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Comparative analysis of natural and supplemental pollination through pollen suspension on neem (*Azadirachta indica* A. Juss) fruit set and morphological characteristics

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Abstract

A supplemental pollination system is crucial when natural pollinators are unavailable or unreliable, as alternative management strategies are necessary to ensure adequate pollination in pollination-dependent trees. Supplemental pollination controls pollen quantity, timing and frequency, resulting in increased yields, improved fruit quality and reduced fruit abortion. In a comparative study of pollination methods, hand pollination using a brush yielded the highest fertilization rate (5.54 ± 0.03 %), followed by a 1 % pollen mixture with 0.2 M basal sucrose solution and 0.01 % Gelrite (5.09 ± 0.21 %). A mixture of 1 % pollen grains with 10 ppm boric acid produced a fertilization rate of 4.75 ± 0.14 %. Hand pollination resulted in the highest fruit production, with 313 ± 43 fruits, while the sucrose solution treatment yielded 287 ± 8 fruits and the boric acid treatment produced 273 ± 12 fruits. In contrast, closed pollination recorded the lowest yield, with only 14 ± 7 fruits. Morphological assessments of neem fruits and seeds revealed that the boric acid treatment (1 % pollen grains and 10 ppm boric acid) achieved the best results, producing the most significant fruits and seeds. Conversely, the sucrose solution and Gelrite treatment showed the lowest values for fruit weight and fruit-to-seed ratio, highlighting the effectiveness of boric acid in enhancing neem fruit and seed morphology.

Keywords

fertilization rate; fruit morphology; neem; pollen suspension; supplemental pollination

Introduction

Neem (*Azadirachta indica* A. Juss), the Indian lilac or Margosa tree, is an evergreen tree native to the Indian subcontinent and South Asia (1). Due to its diverse applications in agriculture, medicine and industry, this species has garnered significant attention (2). The global seed extract market, estimated at over $700 million in 2020, is projected to reach nearly $1.5 billion by 2027, driven by increasing demand for natural and organic ingredients (3). As the largest producer, India significantly contributes to global essential seeds production, reaching approximately 2.5 million barrels by 2020 (4). This growing economic importance highlights the need to optimize neem crop and seed production. Neems' natural regeneration occurs through seeds and root-suckers (1). However, the species exhibits limited natural fecundity due to outbreeding with varying degrees of self-incompatibility (2). Poor fruit set in neem has been attributed to limited factors such as limited population distribution, inadequate pollen and insufficient pollinators (5, 6, 7). Characterized by protandry, neem is an ambophilous species, relying on wind and insects for pollination (8). Its self-incompatibility confirms that neem requires cross-pollination. Notably, natural fruit set in neem is low, at approximately 5 %, due to improper pollen grain landing, pollen grains, low pollen-ovule ratio and pollen wastage. These challenges underscore the potential benefits of supplemental pollination strategies in neem cultivation (9).

Supplemental pollination systems are particularly valuable when natural pollinators are scarce or unreliable, necessitating alternative management solutions to ensure sufficient pollination of pollinator-dependent trees (8). Research revealed that natural pollination failures affected 50 % of fruit yield, while pollinator proximity influenced 8 % (10). Furthermore, the reduction in skewed sex ratio accounted for 10 % of fruit yield. These factors highlight the importance of supplemental pollination to control pollen quantity, pollination timing and frequency, ultimately increasing yields and fruit quality while reducing fruit abortion. Approximately 75 % to 87.5 % of plant communities rely on wind and insect pollination, respectively (11). Pollination limitation occurs when pollen quality or quantity is inadequate or when pollination is entirely absent. Factors contributing to pollen limitation include random fluctuations in pollen receipt, decreased pollinator populations, invasion of biological agents and changes in land use. Pollination limitation is documented in various crops worldwide (12, 13).

In supplemental pollination, pollen grains are manually or mechanically administered onto the flowers' pistil when pollinators are deficient. Supplemental pollination is also referred to as assisted, artificial, controlled, or manual pollination, depending on the context and purpose (14). This method is considered an exclusive option when natural pollination is insufficient (15). In plant breeding, supplemental pollination enables complete control over parentage (16). The increased reproduction success rate is achieved by depositing higher quality pollen grains (i.e., viable, conspecific, compatible) onto the respective stigma. Given the economic importance of neem and the challenges associated with its natural pollination, this study investigates the potential benefits of supplemental pollination in neem cultivation. By addressing issues such as improper pollen landing, infertile pollen grains, low pollen-ovule ratio and pollen wastage, we seek to improve fruit sets and overall productivity in neem orchards.

