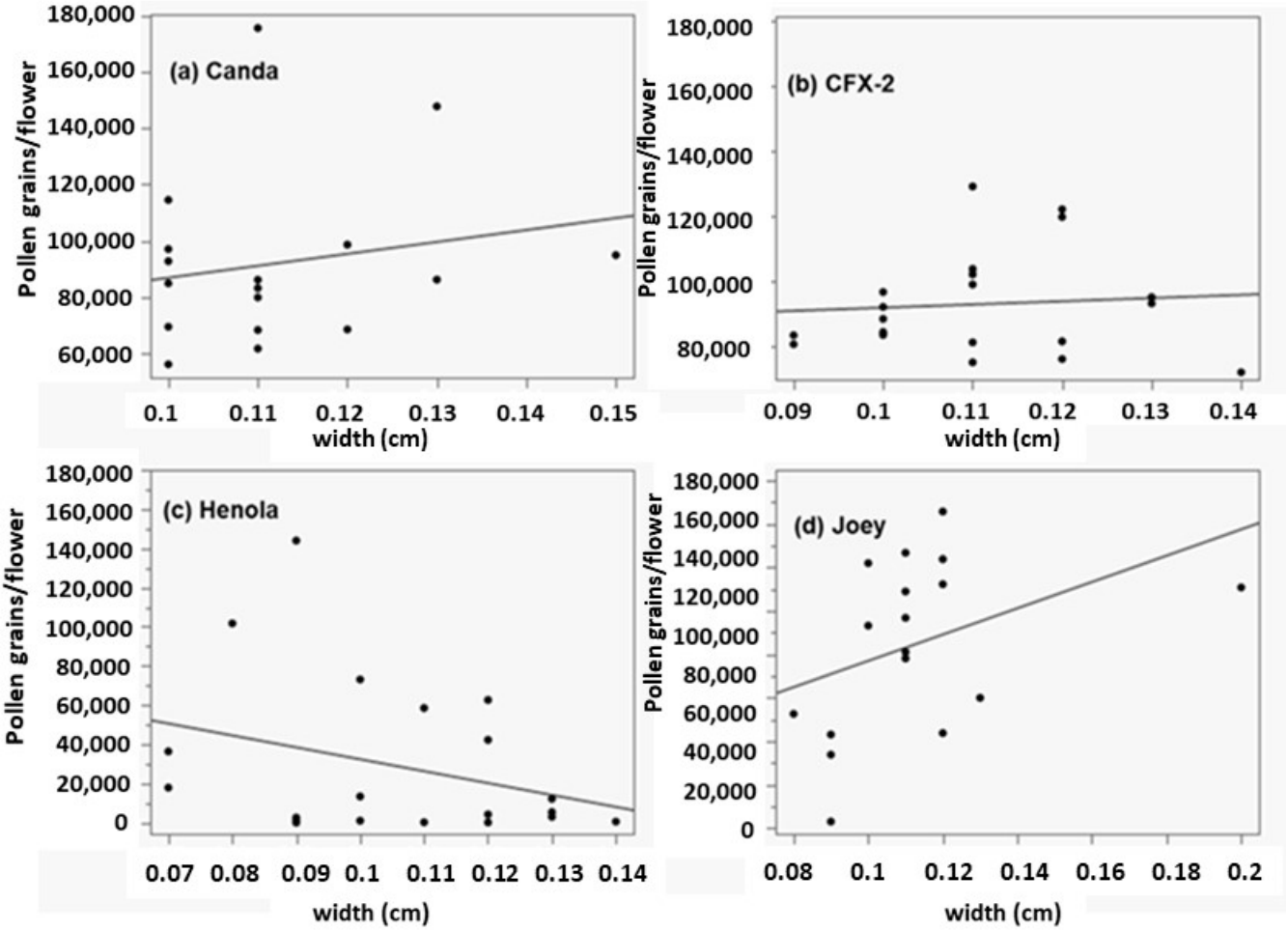

Figure 9. Linear regression showing the relationship between anther width and pollen production: (**a**) Canda (y = 44,789.822 + 422,524.62x; n = 17; r^2 = 0.038; p = 0.46), (**b**) CFX-2 (y = 81,976.305 + 97,682.84x; n = 20; r^2 = 0.007; p = 0.73), (**c**) Henola (y = 93,212.894 – 606,914.64x; n = 20; r^2 = 0.097; p = 0.181), and (**d**) Joey (y = 36,483.659 + 605,804.08x; n = 17; r^2 = 0.162; p = 0.109).

