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Evaluating the precision of staining methods in determining pollen viability and *in vitro* pollen germination in two endemic varieties of *Humboldtia brunonis* Wall. (Fabaceae-Detarioideae)

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Abstract

The genus *Humboldtia*, restricted to the Western Ghats of India and Sri Lanka, is mainly propagated by the seeds. However, the seed set is very low in a few species, which includes *Humboldtia brunonis* and its varieties. One important factor that restricts seed formation may be the viability of the pollen grain employed in cross-pollination operations. Considering this, the present study aims to report the pollen viability and pollen germination of *H. brunonis* var. *brunonis* and *H. brunonis* var. *raktapushpa*. Pollen viability was determined by Fluorochromatic reaction test (FCR) and 3, 3'- Diamino benzidine test (DAB) based on the pollen and *in vitro* pollen germination was determined in different sucrose concentrations of Brewbaker and Kwack (BK) medium. The pollen viability test using DAB revealed that more than 73 % of pollen was viable. However, 68.6 % of pollen in *H. brunonis* var. *brunonis* and 67.8 % of pollen in *H. brunonis* var. *raktapushpa* showed viability under FCR test. More interestingly, at the same time, only 67.27 % of pollen germination was exhibited in the BK medium. Pollen viability by FCR and *in vitro* pollen germination significantly correlates with the results; on the contrary, *in vitro* germination is not correlated with the DAB test. Along with the *in vitro* staining and *in vitro* pollen germination, detailed macromorphology and pollen morphology of the selected taxa are given herewith.

Keywords

Brewbaker and Kwack medium; *Humboldtia* ; Kakkayam; pollen viability

Introduction

The genus *Humboldtia* Vahl of the family Fabaceae was initially named *Batschia* by Martin Henrichsen Vahl (1), a Danish-Norwegian botanist. However, considering the phytogeographical importance of the genus, Vahl himself renamed *Batschia* into *Humboldtia* to honour Alexander von Humboldt- the father of phytogeography. Different species of the same genus have also been named after Vahl by different botanists (2–8). The genus *Humboldtia* comprises 8 species and 2 varieties of medium-sized sub-canopies characterized by its white to pink and red flowers, which are restricted and isolated in the tropical evergreen forest areas of the western coasts of the Indian peninsula and Sri Lanka (9, 10). *Humboldtia laurifolia* Vahl is endemic to Sri Lanka, and the remaining 7 species and 2 varieties are endemic to the Western Ghats of India (11). *H. laurifolia* & *H. brunonis* Wall., are considered major myrmecophytes of the genus. The plant provides both food and

brooding sites for ant species like *Vombisidris humboldticola* Zacharias & Rajan, *Technomyrmex albipes* Smith, and *Tapinoma indicum* Forel (12). A few species, like *H. vahliana, H. bourdillonii,* and *H. brunonis* were also used as traditional medicine for treating urinary infections, stomach aches, and diarrhea (9).

Male gametophytes, or pollen grains, are transient, haploid generations in the life cycle of angiosperms. A crucial prerequisite for microevolution is that pollen competes with one another for the chance to fertilize ovules, which influences reproductive success differentially (13, 14). Viability is not a measure of how many pollen grains may germinate under a certain set of conditions but rather of how many pollen grains are capable of engaging in reproduction under any circumstance since a damaged cytoplasm signifies senescence and gamete death (15, 16). Comparatively, the percentage of pollen that germinates under controlled conditions is measured to determine fertility (17, 18). Rehydrating the apertures and allowing the pollen tube to protrude towards the ovule is necessary for the successful germination of a pollen grain (19, 20). The pollen tube then transfers the male gamete to the female ovule, where it produces a zygote. The percentage of pollen that is able to reproduce under controlled conditions is measured by both pollen grain viability and fertility (21, 22).