Materials and Methods

Location

The experiment was carried out on 100 % flowering neem trees in a 13-year-old neem plantation at Coromandel International Private Limited, Pathamadai, Tirunelveli (8° 38' N, 77° 35' E, 47 m MSL, with an annual rainfall of 680 mm) during 2023. The plantation followed a 4 × 4 m spacing pattern and employed drip irrigated. A randomized block design comprised three blocks of neem trees and seven treatments. The different pollen suspension combinations are presented in Table 1.

Determination of different pollination methods

Bulk pollen was extracted from neem flower anthers using the method described (17). The experiment evaluated three pollination methods: natural, closed and supplemental. The study consisted of 10 treatments with 3 replications. In each replication, 10 trees were pollinated. For natural pollination, hermaphroditic flowers were left intact, allowing honeybees and wind to pollinate them. For closed pollination, buds were bagged without emasculation one day before anthesis. On the day of anthesis, the tied bags were gently shaken to facilitate self-pollination. Supplemental pollination was performed during anthesis (5:00-7:00 am) using pollen-suspended liquids (18).

Determination of fruit set percentage

Fruit set (%) was measured by counting the number of fruits four weeks after pollination (2).

Variations in fruit and seed attributes

The seed and fruit morphology were studied by identifying and tagging twenty-five twigs in eight canopy directions and all the parameters were recorded (2).

Statistical analysis

The analysis used R software version 4.4.0 (ggplot2 package) for Windows. Results were expressed as mean ± standard deviations for three replications (n=30/treatment). Significant differences between treatments were estimated using Duncans' multiple-range tests, with a statistical significance level of $P<0.05$.

Results

Fertilization percentages

Fertilization percentages differed significantly among treatments ($p < 0.05$). Hand pollination with a brush yielded the highest rate (5.54 ± 0.03 %), followed by pollen mixture with sucrose solution and Gelrite (5.09 ± 0.21 %) and pollen with boric acid (4.75 ± 0.14 %). Open pollination resulted in a 3.22 ± 0.17 % fertilization rate, while closed pollination had the lowest rate (0.24 ± 0.05 %) (Table 2).

Table 1. Different pollination methods

Treatment No.	Pollination methods
T_1	Pollen grains (1 %) + Boron (Boric acid 10 ppm)
T_2	Pollen grains (1 %) + 0.2 M Basal Sucrose Solution
T_3	Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Gelrite (0.01w/v)
T_4	Pollen grains (1 %) + 0.2 M Basal Sucrose Solution +Gelatin (1w/v)
T_5	Pollen grains (1 %) + 0.2 M Basal Sucrose Solution +Sago powder (1w/v)
T_6	Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Acacia gum
T_7	Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Agar (1w/v)
T_8	Hand Pollination (Brush)
T_9	Closed pollination
T_{10}	Open pollination (Control)

Number of flowers and fruits

The number of treated flowers ranged from 5503 to 5951 across treatments. Fruit production varied significantly, with hand pollination yielding the most fruits (313 ± 43), followed by Pollen mixture with sucrose solution and Gelrite (287 ± 8) and pollen with boric acid (273 ± 12). Closed pollination produced the fewest fruits (14 ± 7) (Table 2).

Fruit morphology

Statistical analysis revealed significant differences ($p<0.05$) in fruit morphology traits among treatments (Table 3). The Pollen grains (1 %) + Boric acid (10 ppm) treatment and open pollination had the longest fruit length (19.9 ± 1.23 mm), while the shortest length (12.0 ± 1.1 mm) was found in the sucrose solution and Gelrite treatment. The boric acid treatment also had the widest fruit (13.6 ± 0.2 mm), similar to open pollination. The Acacia gum treatment had the lowest fruit width (8.6 ± 0.4 mm). The 100-fruit weight was highest in the boric acid treatment (134.6 ± 16 g) and open pollination (132.7 ± 11 g), with the lowest value (102.7 ± 13 g) in the Gelrite treatment. The highest fruit-to-seed ratio (7.2 ± 1.29) was found in the Agar treatment, while the lowest (4.6 ± 1.44) occurred in the Gelrite treatment.