Figure 10 shows the Ampha Z32 output chart for pollen viability measure presented as a density scatter plot for each of the four hemp varieties, showing percent viable pollen to the top right and non-viable pollen to the top left.

On the output chart, the red vertical line (gate) separates viable from non-viable pollen grains. The data L: 3838 I 10.83% and R: 31,614 I 89.17% for Canda indicate that 3838 data points are on the left side (L) of the vertical gate, and this corresponds to 10.83% of all data points; similarly, 31,614 data points are on the right side (R) of the vertical gate, corresponding to 89.17% of all the data points.

After 24 h of storage at 4 °C, the percentage of viable pollen remained significantly ($F_{3,70}$ = 10.74, p < 0.0001) higher in Henola (Figure 11) compared to the others. After three weeks of storage at 4 °C, hemp pollen viability among the four varieties was not significantly different ($F_{3,75}$ = 1.98, p < 0.124) (Figure 11). However, compared to the viability after 24 h, the viability in this case was reduced but not significantly. There was a 2.9%, 3.4%, and 4.5% decrease for Canda, Joey, and CFX-2, respectively. Also, there was a significant decrease (17.7%) in viability for Henola after three weeks.

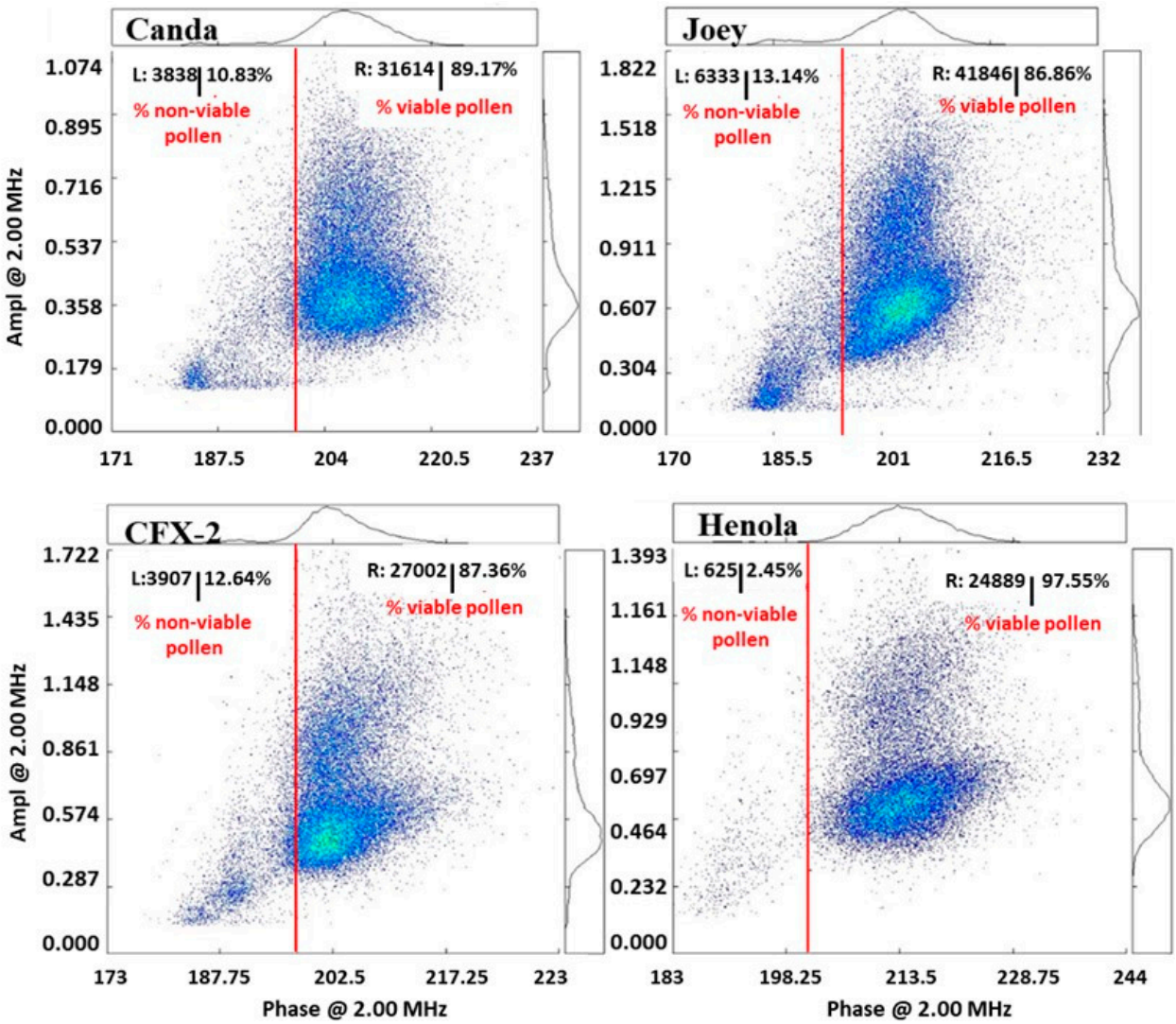

Figure 10. Ampha cytometer chart at 2.00 MHz for four hemp varieties.

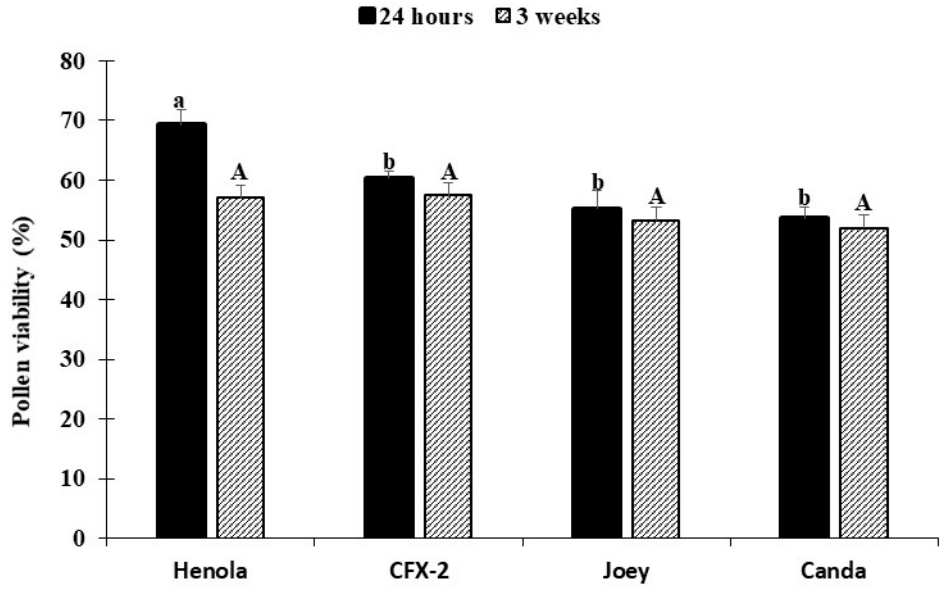

Figure 11. Percentage of viable pollen of four hemp varieties after storage for 24 h and three weeks. Lowercase letters are used to compare percentage viability at 24 h. Upper case letters are used to compare percentage viability after three weeks. Means with the same letters are not significantly different ($p > 0.05$).

The total number of viable pollen gains was computed from the percentage pollen viability and the total quantities of viable and non-viable pollen (Figures 6 and 11). There was a significant difference in the number of viable pollen grains produced ($F_{3,70}$ = 18.3 $p < 0.0001$) among the four hemp varieties. The lowest number was recorded in Henola (Figure 12).

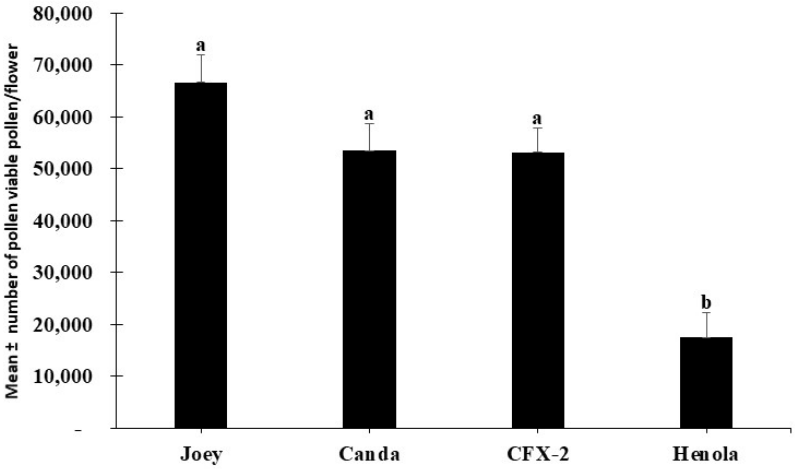

Figure 12. Amount of viable pollen of four hemp varieties at 24 h. Means with the same letters are not significantly different ($p > 0.05$).