In the domain of *in vitro* staining, many viable pollen grains exhibit distinct features that can vary depending on the specific staining method used. In most cases, the cytoplasm of the pollen grain reacts with the stain and turns into vibrant colours. The pollen showing such characteristic colour is called fertile pollen grains, whereas the non-viable pollen does not show any reaction to the stain. Hence, pollen viability and fertility can also be measured by *in vitro* staining; regardless the emergence of pollen tubes through germ pores will happen only during favorable conditions. Infertility can be explained by studying the germination of pollen and growth of pollen tube in a medium (23). Osmotic pressure, the presence of hydrating agents, and the concentration of inorganic compounds in the germinating medium may also affect the effective germination. However, effective pollen germination always depends on the counter effect of the stigma (24).

Pollen germination is a complex biological process that requires more new cells for the initiation and elongation of pollen tubes (25–27). Cell division is the key to provide the new cells at the region of tube initiation. However, in this region, the osmotic potential of the surrounding cells is preferably very important for the progression of pollen tube germination. The better osmotic potential will provide a niche for each and every viable pollen to initiate and develop the tube (28, 29). Pollen germination is an energy-consuming process; it requires the energy currency in the form of ATP to switch on all the biological activities behind germination. During the *in vitro* studies of pollen germination, sucrose was provided as the major carbon source for aerobic respiration, and the adequate amount of sucrose also maintained the preferable osmotic pressure of the medium.

The present study deals with *H. brunonis* var. *brunonis*, which is found in the forests of Kerala and Karnataka with more than 5 populations, and *H. brunonis* var. *raktapushpa* Udayan, Tushar & S. George, which is represented only from the type locality. Both the varieties have lower fruit sets, so the main purpose of the present investigation is to discover the suitable sucrose concentration for the pollen grain germination of *H. brunonis* var. *brunonis* and *H. brunonis* var. *raktapushpa,* in BK medium (25) containing different concentration of sucrose. The study also includes the comparison between the results of *in vitro* pollen germination and the viability determined by the *in vitro* staining techniques such as the FCR (30, 31) and DAB test (32).

Materials and Methods

Study Area

Based on the literature and local Flora (7, 9), the population of the selected taxa for this study was identified and carried out for further investigation from Thamarassery and Kakkayam forest of Kozhikode district, Kerala, India. Both the forest areas are part of Malabar Wildlife Sanctuary, with the elevation range varying from 40 to 1500 M.A.S.L. Flowers of *H. brunonis.* var. *brunonis* Wall. (Fig. 1A) was collected from Thamarassery forest (N 11˚ 30'198" E 76˚ 01'677") with an altitude range from 300 to 500 M.A.S.L and *H. brunonis* var. *raktapushpa* Udayan, Tushar & S. George, (Fig. 1B) was collected from Kuttiady river basins near to Urakkuzhi waterfalls (N 11˚ 33'487" E 75˚ 54'103") at an altitude of 900 M.A.S.L of Kakkayam forest.

Field Study

Five reproductively mature trees were tagged and selected for the study of both varieties. This study was carried out with fresh flower buds collected from the selected populations of *H. brunonis* var. *brunonis* and *H. brunonis* var. *raktapushpa*. The inflorescence had 20–35 flowers in *H. brunonis* var. *brunonis* and 12–20 in *H. brunonis* var. *raktapushpa*. Flowering twigs with mature and immature flower buds were collected from the field before anthesis and transferred to polythene bags. The packed buds were then brought to the lab for further observation and pollen analysis.