Seed morphology

Statistical analysis revealed significant differences ($p<0.05$) in seed morphology and kernel traits among treatments (Table 4). The pollen grains (1 %) + Boric acid (10 ppm) treatment produced the longest seed length (15.33 ± 1.4 mm) and widest seeds (9.24 ± 1.2 mm), outperforming all

Table 2. Effect of different pollination methods on neem fruiting

Treatment	No of flower treated (r=10)	No of fruiting (r=10)	Fertilization percentage
Pollen grains (1 %) + Boron (Boric acid 10 ppm)	5748	273 ± 12 ^{b}	4.75 ± 0.14 ^{b}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution	5503	136 ± 31 ^{e}	2.47 ± 0.01 ^{e}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Gelrite (0.01w/v)	5643	287 ± 08 ^{ab}	5.09 ± 0.21 ^{ab}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Gelatin (1w/v)	5754	210 ± 27 ^{c}	3.65 ± 0.11 ^{c}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Sago powder (1w/v)	5864	199 ± 30 ^{d}	3.39 ± 0.04 ^{d}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Acacia gum	5698	154 ± 11 ^{de}	2.70 ± 0.02 ^{de}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Agar (1w/v)	5865	232 ± 29 ^{bc}	3.96 ± 0.10 ^{bc}
Hand Pollination (Brush)	5645	313 ± 43 ^{a}	5.54 ± 0.03 ^{a}
Closed pollination	5951	14 ± 07 ^{e}	0.24 ± 0.05 ^{e}
Open pollination (Control)	5690	183 ± 16 ^{cd}	3.22 ± 0.17 ^{cd}
Mean			**3.51**
SED			**0.481**
CD (0.05 %)			**1.012**

Results are expressed as mean ± SEM (n = 30). Values in the same row with different letters showed statistically significant differences ($P < 0.05$) according to Duncan test

Table 3. Effect of different pollination methods on neem fruit morphology

Treatments	Fruit Length (mm)	Fruit Width (mm)	100 Fruit Weight (g)	Fruit to seed Ratio
Pollen grains (1 %) + Boron (Boric acid 10 ppm)	19.9 ± 1.23^{a}	13.6 ± 0.2^{a}	134.6 ± 16^{a}	6.9 ± 1.09^{ab}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution	19.3 ± 1.21^{b}	13.3 ± 0.4^{ab}	107.0 ± 29^{e}	5.9 ± 1.32^{d}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Gelrite (0.01w/v)	12.0 ± 1.1^{f}	10.1 ± 0.8^{d}	102.7 ± 13^{f}	4.6 ± 1.44^{e}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Gelatin (1w/v)	19.0 ± 1.43^{b}	10.6 ± 0.9^{c}	119.0 ± 24^{c}	6.0 ± 1.57^{c}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Sago powder (1w/v)	17.6 ± 1.55^{c}	10.0 ± 0.2^{c}	133.3 ± 25^{a}	6.9 ± 1.98^{ab}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Acacia gum	13.0 ± 1.62^{e}	8.6 ± 0.4^{e}	105.0 ± 27^{f}	5.5 ± 1.11^{d}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Agar (1w/v)	15.6 ± 1.18^{d}	8.7 ± 0.6d^{e}	122.3 ± 11^{b}	7.2 ± 1.29^{a}
Hand Pollination (Brush)	17.3 ± 1.11^{c}	10.57 ± 0.9^{c}	114.9 ± 13^{c}	6.2 ± 1.13^{bc}
Closed pollination	14.1 ± 1.20^{d}	10.1 ± 0.6^{d}	112.4 ± 28^{d}	4.4 ± 1.32^{e}
Open pollination (Control)	19.9 ± 1.21^{a}	13.6 ± 0.3^{ab}	132.7 ± 11^{ab}	7.0 ± 1.22^{a}
Mean	**16.76**	**10.898**	**118.39**	**6.06**
SED	**2.92**	**1.93**	**12.06**	**0.99**
CD (0.05 %)	**0.92**	**0.61**	**3.81**	**0.31**

Results are expressed as mean ± SEM (n = 15). Values in the same row with different letters showed statistically significant differences ($P < 0.05$) according to Duncan test.