Figure 13 shows the spherical shape of the hemp seed. Among the four varieties, there was a significant difference ($F_{3,116}$ = 72.9 $p < 0.0001$) in seed length (Figure 13), with Canda having the longest length (0.44 ±0.003 cm), followed by Joey (0.41 ± 0.005 cm) and CFX-2 (0.40 ± 0.0028 cm), and Henola recorded the shortest length (0.35 ± 0.005 cm). Similarly, there was a significant difference ($F_{3,116}$ = 24.5, $p < 0.000$) in seed width, with the shortest in Henola (0.24 ± 0.004 cm) and then Joey (0.27 ± 0.006 cm) and similar widths in CFX-2 (0.28 ± 0.003) and Canda (0.29 ± 0.003).

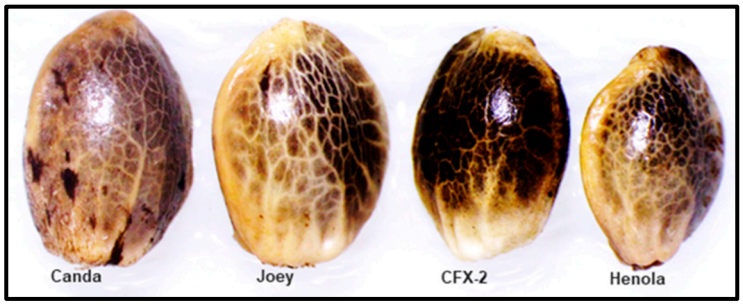

Figure 13. Spherical shape and variation in seed size among four hemp varieties.

4. Discussion

Hemp is primarily a dioecious plant with male and female flowers on separate plants, which is a unique characteristic, as only about 6% of plants exhibit this trait of having separate male and female plants [46]. However, with selective plant breeding, monoecious varieties have become more rampant over time [47]. From our findings, Canda and Joey hemp varieties previously described and purchased as monoecious varieties exhibited the characteristics of dioecious hemp plants with male and female flowers on separate plants (Figure 1A). The display of dioecious traits by Canda and Joey in this study, could have been because there was not enough selective plant breeding or maintenance, which resulted in a reversion to their natural dioecious state. Alternatively, the selection may not have been effective, as monoecious hemp varieties must undergo several generations of intensive

selective breeding to achieve genetic stability and standard performance variables. On the other hand, Henola exhibited the monoecious characteristics of having male and female flowers coexisting on the same plant. Henola is bred in Europe, primarily for higher grain yield. The International Hemp Company is North America's leading supplier of certified hemp seed and owns the exclusive license to Henola and Bialobrzeskie, both monoecious varieties [48].

Hemp has been legalized in the United States and its cultivation is expanding worldwide, providing opportunities for local and commercial growers. Knowledge of floral variation is a prerequisite for successful hemp production and breeding, as the presence of just one male plant in or around hemp cultivation for CBD can significantly decrease the crop's overall cannabinoid yield through pollination. The exclusion of male plants before flowering can be a challenge as the male and female plants are morphologically indistinguishable during the vegetative phase. However, this can be achieved at flowering because the male and female plants have distinct morphologies during this developmental stage. As observed and described in this study (Figure 1A), and in others including [41,49], the female plant is short and has dense leafy inflorescences (bushy appearance) compared to the male plant, which is slender and taller and characterized by hanging panicles with fewer leaves and branches. In addition, the male and female plants flower and age at different times. The male plants flower earlier, providing sufficient time for growers to remove male plants as they appear within their farms to prevent cross-pollination.