Morphology

H. brunonis Wall. var. *brunonis*

Larger shrubs or small trees, 2–12 m tall; branchlets pubescent when young, glabrous at maturity. Stipules 1–2 × 0.25–0.5 cm, linear, prominently parallel-veined, glabrous; appendages broadly reniform, divergently veined, glabrous, glandular, persistent. Leaves 4-foliolate, up to 25 cm long; leaf rachis 3–5 mm long or almost sessile; rachis 2 cm long, obscurely winged, shallowly canaliculate above, dark brown tomentose; leaflets 10–20 × 5–8 cm, obovate or elliptic to narrowly elliptic, base unequal, acuminate at apex, veins prominently reticulate, glabrous. Inflorescence racemes, up to 15 cm long, axillary. Flowers 2–3 cm long, white, pink; pedicels 4–5 mm long, pilose, bracts 3 × 1.5 mm, ovate, acute, light brown pubescent without;

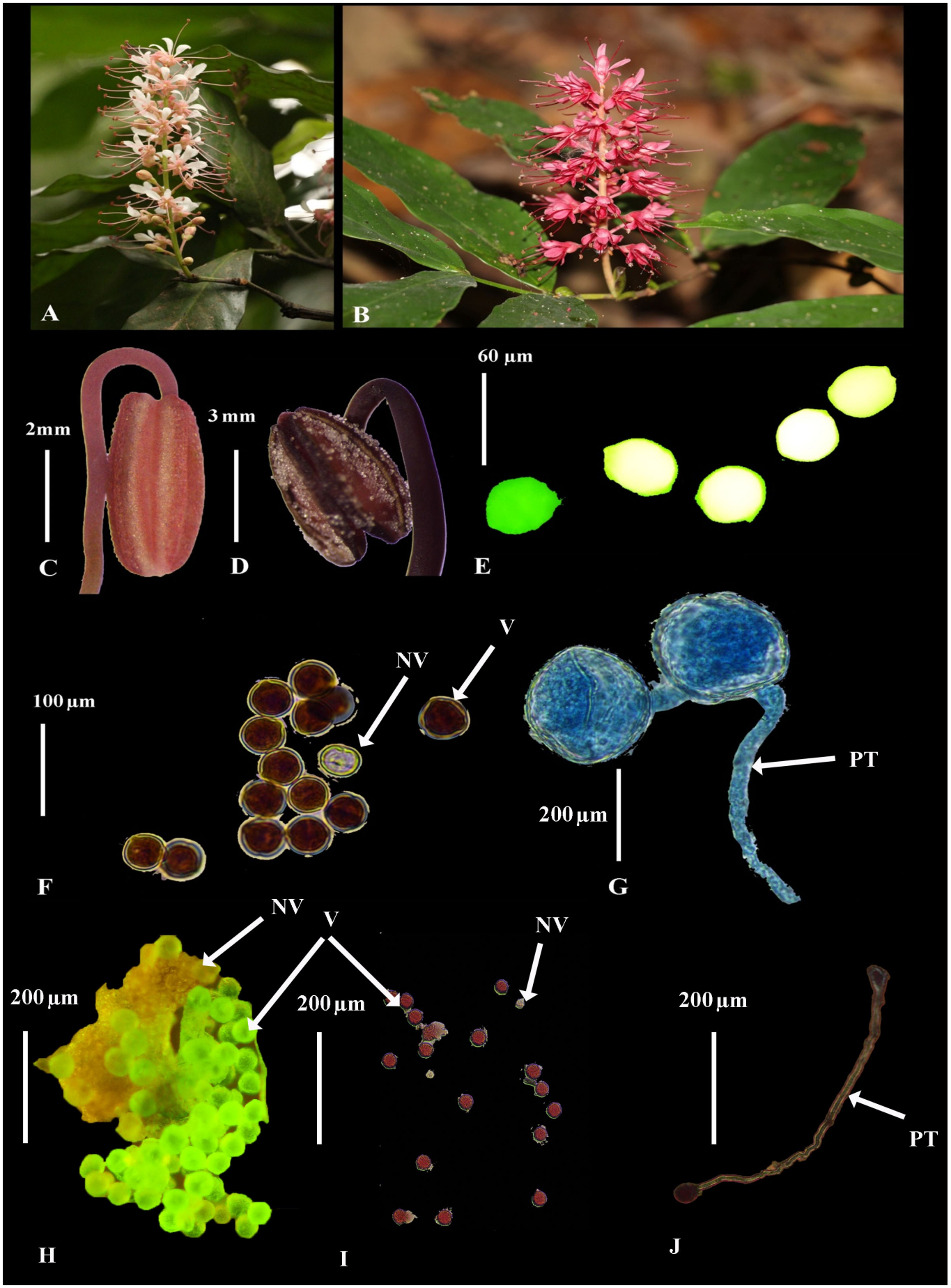

Fig. 1. Morphology, pollen viability and *in vitro* pollen germination of the varieties of *Humboldtia brunonis* Wall. **A, C, E, F & G:** *H. brunonis* Wall. var. *brunonis*. **A.** Flowering twig. **C.** A stamen before anthesis. **E.** Pollen viability by Fluorochromatic reaction test (FCR). **F.** Pollen viability by 3,3'- Diamino benzidine test (DAB). **G.** *In vitro* pollen germination stained with aniline blue. **B, D, H, I, J:** *H. brunonis* var. *raktapushpa* Udayan, Tushar & S. George: **B**. Flowering twig. **D.** A stamen on the day of anthesis. **H.** Pollen viability by Fluorochromatic reaction test (FCR). **I.** Pollen viability by 3,3'- Diamino benzidine test (DAB). **J.** *In vitro* pollen germination in 10 % sucrose. PT- Pollen Tube, V- Viable Pollen, NV- Non-viable Pollen.