Table 4. Effect of different pollination methods on neem seed morphology

Treatments	Seed Length (mm)	Seed Width (mm)	100 Seed Weight (g)	Kernel Weight (g)	Seed to kernel ratio
Pollen grains (1 %) + Boron (Boric acid 10 ppm)	15.33 ± 1.4^{a}	9.24 ± 1.2^{a}	23.14 ± 3.2^{a}	9.73 ± 1.1^{a}	3.33 ± 0.2^{a}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution	13.33 ± 1.5^{c}	6.33 ± 1.5^{c}	17.33 ± 2.5^{c}	7.53 ± 1.3^{c}	2.43 ± 0.5^{c}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Gelrite (0.01w/v)	12.99 ± 1.0^{cd}	7.41 ± 1.7^{cd}	18.85 ± 2.7^{cd}	8.22 ± 1.7^{cd}	2.63 ± 0.7^{cd}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Gelatin (1w/v)	11.12 ± 1.8^{d}	7.01 ± 1.1^{d}	17.33 ± 1.8^{d}	7.21 ± 1.7^{d}	2.25 ± 0.9^{f}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Sago powder (1w/v)	13.00 ± 1.4^{bc}	7.00 ± 1.0^{bc}	19.33 ± 2.3^{bc}	8.16 ± 1.9^{bc}	2.43 ± 0.8b^{c}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Acacia gum	10.33 ± 1.1^{e}	6.66 ± 1.8^{e}	17.66 ± 2.7^{e}	7.46 ± 1.0^{e}	2.41 ± 0.6^{e}
Pollen grains (1 %) + 0.2 M Basal Sucrose Solution + Agar (1w/v)	13.00 ± 1.2^{c}	6.00 ± 1.6^{f}	20.66 ± 3.1^{c}	8.80 ± 1.1^{c}	2.45 ± 0.4^{c}
Hand Pollination (Brush)	13.66 ± 1.7^{bc}	9.23 ± 1.3^{bc}	15.33 ± 1.0^{bc}	10.70 ±1.2^{bc}	3.10 ± 0.2b^{c}
Closed pollination	11.21 ± 1.5^{d}	7.32 ± 1.1^{d}	13.32 ± 2.0^{d}	8.98 ± 1.2^{d}	2.65 ± 0.1^{d}
Open pollination (Control)	14.80 ± 1.2^{b}	7.33 ± 1.1^{b}	21.67 ± 1.2^{b}	6.13 ± 1.6^{b}	2.57 ± 0.8^{b}
Mean	**12.87**	**7.35**	**18.14**	**8.29**	**2.62**
SED	**0.34**	**0.79**	**0.42**	**0.11**	**0.34**
CD (0.05 %)	**1.09**	**2.51**	**1.32**	**0.34**	**1.09**

Results are expressed as mean ± SEM (n = 30).Values in the same row with different letters showed statistically significant differences ($P< 0.05$) according to Duncan test.

other treatments. In contrast, the Acacia gum and Agar treatments had the shortest (10.33 ± 1.1 mm) and narrowest seed (6.00 ± 1.6 mm). The 100-seed weight was highest in the boric acid treatment (23.14 ± 3.2 g), followed by open pollination (21.67 ± 1.2 g) and lowest in closed pollination (13.32 ± 2.0 g). Hand pollination (brush) yielded the heaviest kernels (10.70 ± 1.2 g), while open pollination had the lightest kernel (6.13 ± 1.6 g). The seed-to-kernel ratio was highest in the Boric acid treatment (3.33 ± 0.2) and lowest in the Gelatin treatment (2.25 ± 0.9).

Discussion

Effect of different pollination methods in neem

Our study reveals that supplemental pollination is more effective than natural pollination in neem. The study indicates that Neem trees are obligate outbreeders, self-incompatible and reliant on xenogamous pollination. Autogamy, geitonogamy and apomixis are excluded as fertilization mechanisms. Natural pollination resulted in a 6.3 % fruit set, whereas hand and supplemental pollination showed slightly lower fruit sets (5.45 % and 5.09 %, respectively). This unexpected outcome suggests that while supplemental pollination can be adequate, other factors such as environmental conditions, pollination timing, or tree physiology may influence fruit set in Neem trees. The low pollen-to-ovule ratio in this study suggests that neem is a xenogamous species reliant on cross-pollination (19). Insects play a crucial role in pollinating Neem flowers, while wind pollination is only adequate in dense populations due to rapid pollen density decrease over short distances. Additionally, neem is completely self-incompatible. These findings align with research and are consistent with the obligate outbreeding pattern observed in the *Veratrum album*, further supporting our conclusions about neems' reproductive biology (18, 20).