Generally, most insect-pollinated plants differ from wind-pollinated plants in that insect-pollinated plants produce nectar, have brightly colored flowers, and produce large sticky pollen grains, with some possessing feeding and pollinating anthers (anther dimorphism). The pollinating anthers have been reported to be longer and produce more viable pollen grains than the feeding anthers [50,51]. It has been argued that the longer anthers were to enable successful pollination, while the shorter anthers were to fulfill the demand as a food resource for pollinators. In contrast, wind-pollinated plants such as hemp do not produce nectar but produce abundant small lightweight pollen grains from their anthers [52]. All hemp varieties in this study had five anthers and did not exhibit anther dimorphism. Anther size is vital since it directly influences the amount of pollen produced, which can significantly influence pollination efficiency, reproductive success, and eventually crop yield [53]. In this study, Henola, Joey, and Canda varieties recorded the longest anther length; however, only Joey showed a positive and significant correlation between anther length and the number of pollen grains. Similarly, other studies [53–55] have documented a positive correlation between anther size and the quantity of pollen produced. These findings support the suggestion that increased anther length results in an increase in pollen with higher viability, as larger anthers typically have more space to produce and store a greater quantity of pollen grains [56]. In this study, Henola, the monoecious variety, produced the smallest number of pollen grains despite its long anther length; it was also the variety with the most viable pollen. Similar results of reduced pollen numbers in monoecious varieties have been reported elsewhere [32]. It has also been reported that pollinators preferred pollen from plants with smaller pollen grains and rarely collected pollen from those with larger grains, as bigger grains had relatively reduced nutritional content [57]. Our findings do not support this because the Joey variety had the same anther length and the same pollen size as Henola and was reported by [52] to be the most preferred by bees, despite expressing lower protein, amino acid, and saturated and monosaturated fatty acid content compared to Henola. It is worth noting that although bees collect pollen from hemp flowers, they are not attracted to the female flowers.

Knowledge of the viability of pollen is vital as it provides insight for species fitness and the survival of the next generation because of its direct connection to fertilization efficiency

and the number of offspring. Several factors such as environmental conditions and storage duration affect pollen viability. Mature flower buds in this study were harvested between 9:00 and 11:00 a.m., when pollinators were reported foraging in the field [52]. After 24 h of hemp pollen storage at 4 °C, the percentage of viable pollen remained significantly higher in Henola compared to the other varieties. The high pollen viability reported in this study for Henola may be associated with its high protein, amino acid, and saturated and monosaturated fatty acid contents, which we had previously reported and documented as factors contributing to pollinator attractiveness [52]. After three weeks of storage at 4 °C there was a 2.9%, 3.4%, 4.5%, and 17.7% decrease for Canda, Joey, CFX-2, and Henola, respectively. Our findings indicate that storing hemp pollen at 4 °C for 24 h may significantly retain the lifespan and germination potential of pollen of some hemp varieties. A storage temperature of 4 °C is suitable for the short-term storage (three weeks from harvesting) of hemp pollen to maintain a range from 52 to 58% viability. According to [58], CFX-2 pollen samples tested via in vitro germination right after collection had an average viability of 38.69%, and pollen stored at −4 °C did not germinate regardless of storage time. In addition, pollen stored at room temperature rapidly declined in viability, reaching 0% germination two weeks after anther dehiscence. The authors suggest pollen viability to be more sensitive to environmental degradation.

Studies investigating pollen viability across different plant species vary depending on storage duration and temperature. Hazelnut (*Corylus avellana*), and switchgrass (*Panicum virgatum* L.) are both perennial and wind-pollinated plants with monoecious flowers. In the former, 30 °C was the ideal temperature for high pollen viability (>40–50%) for three weeks of storage [59], while for switchgrass pollen viability decreased rapidly under sunny conditions, with a complete loss of viability in 20 min [60]. Under cloudy conditions with cooler temperatures, there was a complete loss of pollen viability at approximately 150 min. Variation in pollen viability was also reported for perennial and insect-pollinated flowering plants. For example, high pollen viability was reported from herbaceous peony (*Paeonia lactiflora*) stored at 4 °C for up to one week [61]. The pollen viability of rain lily (*Zephyranthes* sp.) decreased progressively from morning to evening and storage at 5 °C maintained viability for 4–8 days [62]. The viability of pollen from almond (*Prunus dulci*) cultivars did not significantly decrease after two months of storage at 4 °C [63]. These examples underline the variability in pollen viability under different environmental and storage conditions.