bracteoles 2, connate below, 5 × 2.5 mm, obovate or obovate-oblong, obtuse at apex, brown pubescent on both surfaces. Calyx tube 8–10 mm long, brown pubescent; lobes 4, 4 × 1.5 mm, concave, imbricate, ovate, acute or obtuse at the tip, densely brown pubescent. Petals 3, white or pink, 1.5 cm long, ovate, shortly clawed, veined, glabrous. Stamens 5, deep pinkish, filaments 2 cm long, glabrous; anthers 2–3 mm long, oblong rounded at both ends. Ovary 5 mm long, obliquely linear, densely brown pubescent, 1–3 ovule; style 10 mm long, filiform, pilose at base; stigma capitate. Pods 5–8 × 2–4 cm, green when young, brown when mature, 1–2 seeds, round, ridged on the surface, brown (Fig. 1A & C).

H. brunonis var. *raktapushpa* Udayan, Tushar & S. George

Larger shrubs or small trees, 2–12 m tall; branchlets pubescent when young, glabrous at maturity. Stipules 1–2 × 0.25–0.5 cm, linear, prominently parallel-veined, glabrous; appendages broadly reniform, divergently veined, glabrous, glandular, persistent. Leaves 4-foliolate, up to 25 cm long; leaf rachis 3–5 mm long or almost sessile; rachis 2 cm long, obscurely winged, shallowly canaliculate above, dark brown tomentose; leaflets 10–20 × 5–8 cm, obovate or elliptic to narrowly elliptic, base unequal, acuminate at apex, veins prominently reticulate, glabrous. Inflorescence racemes, up to 15 cm long, axillary. Flowers 2–3 cm long, bright red; pedicels 4–5 mm long, pilose, bracts 3 × 1.5 mm, ovate, acute, light brown pubescent; bracteoles 2, connate below, 5 × 2.5 mm, obovate or obovate-oblong, obtuse at apex, brown pubescent on both surfaces. Calyx tube 8–10 mm long, brown pubescent; lobes 4, 4 × 1.5 mm, concave, imbricate, ovate, acute or obtuse at the tip, densely pubescent. Petals 3, bright red, 1.5 cm long, ovate, shortly clawed, veined, glabrous. Stamens 5, deep pinkish, filaments 2 cm long, glabrous; anthers 2–3 mm long, oblong rounded at both ends. Ovary 5 mm long, obliquely linear, densely brown pubescent, 1–2 ovule; style 10 mm long, filiform, pilose at base; stigma capitate. Pods 5–8 × 2–4 cm, green when young, brown when mature, 1–2 seeds, round, ridged on the surface, brown (Fig. 1B & D).