Effect of supplemental pollination on neem fruit set

Hand pollination using a brush yields the highest fertilization percentage (5.54 ± 0.03 %), followed by the pollen mixture treatment with sucrose and Gelrite (5.09 ± 0.21 %) and the pollen treatment with boron (4.75 ± 0.14 %) (Fig. 1a). The superior efficiency of hand pollination can be attributed to precise pollen transfer, minimizing wastage and increasing fertilization likelihood. Studies have consistently demonstrated that hand pollination increases fertilization rates in several tree species, particularly those with low natural pollination success. In mango (*Mangifera indica*), manual pollen application improved fruit set by up to 30 % compared to natural pollination reported (21). In contrast, pollen mixtures with sucrose or boron may dilute pollen or affect viability, reducing fertilization efficiency. While sucrose and boron enhance pollen germination and tube growth, their efficacy depends on concentration and conditions, potentially explaining the lower fertilization rates in these treatments (21).

Sucrose and boron are critical in pollen germination and pollen tube elongation. Boron is essential for pollen viability, facilitating carbohydrate metabolism and cell wall development**.** Similarly, sucrose is an osmotic stabilizer, promoting pollen hydration and tube growth**.** However, the efficiency of these compounds depends on concentration, as excessive sucrose can lead to pollen desiccation and high boron levels may become toxic**,** reducing fertilization efficiency (22). These findings align with research in other species. For example, research indicates that Japanese pear fruiting was optimized using spray solutions containing xanthan gum with either pectin methylesterase (PME) or polygalacturonase (PG) (23). Similarly, research indicates supplemental pollination compared to natural pollination. Supplemental pollination has also improved fruit sets in other species (24). Research suggests that significant increases in fruit set in *Catamarca argentina* were found when pollen was manually collected and applied (25). Similar results have been reported in pistachio and custard apples, highlighting the effectiveness of supplemental pollination across various species(26, 27).

Seed and fruit morphology

The significant differences in fruit and seed morphology among treatments highlight the positive impact of supplemental pollination and nutrient additions, particularly Boric acid, on neems' reproductive traits (Fig. 1b-j). The treatment of pollen grains (1 %) + boric acid (10 ppm) consistently outperformed others regarding fruit length, width and seed dimensions. Borons' critical role in improving fruit set and seed quality is attributed to enhanced pollen tube growth, increased fertilization efficiency and improved nutrient transport (28). Boron facilitates carbohydrate transport and cell wall synthesis, explaining this treatments' increased fruit and seed size (29). Open pollination also performed well due to natural pollinators and favourable environmental conditions. The lowest fruit and seed traits were observed in treatments with basal sucrose solution and gel-based additives like Gelrite, Acacia gum and gelatine. These additives may have attributed pollen viability or nutrient absorption despite sucroses' benefits for hydration and viability (30). The combination with gels may have reduced pollen tube growth due to altered osmotic potential or impaired nutrient availability. Previous studies support these findings and demonstrate the adverse effects of high sucrose and additive concentrations on pollen performance and plant reproductive success (31).

The large fruit-to-seed ratio in the Agar treatment suggests a disproportionate fruit size increase relative to seed development. This may be due to the gel mediums' impact on nutrient distribution or delayed seed formation (32). This imbalance between fruit and seed development is consistent with findings in other species where altered media or nutrient treatments affect resource allocation. Hand-pollinated (brush) yielded the heaviest kernels, demonstrating the effectiveness of precise pollination techniques in enhancing seed filling and nutrient allocation. Controlled pollen application in hand