Usually, the number of pollen grains produced is greater when compared to the amount of viable pollen, since only a fraction of the total pollen grains produced are capable of fertilizing an ovule. Pollen dispersal from one plant to another translates into gene flow, leading to the development of mature seeds. Therefore, any factor that negatively affects pollen dispersal and pollination will decrease the chances of gene flow occurring. Generally, anemophilous plants produce copious amounts of pollen; for example, a single hemp flower can generate thousands of pollen grains [64], and hemp pollen can travel long distances by wind. The abundant production offsets the low efficiency of wind pollination [53] and ensures successful pollination since some pollen is dispersed to the surrounding environment. All four hemp varieties in this study showed a pollen size within the range of 22.04 µm to 27.83 µm, similar to other reports [65]. It is important to note that pollen viability and pollen size may have a direct impact on pollen dispersal, as small, lightweight pollen is easily dispersed [66] and spread over long distances by wind. In this study, Joey and Henola recorded the largest pollen size and Canda the smallest. It can be speculated that pollen from the latter may be dispersed further and more efficiently by wind. Since it may only require a single pollen grain to fertilize a female flower, the small pollen size in addition to more pollen produced by Canda and the high viability of Henola

pollen would likely increase the chances of fertilization and cross-pollination of their pollen with other hemp plants; this is undesirable in the cultivation of hemp for CBD production. In the United States, there is no national mandate for hemp growers to maintain buffer zones to mitigate cross-pollination; however, it is required in some jurisdictions [67]. In Europe and Canada, a standard buffer zone of 5 km is required [32]. However, according to other published reports, this was never validated experimentally and may have been the result of collective empirical observations [32]. In addition, research has shown that hemp pollen can travel much further than 5 km. For instance, the occurrence of hemp pollen from North Africa has been recorded in southwestern Europe, a distance of several thousand kilometers [68]. Other crops such as maize, with a relatively larger pollen size (between 80 and 125 µm) [69] compared to hemp pollen (22 to 28 µm), were transported over distances as far as 70 km. In Germany, regulations mandate 0.15 km buffer zones adjacent to conventional maize fields and 0.3 km adjacent to organic fields [70]. These recommended buffer zones may be effective because the amount of pollen transported decreases logarithmically with increasing distance from the source [71]. Therefore, the risk of cross-pollination could be insignificant beyond the buffer zone from a pollen source. However, further research is needed to determine buffer zone distances for hemp, as this might vary with variety or the botanical classification of the variety.

The seeds of all four hemp varieties were spheroidal in shape, with seed length and width within the range reported for other hemp varieties [72]. Henola is a monoecious and dual-purpose variety bred for fiber and seed production, compared to CFX-2, Canda, and Joey, which are dioecious and grown predominantly for grain [48,73]. Ineffective fertilization and lower seed set and productivity have been linked to a lack of viable pollen [74]. Some studies have documented the seed yield of Henola to average around 3.25 t/ha [75,76], a value higher than the average grain yield of 2.21 t/ha reported for CFX-2, Canda, and Joey [77], and also a higher Henola seed yield compared to other varieties [78]. This could be because pollination effectiveness and the resultant seed yield are due to increased pollen viability rather than increased pollen quantity [74,79]. Despite its small seed size, the lipid content, palmitic acid (C16:0), and the major fatty acid linolenic acid (C18:3 n3) were higher in Henola seed compared to the seeds of two monoecious (Futura 75 and Futura 83) hemp varieties [80]. Overall, seed metrics do not appear to be the primary target in variety selection by growers. Chemical composition and pollination efficiency may be the most important traits.

5. Conclusions

Based on the results from this study, it can be concluded that even though hemp has been bred for monoecious varieties because they produce higher yields, these varieties could revert to their natural dioecious state. The findings highlight the existence of variations in the floral morphology of hemp varieties that can influence the quantity and quality of pollen produced. These attributes can significantly influence pollen viability, dispersal, and pollination efficiency. For instance, the small pollen size, in addition to more pollen produced by Canda, a dioecious variety, and the high pollen viability of the monoecious variety Henola, could increase the chances of fertilization and cross-pollination. In addition, pollen viability remained significantly higher in Henola compared to the other varieties after 24 h of storing at 4 °C. Overall, these findings provide information for growers and researchers on hemp breeding and cultivation practices that may contribute to the prevention of cross-pollination.

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