Pollen Morphology

Pollen grains tricolpate with elongated furrows on the surface; average grain size 33.73 × 35.69 µm, ellipsoidal pores, spherical to subprolate in equatorial view and angular in polar view, exine thickness 3.45 µm, rugulate ornamentation on the surface. In both the varieties of *Humboldtia brunonis,* the pollen does not show any significant variation.

Pollen Viability

To determine pollen viability, mature pollen was extracted from the flowers for 3 consecutive days (i.e., the previous day of flower opening, the day of flower opening, and the second day of flower opening). For each observation, anthers of 3 fresh flowers per inflorescence were obtained from 4 different inflorescences of 5 selected trees. The cytoplasm of each pollen grain will be both physiologically and enzymatically active when the pollen is viable. The rate of this activity can be measured and calculated mathematically with the help of *in vitro* staining techniques, *i.e*., FCR and DAB (Fig. 1E, F, H & I). These stains react with the peroxidase present in the pollen produced by themselves through effective biochemical activities. Thus, the effective or viable pollen will react with the dye and give the characteristic colours. Extracted pollen grains of both varieties were stained with FCR and DAB in different clear glass slides and carefully mounted with a cover slip. The observations on FCR were made using a Nikon digital camera attached to a Nikon H600L fluorescent microscope in the magnification of 10X under the green filter, and the observations of DAB test was made in the similar way which excluded the fluorescent filter (30, 31).

According to researchers (15), the FCR principally works on the plasmalemma within the vegetative cells. This method generally provides an excellent guide to potential germinability. The FCR test only takes up to 30 min. However, when it is exposed to light for a longer period of time, fluorescein will leak from the pollen grains and thus may be exposed to non-viable pollen. Hence, the duration of the light exposure and the concentration of the stain solution play a crucial role in finding the viability. In the FCR test, viable pollen grains incubated in the stain for 10 min in a dark humidity chamber will emit a bright yellow or yellow-green fluorescence under an epifluorescence microscope, whereas the non-viable pollen grains are faintly stained and do not emit any fluorescence or will show significantly reduced fluorescence. At the same time, in the DAB test, the viable pollen grains turned into a rust-brown colour and the non-viable did not absorb any stain.

In vitro Pollen Germination

Pollen grains from the fresh flowers were separated from the anthers and carefully placed in a petri dish containing BK medium (25) with different concentrations of sucrose (5 %, 10 %, 15 %, and 20 %). The pollen grains were exposed to the BK medium for 8 hrs. After the incubation, the pollen grains were stained with 1 % aniline blue (33), covered by a coverslip, and then observed under a light microscope (Fig. 1G & J). To determine the pollen germination rate, 50 pollen grains were observed in each replica of both varieties.

Statistical Analysis

The data obtained were analyzed using one-way ANOVA in GENSTAT® Version 9 (34). Least significant differences (LSD) were used to determine the significant difference between the stains.

Results and Discussion

Pollen viability was evaluated by FCR; the viable pollen shows ephemeral fluorescence activity depending upon the concentration of dye absorbed and the duration of light exposed. *H. brunonis* var. *brunonis* has 68.6 %, and *H. brunonis* var. *raktapushpa* has 67.8 % (Table 1, Fig. 1E & H) viable pollen grains, respectively. Viability studies using the DAB test revealed that 77.1 % of pollen grains were viable in *H. brunonis* var. *brunonis,* at the same time in *H. brunonis* var. *raktapushpa,* 73.5 % pollen grains showed the characteristic rust brown colour (Fig. 1F & I). Maximum

Table 1. *In vitro* pollen viability of *Humboldtia brunonis* var. *brunonis* and *H. brunonis* var. *raktapushpa* assessed by FCR and DAB staining.