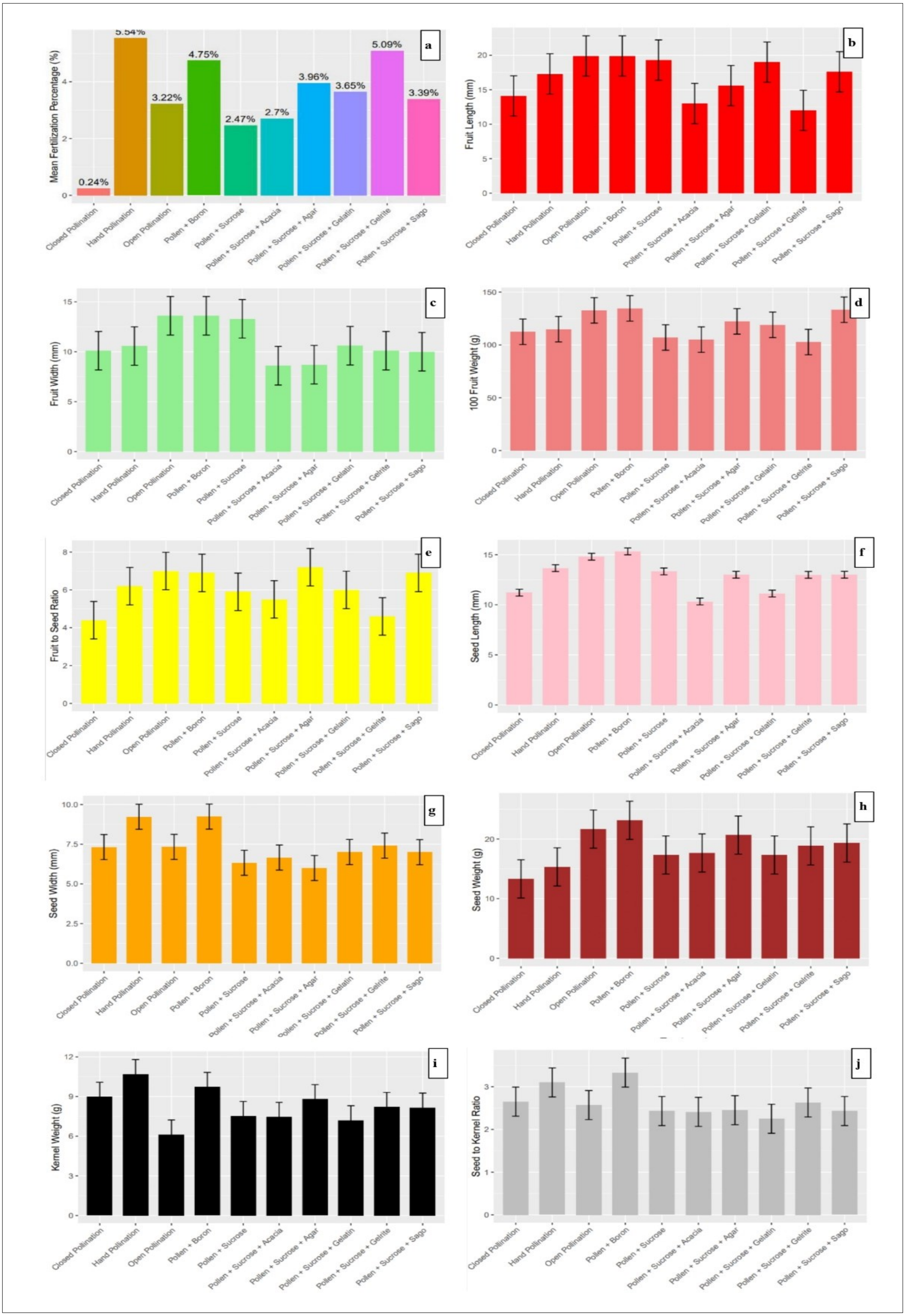

Fig. 1. Effect of different pollination on neem seed production.

pollination increases fertilization success by ensuring optimal pollen adhesion and distribution on stigmas (33). In contrast, open pollination resulted in lower kernel weights, likely due to natural variations in pollen quantity or quality caused by environmental factors. Despite this, open pollination performed well in other traits.

Conclusion

Boron significantly enhances fruit and seed morphology in neem. Boric acid treatment consistently outperformed others, highlighting its importance in reproductive success. However, sucrose solutions or gel-based media had mixed effects. Further research is needed to optimize pollination and nutrient application strategies for improved neem productivity.

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Authors' contributions

MM and KK contributed to the conceptualization of the study. MG, MM, KK and MT carried out a formal analysis. KK managed funding acquisition. MM, KK, MT, SV and PD developed the methodology. MM and KK undertook supervision and validation.

Compliance with ethical standards

Data Availability Statement: All data analyzed and generated during this study are included in this article.

Competing Interests: All the authors declare no conflict of interest.

Ethical issues: None

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