	H. brunonis* var. *brunonis			***H. brunonis* var. *raktapushpa***		
Dye used	**Day before anthesis (%)**	**On the day of anthesis (%)**	**Day after anthesis (%)**	**Day before anthesis (%)**	**On the day of anthesis (%)**	**Day after anthesis (%)**
FCR	25.4±4.5	68.6±1.4	48.4±2.3	20.7±3.8.	67.8±1.2	50.2±2.0
DAB	28.2±4.5	77.1±2.5	53.3±2.0	30.4±4.3	73.5±2.4	62.4±01.53

FCR: Fluorochromatic reaction test; **DAB**: 3, 3'- Diamino benzidine test

percentage of viability was observed soon after the flower opened. The viability of the pollen grains slowly decreased after the day of anthesis.

In the FCR test, the viable pollen grains will absorb the dye and produce a polar component called fluorescein, which will give the fluorescence activity to the pollen grain, and it can be observed via a fluorescent microscope (35). On the other hand, the DAB test shows rust brown pollens grains if they are viable. This specific change was stipulated by the peroxidase activity (36); hence, they are counted as viable pollen grains.

In vitro Pollen Germination

In vitro pollen germination studies indicate that 63.5 % of pollen grains, on average, were germinated in both varieties and achieved a tube length of 786±5 µm in 10 % sucrose-containing BK medium (Table 2). The percentage of pollen germination other than 10 % of sucrose is remarkably low. The considerable decrease in the percentage of germination on the other sucrose concentration indicates that 10 % of sucrose is a sufficient amount of carbon source, and it maintains the optimum osmotic pressure of the medium for better germination. After 8 hrs of incubation in the BK medium, 67 % of pollen grains were germinated with a 791 µm pollen tube in *H. brunonis* var. *brunonis* (Fig. 1G & J).

In a culture medium, binucleate pollen germinates first (37). This was also true for binucleate *Humboldtia* pollen. The highest percentage of pollen germination was noted for other *Humboldtia* species in BK medium containing 5 % sucrose, i.e., 76 % in *H. decurrens* Bedd. ex Oliv. (5 % as 0.5 %), 72 % in *H. sanjappae* Sasidh. & Sujanapal (5 % as 0.5 %), and 73 % in *H. vahliana* Wight (2.5 % as 0.25 %), were found (38–40). In the methodology (38–40), the sucrose concentration was mentioned as 2.5 %, 5 %, and 10 %. However, results from the present study show that the pollen grains germinate in a 10 % sucrose solution, which reveals the differences between those of the previous studies. As mentioned, the pollen germinated there in a 5 % sucrose solution. In other *Humboldtia* species, 10 % sucrose solution did not germinate the pollen grains, whereas in the present study, most of the pollen grains were germinated in a higher concentration of sucrose solution (10 %). Thus, the results support the suitable sucrose concentration variation between species to species in the same genus (41). Pollen germination is a collection of biochemical events (42). It requires boron and calcium, as they play a crucial role in pollen germination and tube extrusion. Primarily, boron stimulates pollen germination. Even though the protrusions through the germ pore may be independent, the tube growth is regulated by the presence of boric acid in the BK medium. Secondly, the role of calcium is concerned with the 'crowding effect/population effect' of pollen grain (25), which has been found in many taxa (43).

Table 2. *In vitro* pollen germination and pollen tube growth using Brewbaker and Kwack medium (BK Medium).

Concentration of sucrose	**Percentage of *in vitro* pollen germination in *H. brunonis* var. *brunonis* (%)**	**Percentage of *in vitro* pollen germination in *H. brunonis* var. *raktapushpa* (%)**	**Pollen tube length of *H. brunonis* var. *brunonis***	**Pollen tube length of *H. brunonis* var. *raktapushpa***
5 %	5.20±1.10	4.6±1.20	47±3 µm	24±6 µm
10 %	67.2±1.10	60.4±0.54	786±5 µm	731±3 µm
15 %	26.1±4.37	18.7±1.43	112±6 µm	79±5 µm
20 %	7.30±2.0	6.6±2.50	56±5 µm	29±7 µm

Statistical Analysis

The correlation of pollen viability and pollen germination (67.2 % & 60.4 %) between both the varieties (*H. brunonis* var. *brunonis* & *H. brunonis* var. *raktapushpa*) has shown similar results in the FCR test (68.2 % & 67.8 %), but the results are varying in DAB test (77.1 % & 73.5 %). The comparison of pollen germination and FCR resulted in p-values less than 0.05 (0.038, 0.028). This suggests that there are statistically significant differences in the means of pollen germination and FCR compared to the DAB test (Table 3). Therefore, the correlation between pollen germination percentage and the FCR test is more significant than the DAB test. In a BK medium containing 10 % sucrose solution, the DAB test showed significantly higher pollen viability than the pollen germination test; however, there were no significant differences ($p<0.05$) between the DAB test and *in vitro* pollen germination. As a result, when compared to the FCR, the DAB test does not significantly differ for the majority of the parameters being measured. The current investigation demonstrated statistically significant differences in the means of the FCR test and pollen germination in the BK medium when compared to at least one other group based on comparisons of test means. In contrast to the other groups, there was no statistically significant difference in the DAB test mean. The results of the present study indicate that the factors under study

Table 3. One-way ANOVA test for *in vitro* pollen germination of two varieties of *Humboldtia brunonis* using DAB and FCR staining methods.

Source	Sum of squares	Degrees of freedom	Mean square	F	Significance level (p <0.05)
***In vitro* pollen germination**					
Between Groups	67.697	7	9.671	2.873	0.038
Within Groups	53.860	16	3.366		
Total	121.557	23			
DAB					
Between Groups	22.901	7	3.272	0.445	0.859
Within Groups	117.688	16	7.355		
Total	140.588	23			
FCR					
Between Groups	69.697	7	9.943	3.115	0.028
Within Groups	51.073	16	3.192		
Total	120.676	23			

DAB = 3, 3'- Diamino benzidine test; **FCR** = Fluorochromatic reaction test.

had varying effects on the groups, and they highlight the need for more research to determine the underlying mechanisms causing these variations. However, the disparities between the two stain values regarding *in vitro* germination raised doubts about the mathematical validity of *in vitro* staining. Using a suboptimal medium comparison for *in vitro* germinability, FCR is able to predict a higher viable level of the pollen grain.

The differences in the rate of pollen viability and germination are due to the significant impact of some crucial post-pollination events, and the pollen adhesion and hydration are major events after pollination provided by the stigma or the medium, and it favors the suitable conditions for germination (35, 44). On the other hand, it gives the idea that pollen germination is a cumulative factor. This study indicates that pollen viability and pollen germination are fundamentally different phenomenon. Pollen viability is an individual factor, although pollen germination was exonerated by the suitable medium or the receptive stigma. Even though *in vitro* staining with FCR and DAB gives an idea about pollen viability, it can only be validated by pollen germination. In many studies, pollen viability and *in vitro* pollen germination do not significantly correlate with each other. However, in the case of *H. brunonis* and its varieties, the FCR test shows statistically significant results.

Conclusion

Pollen germination in a 10 % sucrose-containing sample gives the better result with germination rates of 67.2 % & 60.4 % on *H. brunonis* var. *brunonis* & *H. brunonis* var. *raktapushpa* respectively. This means that 10 % is the threshold amount of sucrose for the better germination of pollen grains in these two varieties of *Humboldtia*. Along with this, the values of the FCR test using FDA (fluorescein diacetate) was closer to the percentage of *in vitro* germination. It gives the idea that the FCR test for pollen viability is more reliable than the DAB test (73–77 %) for at least the two selected varieties of *Humboldtia*.

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Authors' contributions

AR and SAK conceived the idea and planned experiments. AR performed the experiments under the supervision of SAK. All authors analyzed the research data and drafted the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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